Regulation of Protein Traffic in the TGN/ Endosomal System by Acidic Cluster Sorting Motifs

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For Lydia,

My love and passion

Table of Contents

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Abstract

Mammalian proprotein convertases (PCs) are a group of endoproteases that process molecules ranging from growth factors, hormones and serum proteins to bacterial toxins and virus envelope glycoproteins (Seidah *et al.*, 1998; Steiner, 1998). The processing of these substrates is critical for their bioactivities, which is important for the physiological functions of cells or organisms. Moreover, studies on processing of bacterial toxins and viral envelope glycoproteins have provided useful information for novel anti-pathogen strategies.

Biochemical and cellular studies have provided valuable information in understanding the function of these proteases. Both tissue and cellular distributions of the different PCs have indicated that these enzymes have distinct functions *in vivo*. For instance, PC1/3 and PC2 are specifically expressed in neuorendocrine cells and process prohormones during their maturation in mature secretory granules (Seidah *et al.*, 1998). Although PC6B and furin are ubiquitously expressed and they display nearly identical enzymatic characteristics, my studies presented in this thesis show that these two proteases are concentrated into distinct subcellular compartments. PC6B is localized into a late Golgi or post-TGN/endosome compartment distinct from furin (Chapter 2). Therefore, PC6B and furin may have different functions in processing substrates *in vivo*. My findings are consistent with previous genetic studies showing that deficiency of furin cannot be complemented by endogenous PC6B (Roebroek *et al.*, 1998).

The PC6B cd contains information sufficient for the intracellular localization of the protease, and includes two novel sorting motifs that coordinate with each other to

maintain the intracellular distribution of PC6B (Chapter 2). Both motifs contain multiple acidic residues and belong to a group of sorting signals named acidic cluster (AC) motifs, which are present on many membrane proteins such as furin, M6PR and VZV gE (Molloy *et al.*, 1999; Voorhees *et al.*, 1995; Zhu *et al.*, 1996). In addition, the PC6B-cd AC1 and the furin AC selectively bind to a novel cytosolic factor (PACS-1). PACS-1 associates with the AP-1/clathrin sorting machinery and is necessary for the correct localization of furin and PC6B (chapter 2).

PACS-1 belong a gene family including two broadly expressed human PACS genes (human PACS-1 and human PACS-2, Chapter 3). Both human PACS proteins share capacities in binding to cargo molecules and clathrin adaptor complexes (Chapter 3), suggesting they share similar roles in membrane protein trafficking. Biochemical studies identified a sequence in PACS-1 that binds to AP-1 complex. Mutation of this determinant blocked the interaction between PACS-1 and AP-1 complex, and behaved as a dominant negative when overexpressed in cells. The overexpressed PACS-1 mutant mislocalized furin and PC6B from the correct subcellular compartments (Chapter 4).

Together, these data illustrate a general mechanism of protein sorting in the TGN/endosomal system. Studies on this mechanism are not only useful for understanding the function of the endoproteases, but also provide tools to study the other potential AC motif/PACS-1-mediated sorting events.

List of Figures

List of Tables

Abbreviations

α_1-PDX	alpha-1-antitrypsin Portland
AC	acidic cluster
ACTH	adrenocorticotrophic hormone
ALP	alkaline phosphatase
AP	adaptor complex
ARR	atrophin-related region
ARF	ADP ribonucleatide factor
BBS 1	Bardet-Biedl syndrome
BFA	brefeldin A
CCP	clathrin coated pit
CD	cytoplasmic domain
CD-MPR	cation dependent mannose-6-phosphate receptor
CI-MPR	cation independent mannose-6-phosphate receptor
CK2	casein kinase 2
CGN	cis-Golgi network
CNS	central nervous system
COP	coatomer protein
CPD	carboxypeptidase D
CPE	carboxypeptidase E
DRPLA	Dentatorubral pallidoluysian atrophy
EGF	epidemic growth factor
Eps	EGF receptor phosphorylated substrate
EVR 1	Vittroretinopathy exudative familial
Fbr	furin binding region
GAP	guanine nucleotide exchange factor
GEF	guanine activating protein
GST	glutathione-S-transferase
HA	hemaglutinin
HBBS	Hank's balanced salt saline
HC	human chromosome
IDDM 4	Insulin independent diabetes mellitus 4
ISG	immature secretory granule
LAMP	lysosome-associated membrane protein
LAP	lysosomal acid phosphatase
LDL	low density lipoprotein

LIMP	lysosomal integral membrane porteins
MAb	monoclonal antibody
MEN 1	spinocerebellar ataxia Multiple endocrine neoplasia 1
MSG	mature secretory granule
MTOC	microtubule organization center
PA	phosphatidic acid
PACS	phosphorylated acidic cluster sorting protein
PAM	peptidylglycine α-amidating monooxygenase
PBS	phosphate buffler of saline
PC	prohormone convertase
PM	plasma membrane
POMC	pro-opiomelanocortin
PtdIns	phosphatidylinositol
RT-PCR	reverse transcription - polymerase chain reaction
SDS	sodium dodecyl sulfate
SCA	spinocerebellar ataxia
Tac	T cell receptor alpha chain
Tf	transferrin
TGN	*trans*-Golgi network
TMD	transmembrane domain
TRX	thioredoxin
RIA	radioimmunoassay
VMD 2	vitelliform macular degeneration type 2
VRN	Vitreoretinopathy neovascular inflammatory
VPS	vacuolar protein sorting
VZV-gE	varicellar zoster virus glycoprotein E

Introduction

In a mammalian cell, thousands of proteins must be targeted to specific subcellular locations in order to conduct their physiological functions. Some proteins are present in the cytosol whereas others are associated with the membranes of various organelles. In order to maintain the ordered structure in a cell, the coordination of movement or "trafficking" of molecules between different membrane-bound compartments is critical. There are two major membrane trafficking pathways in mammalian cells: the secretory and the endocytic pathways. Along the secretory pathway, newly synthesized proteins are translocated into the endoplasmic reticulum (ER), folded, and transported to the Golgi complex where further post-translational modifications are carried out. Proteins are then transported to the *trans* Golgi network (TGN), where sorting occurs. From the TGN, proteins are either secreted or transported to other intracellular compartments. These compartments include endosomes, lysosomes, and, the specialized secretory granules of endocrine/ neuroendocrine cells. The endocytic pathway is principally involved in the uptake and degradation of extracellular molecules. This pathway is composed of a highly dynamic early endosomal system and late endosomal/lysosomal compartments. Both the secretory and the endocytic pathways connect with each other in the TGN and endosomal system.

My thesis studies focus on the intracellular sorting of two mammalian proprotein convertases (PCs), PC6B and furin, in the TGN/endosomal system. Mammalian PCs are a group of Ca^{2+}-dependent serine endoproteases which are resident in both secretory and endocytic pathways. Mammalian PCs can process a broad range of substrates including precursors of growth factors, hormones, virus envelope glycoproteins, and bacterial toxins. The processing of these substrates is critical for their function *in vivo*. While

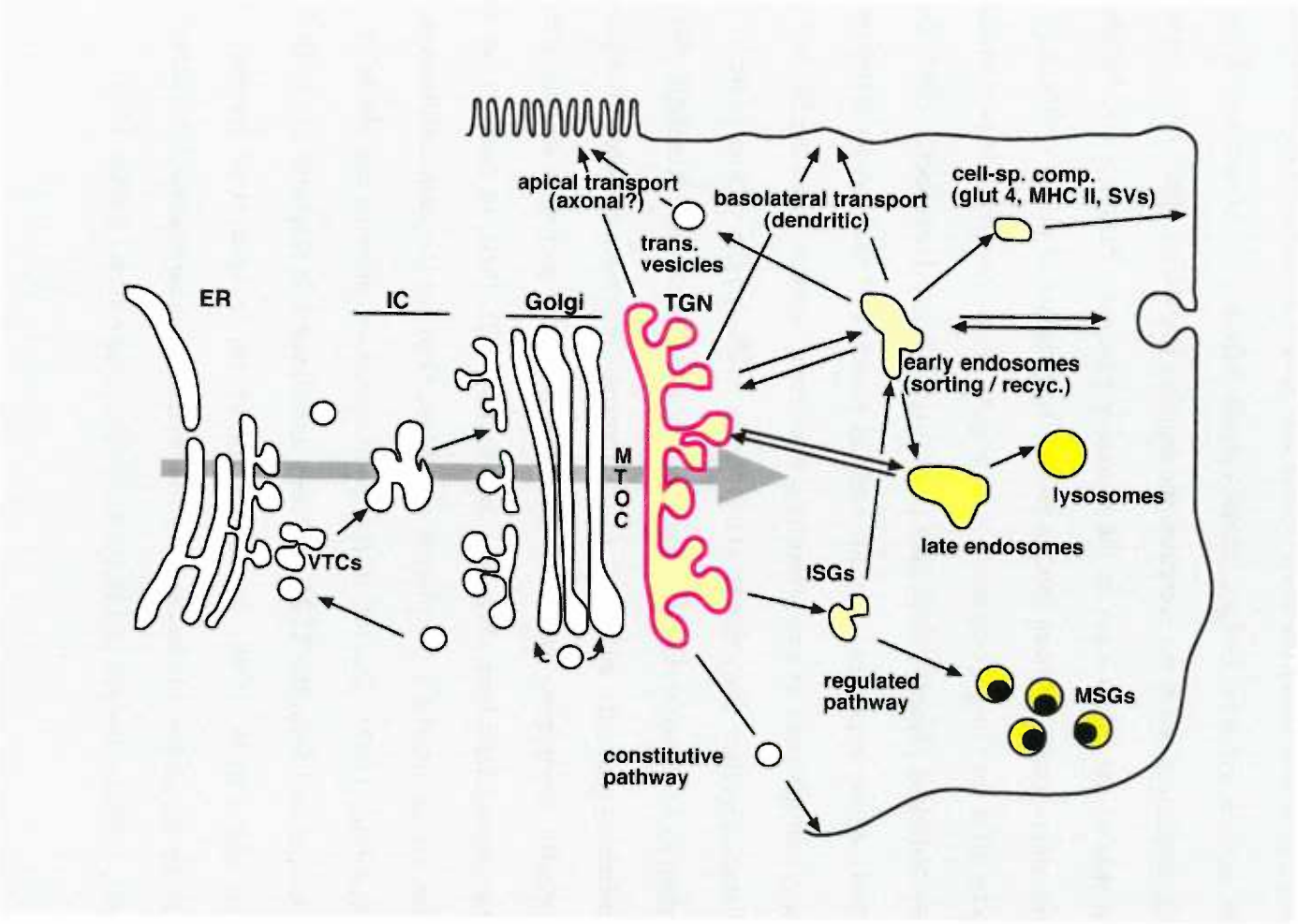
PC6B and furin show similar enzymatic properties *in vitro*, genetic analyses show that they have different functions *in vivo*. Moreover, cellular and molecular studies have shown that the intracellular localization of mammalian PCs is critical for the proper functions of these enzymes. In mammalian cells, both PC6B and furin are ubiquitously expressed. Biochemical studies suggest that PC6B and furin share highly similar cleavage site specificities. However, the deficiency of furin in mice by gene knockout cannot be complemented by endogenous PC6B, suggesting their distinct functions *in vivo*. In order to understand the functional difference between these two enzymes, the intracellular localization of PC6B was studied to in comparison with that of furin. Moreover, studies show that intracellular sorting of various membrane proteins relies on the interaction between the sorting signals incorporated in their cytoplasmic domains (cds) and the corresponding cellular sorting machinery. In order to understand the intracellular sorting of PC6B and furin, the molecular mechanisms involved in the sorting of both enzymes were further characterized. A general review of membrane trafficking will be presented and specific aspects that relate to my research will be discussed.

1.1. General back ground of protein transport

1.1.1 Compartments

Since the outline of the secretory pathway was delineated by Palade and his colleagues thirty years ago, the presence of multiple transport steps between a series of organelles has been well established in eukaryotic cells (Figure 1). Newly synthesized secretory proteins originate predominantly in the ER, and are sorted through the ER and Golgi intermediate compartment (ERGIC) into the *cis* Golgi network (CGN), which is located on the *cis* face of the Golgi complex. Proteins are then transported through the Golgi complex into the TGN, a network complex of vesicles on the *trans* face of the Golgi

Figure 1. Intracellular trafficking in mammalian cells



complex (Figure 1). Movement of protein between the compartments occurs by the budding and fusion of transport vesicles (Palade, 1975; Rothman, 1996). The Golgi complex consists of many flattened tubules and spherical vesicles known as "cisternae". The *cis*, *medial*, and *trans* Golgi cisternae contain different sets of enzymes that introduce different modifications to the secretory and membrane proteins. Studies have revealed the central role of the Golgi stack in the secretory pathway (Farquhar and Palade, 1981). Electron microscopy has been used to follow the progress of newly synthesized proteins and to localize the Golgi compartments in which various post-translational modifications (mainly N-linked glycosylation) take place (Abeijon and Hirschberg, 1992; Dunphy *et al.*, 1985). Many secretory proteins, such as proteins of the plasma membrane (PM), secretory storage vesicles and lysosomes, have been found to leave the ER and cross the Golgi stack together. Only during exit from the TGN, are the various proteins separated according to destination (Griffiths and Simons, 1986; Traub and Kornfeld, 1997). While the trafficking proceeds, additional vesicle transport pathways operate in independent and occasionally overlapping flow patterns. Three of the exit pathways are characterized and will be summarized here (Figure 1 and section 1.2). First, in constitutive secretion, proteins are destined for the plasma membrane (PM) or the extracellular space (Traub and Kornfeld, 1997). Second, in regulated secretion, proteins are stored in secretory granules derived from the TGN until they are released in response to external signal(s) (Arvan and Castle, 1998; Tooze, 1998). Third, in lysosomal sorting, lysosomal hydrolases are targeted from the TGN to lysosomes via endosomal compartments (Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998b; Sandoval and Bakke, 1994).

The second major membrane trafficking pathway is the endocytic pathway, which starts at the cell surface. The endocytic pathway is critical in regulating cellular response to extracellular signals. For example, it is used in clearing extracellular signal molecules by

targeting them to lysosomes for degradation (Casanova *et al.*, 1990; Kaetzel *et al.*, 1991; Song *et al.*, 1994), internalizing exogenous nutritional molecules, delivering molecules from the basolateral to the apical surface of polarized cells, and regenerating synaptic vesicles in presynaptic neurons (Bauerfeind *et al.*, 1995; Sudhof, 1995). In addition, bacterial toxins and viruses also use the endocytic pathway to invade cells (Chaudhary *et al.*, 1990; Yoshida *et al.*, 1991). Internalization from the cell surface is the first step in the endocytic pathway. After internalization, proteins are transported by budding and fusion of carrier vesicles through a series of endosomal compartments including early (sorting and recycling) endosomes and late endosomes/prelysosomes. Proteins are then either targeted to lysosomes for degradation or recycle back the cell surface for reutilization. In addition, many TGN localized proteins can be delivered to the cell surface, then transported from the cell surface back to the TGN through the endocytic pathway. Bacterial toxins can also follow a similar pathway from the cell surface to the Golgi compartments.

As described above, transport occurs not just in a forward direction, from the ER to the cell surface, but also in a retrograde manner by endocytosis and Golgi-to-ER transport. The retrograde transport is presumably necessary to maintain the size of the ER and also allows retrieval of ER proteins from the Golgi complex and the recycling of those integral membrane proteins that are included as targeting molecules in transport vesicles (Sollner *et al.*, 1993). Indeed, as has been visualized for some protein toxins, transport of material from the cell surface all the way to the ER is possible (Sandvig *et al.*, 1992).

1.1.2 Coat proteins

Transport of membrane proteins and soluble cargo molecules between intracellular organelles is mediated by the budding and fusion of carrier vesicles. Carrier vesicle

formation requires the assembly of a protein "coat" from soluble cytosolic components. Coat assembly demarcates the bud site on the donor membrane and serves to shape the budding vesicle. Three major classes of coat proteins have been characterized to date although others certainly exist. Clathrin coats were first identified morphologically and later by biochemical characterization. Clathrin-coated vesicles are ~ 50-150 nm in diameter and consist of an outer layer of clathrin lattice assembled from clathrin triskelions and an inner shell made by adaptor protein (AP) complexes (Keen *et al.*, 1979). The clathrin triskelions consist of three heavy chains and three light chains, which can self-assemble into a cage-like structure (Robinson, 1992). AP complexes that interact with clathrin heavy chains were first described as factors that promote the formation of coat structures from the purified clathrin trimers (Keen *et al.*, 1979). Structural analyses show that a globular domain in the clathrin heavy chain, consisting of a seven bladed β-propeller structure, interacts with β adaptins of AP complexes (ter Haar *et al.*, 1998, Dell'angelica, 99). AP complexes are heterotetramers composed of two large subunits (α and β adaptins, ~100kD each), one medium chain (μ, ~50kD), and one small chain (σ, ~21kD). The structure of an adaptor complex is similar to a "Mickey Mouse" head with a large core region formed by μ subunit, σ subunit and the N-termini of both α and β adaptins; and two smaller appendages formed by the C-termini of α and β adaptins (Heuser and Keen, 1988; Kirchhausen *et al.*, 1989; Zaremba and Keen, 1985). To date, four AP complexes have been identified in mammalian cells: AP-1, AP-2, AP-3, and recently, AP-4 (Table 1). Each of these contains two large adaptins (γ and β1, α and β2, δ and β3, ε and β4 respectively), one medium chain (μ1, μ2, μ3 and μ4) and one small chain (σ1, σ2, σ3, and σ4) (Ahle *et al.*, 1988; Dell'Angelica *et al.*, 1999a; Dell'Angelica *et al.*, 1997a; Robinson, 1987). Different isoforms of γ, β, μ and σ subunits have been identified for AP-1 and AP-3 complexes, some of which are tissue specific. (Dell'Angelica *et al.*, 1997b; Simpson *et al.*, 1997). As suggested by the nomenclature,

the μ, σ, and β subunits of different AP complexes are highly related, whereas α, γ, δ, and ε are quite distinct (Bonifacino and Dell'Angelica, 1999; Robinson, 1994). In mammalian cells, AP complexes show a differential subcellular localization. AP-2 is found primarily on the PM and is involved in the internalization from the cell surface (Ahle *et al.*, 1988; Robinson, 1987). Other APs (AP-1, AP-3, and AP-4) are localized in the perinuclear region and in punctate vesicular structures throughout the cytoplasm, probably corresponding to the Golgi, post-Golgi compartments and endosomes (Ahle *et al.*, 1988; Dell'Angelica *et al.*, 1999a; Dell'Angelica *et al.*, 1997b). Both AP-1 and AP-3 have been shown to play a role in the TGN sorting.

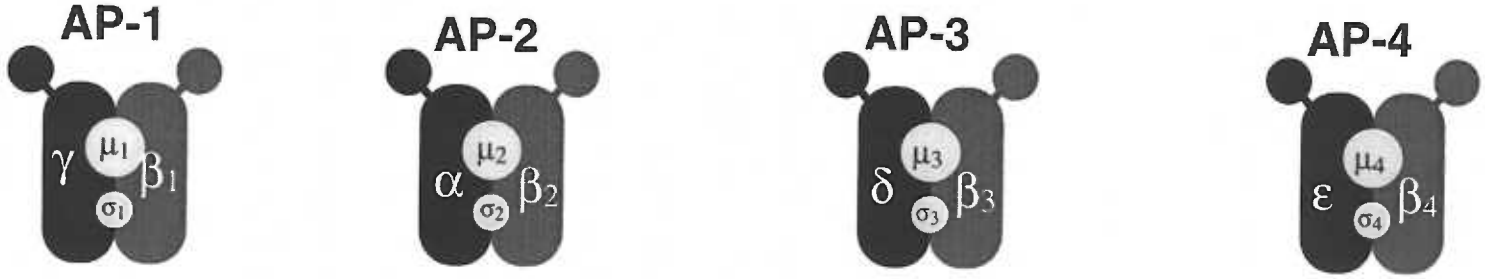
The coat constituents of two additional classes of vesicles that mediate membrane trafficking in the early secretory pathway have been defined recently. COP-coated vesicles assemble onto the *cis*- and *medial*-cisternae of the Golgi and onto the ERGIC between the ER and Golgi (Balch *et al.*, 1994; Kreis and Pepperkok, 1994). The ER-derived 60-65 nm vesicles are covered by a 10nm fuzzy COPII coat, which is involved in anterograde transport from the ER to the Golgi stack (Salama and Schekman, 1995). In yeast, the COPII coats have been shown to be composed of three components: the small GTP binding protein Sar1p, the Sec23/24p complex, and the Sec13/31p complex (Salama *et al.*, 1993). Sar1p is a GTP-binding protein, and Sec23p is a GTPase activating protein (GAP) specific for Sar1p (Yoshihisa *et al.*, 1993). Sec24p has no effect on the GAP activity, but is necessary for vesicle formation (Salama and Schekman, 1995). Sec13p and Sec31p contain six to seven WD-repeats, presumably involved in protein-protein interactions (Salama and Schekman, 1995). COPII coated vesicles are involved in ER-to-Golgi transport. Sec23p interacts with an ER-exporting signal, di-phenylalanine, on cargo molecules, and these enable COPII-coated vesicles budding from the ER to selectively

carry secretory cargo to the Golgi while excluding ER-resident proteins (Barlowe *et al.*, 1994; Rexach *et al.*, 1994).

Table 1. ***Subunit composition of the mammalian assembly proteins.***

The yeast homologues are also listed. Four yeast gene products (Apl4p, Apl2p, Apm1p and Apa1p) may represent the homologues of AP-2 subunits. A clear function in transport, however, has only been demonstrated for the yeast AP-3 complex (Cowles *et al.*, 1997). [a]Here are listed the interactions of adaptor protein complexes (APs) with clathrin, sorting signals, and other cytosolic factors. APs also interact with other components. [b]In neuroendocrine cells, AP-1 is also found on immature secretory granules (Dittie *et al.*, 1997). Under *in vitro* conditions AP-2 binds to late endocytic structures (Traub *et al.*, 1996; West *et al.*, 1997). The dual localization of AP-3 is not understood. Presumably, the APs exhibit the same sorting function. Reference [c](Page *et al.*, 1999); [d](Le Borgne *et al.*, 1993); [e](Owen *et al.*, 1999); [f](Dell'Angelica *et al.*, 1998; Dell'Angelica *et al.*, 1997a; Simpson *et al.*, 1996; Simpson *et al.*, 1997); [g](Dell'Angelica *et al.*, 1999a).

COPI-coated vesicles are a population of 70-75 nm vesicles generated from the Golgi membrane (Malhotra *et al.*, 1989). They have been shown to mediate intra-Golgi transport as well as Golgi-to-ER retrograde transport (Rothman, 1994). In addition, a COPI coat is also found on the endosome compartments and is involved in early to late endosome transport (Gruenberg and Maxfield, 1995). The COPI-coat complex is composed of seven subunits α, β, β, γ, δ, ε and ζ (Rothman, 1994). In early studies, COPI was found to be required for intra-Golgi transport and ER-to-Golgi anterograde transport (Balch *et al.*, 1994; Zhang *et al.*, 1994). Some data suggest a sequential role of COPII- followed by COPI-coated vesicles in the ER-to-Golgi anterograde transport (Aridor *et al.*, 1995; Scales *et al.*, 1997). However, in yeast, the COPI coat can recognize the di-lysine motif KKXX (an ER retrieval signal), supporting a role for the COPI-coated vesicles in retrograde transport (Cosson and Letourneur, 1997; Letourneur *et al.*, 1994;



Adaptor	Subunits		Interactions[a]	Localization[b]	Function
	Mammals	Yeast			
AP1	γ	Apl4p	γ-synergin, p34[c]	TGN, early endosomes[d]	Biosynthetic transport to endosome
	β1	Apl2p	Clathrin, di-leucine motifs		
	μ1	Apm1p	Tyrosine based motifs		
	σ1	Apa1p	Unknow		
AP2	αa,αc		ps15, Epsin, Dynamin, AP180, Amphiphysin, Auxclin,Synaptotagmin[e]	Plasma membrane endosomes?	Endocytosis
	β2		Clathrin, di-leucine motifs		
	μ2		Tyrosine based motifs		
	σ2		Unknown		
AP3	δ	Apl5p	Unknown	TGN? endosomes[f]	Biosynthetic intracellular transport to lysosomes/melanosomes Synaptic vesicle formation
	β3A/β3B (or β-NAP)	Apl6p	Clathrin? di-luecine motifs		
	μ3A/μ3B	Apm3p	Tyrosine based motifs		
	σ3A/σ3B	Aps3p	Unknown		
AP4	ε		Unknown	TGN? endosomes[g]	?
	β4		Unknown		
	μ4		Tyrosine-based motifs		
	σ4		Unknwn		

Lewis and Pelham, 1992; Munro and Pelham, 1987; Semenza *et al.*, 1990). Cell-free transport assays have also shown that COPI is required for Golgi-to-ER but not ER-to-Golgi transport (Barlowe, 1997; Spang and Schekman, 1998). Furthermore, both anterograde and retrograde cargo molecules have been observed in separate populations of the COPI-coated vesicles on the Golgi by electronic microscopy (Orci *et al.*, 1997). Together, these data suggest dual roles for COPI coat complex in the transport in the early secretory pathway.

1.1.3 Regulation of coat protein assembly

Cell-free assays that reconstitute coated vesicle budding, in some cases using purified cytosolic components, have provided detailed insight into the mechanism of coated vesicle budding. ADP-ribonucleotide factors (ARFs) are a group of GTP binding proteins involved in coat assembly. The GDP to GTP transition induces a conformational change on ARF1, which allows ARF1 to interact with the lipid bilayer (Paris *et al.*, 1997). The GTP bound ARF1 then triggers nonclathrin (COPI coatomer) and clathrin (AP-1 and AP-3 adaptor complexes) coat recruitment onto the donor membrane (Ooi *et al.*, 1998; Rothman, 1996; Schekman and Orci, 1996). The GTP binding protein Sar1p, which shares 30% homology with ARF1, probably plays a similar role in COPII coat assembly. The current model of COPI coated vesicle formation suggests that phospholipase D (PLD), activated by ARF1, hydrolyzes phosphatidylcholine to phosphatidic acid (PA) and choline. The local production of the negatively charged PA recruits COPI to membranes and at the same time drives membrane curvature facilitating budding (Ktistakis *et al.*, 1995; Ktistakis *et al.*, 1996). PLD activities have been studied in several other steps of transport where ARF1 could be involved. It has been shown to be involved in ER-to-Golgi transport (Roth *et al.*, 1999), to stimulate the release of nascent secretory vesicles from the TGN (Chen *et al.*, 1997b), and to mediate the mistargeting of AP-2

adaptor to an endosomal compartment (Chen *et al.*, 1997b). Further studies will be required for understanding the role of PLD in membrane trafficking.

After vesicle formation, a vesicle-associated GTPase-activating protein (GAP) stimulates the hydrolysis of bound GTP to release ARF1/GTP and to trigger coat dissociation prior to vesicle fusion (Pelham, 1991; Rothman, 1996; Serafini *et al.*, 1991). A group of guanine nucleotide exchange factors (GEFs) facilitate the GDP to GTP exchange for ARFs (Rothman, 1996). Biochemical studies show that a fungal metabolic product, named brefeldin A (BFA) (Orci *et al.*, 1991; Takatsuki and Tamura, 1985; Tamura *et al.*, 1968) inhibits GEF activity specific for ARF1. Therefore, BFA can prevent vesicle formation and budding and, where ARF1 is involved, block transport in the secretory pathway.

In addition to preventing secretion, BFA also causes reversible effects on the morphology of multiple subcellular compartments as shown in Figure 2 (Klausner *et al.*, 1992). Upon BFA treatment, many tubular structures are observed on the different compartments such as Golgi and endosomes. Although the mechanism is not clear, the tubular-structure is proposed to be caused by the failure of vesicle formation, budding, or both. The tubular structures from the Golgi stack extend along microtubules. These extended tubular structures eventually fuse with the ER; and Golgi marker proteins diffuse from the perinuclear region (Lippincott-Schwartz *et al.*, 1990b; Lippincott-Schwartz *et al.*, 1989). Moreover, upon BFA treatment, similar tubular structures can be seen in the TGN and early endosomal compartments. These structures extend along microtubules and fuse with each other, and result in condensation of TGN markers onto the microtubule organization center (MTOC) and the elongated tubular staining of endosomal markers (Lippincott-Schwartz *et al.*, 1991; Prydz *et al.*, 1992; Reaves *et al.*, 1993). In contrast, the

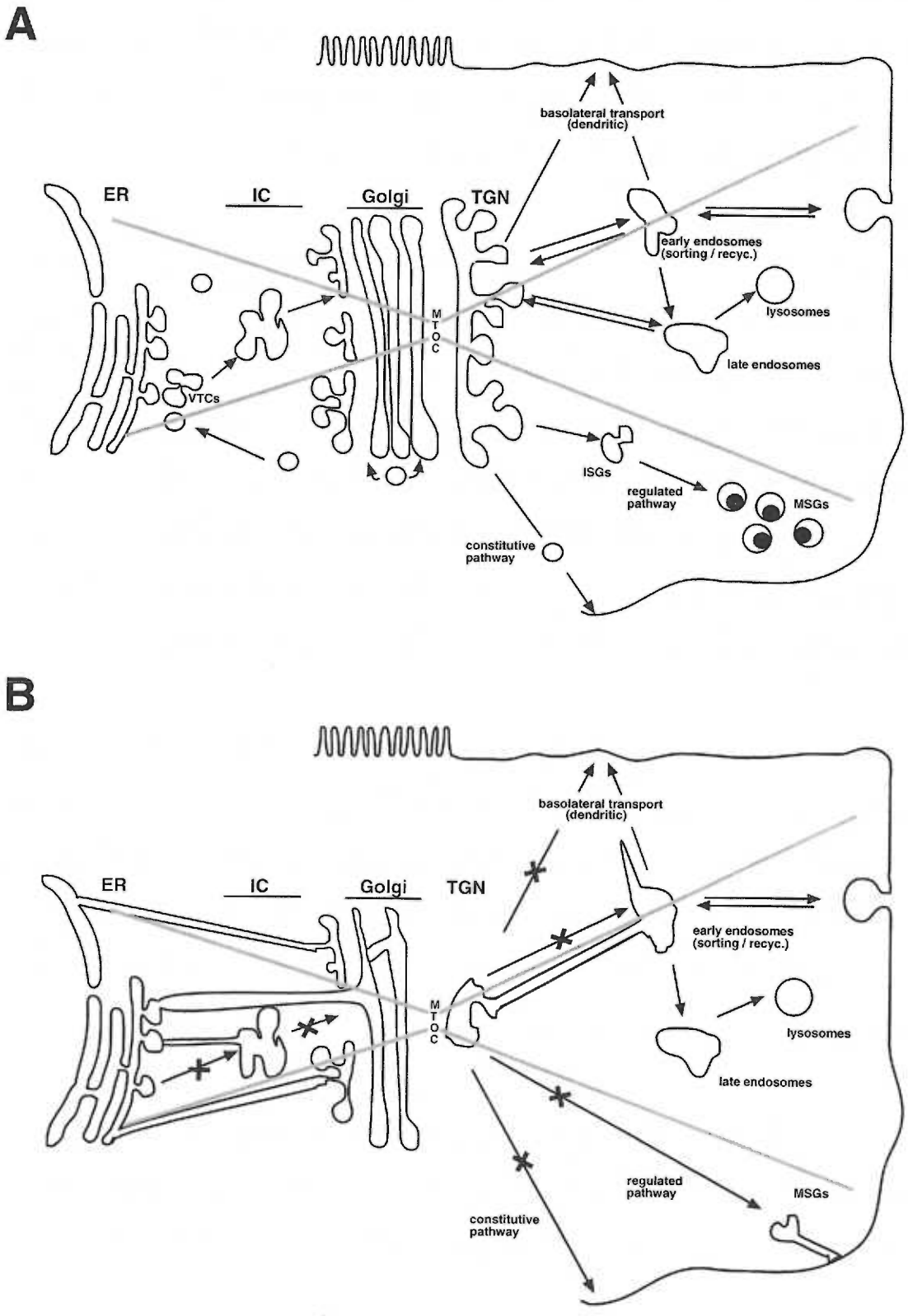
late endosomes and lysosomes seem to be stable during BFA treatment (Lippincott-Schwartz *et al.*, 1991).

Figure 2. ***A model for the observed effects of BFA on intracellular membrane compartments.*** (A) In normal cells, intracellular sorting pathways along both biosynthestic and endocytic pathways are illustrated. The arrows represent the defined pathways with well-understood mechanisms of vesicular transport include clathrin-coated vesicles and non-clathrin –coated vesicles. The TGN functions as a sorting center in the biosynthetic pathway hosting constitutive, regulated, and endosomal-sorting secretory pathways. The existence of transport pathways between the TGN and early endosomes is suggested by the results presented previously (Wood *et al.*, 1991). (B) After addition of BFA, cisternal elements of the Golgi stack and intermediate compartment (IC) are redistributed along microtubules (MT) and fuse with the ER. The TGN extends MT-dependent tubular processes, which fuse with early endosomes. Endocytosis and transport to late endosomes and lysosomes (Lys) appears to be unaffected.

1.1.4. Localization signals of secretory proteins

In the secretory pathway, localization signals that interact with vesicle coats can be found on the cytoplasmic domains (cds) of sorted membrane proteins. Several such signals have now been identified that influence the distribution of proteins between compartments in the secretory pathway. Two types of ER-exporting motifs have been identified on transmembrane proteins. The first type of motif is the di-acidic motif (Asp-X-Glu, where X represents any amino acid) present on the cds of vesicular stomatitis virus glycoprotein (VSV-G) and other secreted cargo molecules. These motifs are required for efficient ER-export (Nishimura and Balch, 1997). Studies of an ERGIC marker (ERGIC-53) and several vesicle proteins (Bet1p and Bos1p) have revealed a second type of ER-export motif consisting of di-phenylalanine on the C-termini of protein cds (Obar *et al.*, 1990). This motif is essential for efficient ER-exit of the proteins.

Figure 2 BFA effects on different intracellular compartments



The export of soluble cargo molecules from the ER is mediated by cargo receptors (Aridor and Balch, 1996; Kuehn and Schekman, 1997). ERGIC-53 contains a cytoplasmic di-lysine motif (KKXX) for retrieval back to the ER (Obar *et al.*, 1990) in addition to determinants for ER-retrieval, and these are necessary for the continuous recycling of ERGIC-53 in the early secretory pathway (Dominguez *et al.*, 1998; Kappeler *et al.*, 1997; Springer and Schekman, 1998). Since ERGIC-53 can bind to mannose residues, it has been proposed to bind the high-mannose oligosaccharides of secretory proteins in the ER, and function as a receptor for these cargo molecules to move them to the Golgi (Arar *et al.*, 1995). In addition, members of the transmembrane p24 protein family (including Emp24 in yeast) have been proposed to be cargo receptors for mediating the ER-exit of soluble cargo (Schroder *et al.*, 1995). A di-phenylalanine motif is present on the cds of most p24 members, and is recognized by Sec23p of COPII coat to recruit soluble cargo into the ER-budded vesicles. Moreover, two mammalian members in the p24 family also contain a K(X)KXX-like ER-retrieval motif, which likely plays a role in COPI-mediated ER-retrieval to enable reutilization of the p24 proteins.

Retrieval signals are also found on ER proteins. The C-terminal KEDL/HDEL signal, used for the retrieval of lumenal ER proteins from the Golgi complex, is recognized by Erd2, a yeast KEDL receptor. Like the p24 family members, the Erd2 cd contains a putative di-lysine motif (KKXX), which allows Erd2 to bind to COPI coat and to recruit soluble ER proteins into the retrograde transport vesicles (Lewis and Pelham, 1992; Munro and Pelham, 1987; Semenza *et al.*, 1990).

Several sorting signals have also been characterized that influence the distribution of proteins between post-Golgi compartments, i.e., the TGN, endosomes, lysosomes, storage vesicles, and (in polarized cells) the basolateral domain of the PM. Typically, the signal

consists of a short peptide sequence. The best characterized signals are tyrosine-based motifs (YXXΦ) that direct endocytosis of proteins from the cell surface (Section 1.4 and Table 2). Intriguingly, the fates of internalized proteins are often controlled by signals that are very similar to, and even overlap with, the endocytosis signals themselves. Thus, some tyrosine-based motifs can, when appended to a reporter molecule such as the Tac antigen, mediate both endocytosis and targeting to lysosomes (Letourneur and Klausner, 1992). In contrast, the sequence YQRL (found in the protein TGN38), which also acts as an endocytosis signal, targets the same reporter protein to the TGN. Mutation of the arginine residue to an aspartic acid abolishes the TGN localization but not endocytosis; the protein simply recycles to the cell surface (Humphrey *et al.*, 1993). It is well known that sequences containing a tyrosine-based motif can selectively associate with different AP complexes for distinct sorting steps in the TGN/endosomal system. Thus, whether the similarity of these signals reflects their recognition by a related family of AP complexes is likely critical for their functions in membrane protein sorting (Section 1.4.1).

The analysis of targeting signals can be complicated. In addition to tyrosine-based motifs, di-leucine-like motifs (containing two leucines next to each other or a leucine and a hydrophobic residue such as isoleucine and valine, Section 1.4.1) and acidic clusters (containing multiple acidic residues in a stretch, Section 1.4.2) have also been characterized in protein sorting in the TGN/endosomal system (Section 1.4). A single protein may have multiple signals, either overlapping or distinct, and the function of these signals may be affected by phosphorylation. Quite different signals may serve to target proteins to the same organelle, an example being the tyrosine-based and di-leucine-like motifs that both direct proteins to lysosomes (Letourneur and Klausner, 1992). Furthermore, the same signal may function at more than one location, for example, to specify transport to the basolateral domain of the PM from both endosomes and the TGN

in polarized cells (Matter *et al.*, 1994). The localization of proteins to post-Golgi compartments is mainly determined by signals in their cds, which mediate specific transport rather than simple retention (Pelham and Munro, 1993). A detailed discussion of these complexities is presented in the section 1.4.

Table 2. ***Protein sorting motifs and their functions.***

Proteins are grouped according to their sorting properties. The last four proteins are cytoplasmic viral proteins that showed interaction with adaptors. Abbreviation: E endocytosis, B basolateral in polarized cells; L lysosomal sorting; MIIC MHC class II compartments; Y, tyrosine motifs; LL di-leucine-like motif; CK2, casein kinase 2 phosphorylation site; acidic motif. a). (Pearse, 1988); b) (Beltzer and Spiess, 1991; Fuhrer *et al.*, 1991; Geffen *et al.*, 1991); c) (Nesterov *et al.*, 1995a; Nesterov *et al.*, 1995b); d) (Bremnes *et al.*, 1998a; Ohno *et al.*, 1996; Ohno *et al.*, 1995); e) (Heilker *et al.*, 1996); f) (Boge *et al.*, 1998; Ohno *et al.*, 1997); g) (Bremnes *et al.*, 1998a; Dietrich *et al.*, 1997; Ohno *et al.*, 1995; Rapoport *et al.*, 1998); h) (Bradshaw *et al.*, 1997; Chuang *et al.*, 1997; Shiratori *et al.*, 1997); i) (Honing *et al.*, 1996; Honing *et al.*, 1998; Ohno *et al.*, 1996; Ohno *et al.*, 1995; Rapoport *et al.*, 1998); j) (Honing *et al.*, 1998; Sosa *et al.*, 1993); k) (Ohno *et al.*, 1996; Ohno *et al.*, 1995); l) (Honing *et al.*, 1998); m) (Honing *et al.*, 1998); n)(Bremnes *et al.*, 1998a; Rodionov and Bakke, 1998); o) (Glickman *et al.*, 1989); p) (Honing *et al.*, 1998; Honing *et al.*, 1997; Sosa *et al.*, 1993); q) (Boll *et al.*, 1996; Ohno *et al.*, 1996; Ohno *et al.*, 1995; Rapoport *et al.*, 1997; Stephens *et al.*, 1997); r) (Dittie *et al.*, 1997; Teuchert *et al.*, 1999a; Teuchert *et al.*, 1999b); s) (Eng *et al.*, 1999a); t) (Alconada *et al.*, 1996; Zhu *et al.*, 1996); u) (Le Gall *et al.*, 1998; Piguet *et al.*, 1998); v) (Le Gall *et al.*, 1998; Piguet *et al.*, 1998); w) (Le Gall *et al.*, 1998; Piguet *et al.*, 1998).

In contrast to the proteins of the TGN and ER, there is little evidence that the enzymes of the Golgi stack recycle through distal compartments. Furthermore, no cytoplasmic sorting signal has been detected in these proteins. Analyses of deletion mutants and chimeras have shown that their TMDs are both necessary and sufficient for Golgi retention (Munro, 1998). For example, the first TMD (m1) of the M glycoprotein of avian coronavirus is sufficient to retain the protein in the *cis*-Golgi. When the TMD of the

Table 2. Sorting motifs on proteins in the TGN/endosome system

Proteins	Sorting	Motifs		
		Y	LL	AC
LDL receptor[a]	E B	NPVY		
ASGP[b]	E B	YQDL		
EGF receptor[c]	E B	YRAL		DEEDMDDVVDADE
Transferrin receptor[d]	E B	YTRF		
HA(Y543)[e]	E B	YRIC	CRICLL	
HIV envelope[f]	E B	GYSPL		
		YHRL		
CD3γ[g]	E		DKQTLL	
CTLA-4(CD152)[h]	E	TTGVY		
Lamp-1[i]	E B L	GYQTI		
LAP[j]	E B L	GYRHA		
CD68[k]	E L	STYQPL		
Limp-II[l]	E B L		DERAPLI	
Tyrosinase[m]	E B L		EERQPLLMDK	
			DDYHSLL	
Invariant chain[n]	E B MIIC		DDQRDLI	
			EQLPML	
CI-MPR[o]	E B TGN	YKYSKV	LLHV	GDEQDSEDEVLT
				HDDSDEDLL
CD-MPR[p]	E B TGN	YRGV	DHLLPM	GDDQLGEESEERD
TGN38[q]	E B TGN	SDYQRL		
Furin[r]	E B TGN	YKGL	LL	EECPSDSEEDE
VZV gE[s]	E B TGN	AYRV		FEDSESTDTEEEF
HIV-1 Nef[t]	E		LL	EE
HIV-2 Nef[u]	E	YSRF		
SIV Nef[v]	E	YGRL	YGRLL	

VSV-G protein, which is efficiently delivered to the PM, is replaced with m1, the resulting chimera is retained in the Golgi (Swift and Machamer, 1991). This chimera also forms large SDS resistant oligomers. The retention of chimera may be due to large sizes of aggregation that would exclude the oligomers from transport vesicles (Nilsson and Warren, 1994; Weisz *et al.*, 1993). Another hypothesis suggests that the retention of Golgi proteins in the appropriate compartment is mediated by the length of their TMDs. Evidence for this has been sought by examining the retention of sialytransferase, an enzyme of the *trans*-Golgi stack. Mutation of any residues in the TMD of the enzyme does not change the efficiency of retention, but the addition or removal of residues show that the efficiency of sialytransferase retention is related to its TMD length (Munro, 1995). On average, Golgi membrane proteins have TMDs that are five amino acids shorter than those on the PM (Bretscher and Munro, 1993; Munro, 1991). Although the mechanism of the retention of Golgi enzymes is not clear, some interaction within the membrane can lead to the selective retention of these proteins (Munro, 1995).

1.2 TGN Sorting

Secretory proteins share a common transport pathway from the ER, through the Golgi stack to the TGN, where they are sorted to different destinations (Figure 1). The TGN, as the major sorting station in the secretory pathway, hosts a group of enzymes (i.e. furin) and receptors (i.e. mannose-6-phosphate receptors, MPRs), required for protein maturation and transport. Although both furin and MPRs are concentrated in the TGN at a steady state, they can be sorted out of the TGN to the PM and endosomes. Their retrieval to the TGN is dependent on the information on their C-terminal cds (section 1.6.4 and 1.2.2, respectively). Such a recycling mechanism ensures a concentration of the proteins in the TGN. Another integral membrane protein TGN38/41, which is a conventional TGN marker with unknown physiological function (Banting and

Ponnambalam, 1997), is also localized to the TGN at steady-state, and cycles between the TGN and the cell surface (Reaves *et al.*, 1993). Interestingly, the TMD of TGN38/41 is a sufficient TGN retention signal for maintaining the TGN concentration (Ponnambalam *et al.*, 1994), which is different from those of furin and MPRs. In addition, the tyrosine-based motif (SDYQRL) within the TGN38/41 cd is necessary for the protein's retrieval from the cell surface by the AP-2/clathrin coat-dependent endocytosis (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Ohno *et al.*, 1995; Ponnambalam *et al.*, 1994; Stephens and Banting, 1998; Stephens *et al.*, 1997).

While an enormous number of proteins pass through the TGN to different destinations, the mechanisms for the TGN sorting are complex. Conventionally, the sorting from the TGN is characterized as following three major pathways (Figure 1). In non-endocrine cells, proteins can be sorted into two pathways: the constitutive secretory pathway and the endosomal/lysosomal sorting pathway. In polarized epithelial cells, distinct apical- and basolateral-sorting pathways from the TGN have been characterized for the constitutive secretion (section 1.3.1). In cells specialized in regulated secretion, such as neuroendocrine cells, proteins can be also sorted into the third pathway, the regulated secretory pathway. Moreover, immature secretory granules (ISGs), the intermediate compartments on the regulated secretory pathway, host the constitutive-like secretion, which can transport proteins from the ISGs to the cell surface.

Recent structural studies provide new information to understand the sorting in the TGN. The data show that the TGN has many extended tubules that are continuous with different *trans* cisternae, and the tubular structures do not connect with each other as a network (Ladinsky *et al.*, 1994). An individual tubule produces buds that are either clathrin coated or covered with a lace-like coat, but never a mixture of the two (Ladinsky *et al.*, 1994).

These observations suggest that sorting of molecules must occur before the formation of such TGN tubules. From each of these cisternae the cargo molecules are then packaged for transport (Ladinsky *et al.*, 1999).

Many studies support the concept that the TGN sorting is an active process. For instance, tyrosine-based and di-leucine-like motifs, as well as several other sequence signals play critical roles for correct sorting of cargo molecules destined to endosomes and lysosomes (Le Borgne and Hoflack, 1998b). However, without the identification of a positive sorting signal, the constitutive secretion is proposed to be a default process. Default sorting has also been used to explain the enrichment of soluble cargo molecules in the condensing vacuoles in the regulated secretory pathway (Tooze, 1998). Despite the discrepancy between active and default sorting, the TGN-export can be blocked at 20°C (Traub and Kornfeld, 1997).

1.2.1 Constitutive Secretory Pathway

The constitutive secretory pathway delivers proteins (e.g. receptors) to the cell surface or secreted proteins (e.g. growth factors) to the extracellular space from the TGN. Most cell surface proteins are transported to the PM through the constitutive pathway. Some proteins, such as transferrin receptor (Tfr), can be targeted to the cell surface via both the constitutive secretory and the endosomal sorting pathway (Strous *et al.*, 1983). The delivery is believed to be a direct process without any intermediate compartment between the TGN and the cell surface (Traub and Kornfeld, 1997). However, in polarized MDCK cells, the recycling endosomes may be involved in the cell surface delivery of some proteins (Matter and Mellman, 1994; Traub and Kornfeld, 1997).

The transport from the TGN to the cell surface can be inhibited at 20°C (Matlin and Simons, 1983). At 20°C, the TGN has many budding structures with cytoplasmic coats, and some of the coats are labeled by clathrin antibody. However, these coats do not contain VSV-G, a well-characterized cargo in the constitutive pathway, suggesting that these coated vesicles do not transport newly synthesized PM proteins (Rothman *et al.*, 1980; Rothman and Fine, 1980; Wehland *et al.*, 1982; Yoshimori *et al.*, 1996). The identity of the coat proteins found on the isolated post-TGN coated vesicles is still unclear (Simon, 1996). Several non-clathrin coat-like proteins, including p62 (Jones *et al.*, 1993), p200 (Narula *et al.*, 1992; Narula and Stow, 1995), and p230 (Kooy *et al.*, 1992), have been identified in association with the TGN membrane. However, none of them has been shown to co-localize with the cargo proteins for constitutive secretion.

In polarized epithelial cells, the tight junctions connect the monolayer of cells, which can separate the tissues or organs from the surrounding environment. The tight junctions also split the PM into two domains: apical and basolateral surfaces. Distinct apical- and basolateral-sorting pathways have been characterized, and the TGN is thought to be the major sorting compartment for directly targeting proteins to each membrane domain. In agreement with this idea, basolateral proteins such as VSVG and apical proteins such as hemaglutinin (HA) can be seen on distinct vesicular profiles on the TGN membrane (Aroeti *et al.*, 1998; Ikonen *et al.*, 1996). In addition, some apical membrane proteins can be delivered first from the TGN to the basolateral surface and from there to the apical surface (Matter *et al.*, 1994; Mostov *et al.*, 1995). Both the tyrosine-based and the di-leucine-like motifs (Casanova, 1992; Matter *et al.*, 1992), as well as another sequence, H/R-X-X-V (Aroeti *et al.*, 1998; Distel *et al.*, 1998), have been shown to function as basolateral targeting signals. Meanwhile, two signals have been proposed for apical sorting, GPI-anchor and N-glycosylation. These signals are probably involved in

association with the glycolipid rafts in the apical domain although the mechanism by which these signals modulate the apical sorting is not clear (Simons and Ikonen, 1997; Weimbs *et al.*, 1997).

Neurons are also polarized cells with axons and soma/dendrites. These two specialized membrane surfaces are believed to correspond to apical and basolateral membranes respectively in epithelial cells. Thus, neuronal cells may use similar sorting signals in transporting from the TGN to the cell surface (Bredt, 1998; Jareb and Banker, 1998). An equivalent transcytotic pathway also appears to be present in neurons (Bonzelius *et al.*, 1994; Ikonen *et al.*, 1993).

1.2.2 Endosome/lysosome sorting pathway

In the TGN, lysosomal hydrolases use phosphorylated mannose residues, a modification occurring in the Golgi stacks, to bind to MPRs (Campbell and Rome, 1983; Geuze *et al.*, 1985). The protein/receptor complexes are then incorporated into the AP-1/clathrin-coated vesicles delivered to the early endosomes and subsequently transported to the late endosomes/prelysosomes. From there, MPR uncouples from lysosomal enzymes and recycles back to the TGN whereas the lysosomal hydrolyses are targeted to the lysosomes (Traub, 1997). In addition, lysosomal hydrolases are also occasionally exported to the cell surface where they are recognized by the cell surface MPRs and retrieved back to the lysosomes through the receptor mediated endocytosis.

In mammalian cells, two MPRs have been characterized: cation-independent (CI) (Lobel *et al.*, 1987; Lobel *et al.*, 1988) and cation-dependent (CD) MPRs (Dahms *et al.*, 1987) based on the requirement of divalent cations in their binding to mannose-6-phosphate. Both MPRs are type I membrane proteins with the N-terminal ligand-binding domains

exposed to the lumen or the cell surface (Dahms and Kornfeld, 1989; Dahms *et al.*, 1989). CI-MPR has three ligand binding domains where as CD-MPR has only one. The binding between MPRs and their ligands is sensitive to pH, which is compatible with the assumption that ligands are bound by both receptors in the TGN or by CI-MPR at the PM, and that dissociation occurs in the acidic endosomal compartments (Hoflack *et al.*, 1987; Tong *et al.*, 1989). Both receptors have multiple functions in transport of lysosomal hydrolyses. First, both receptors are involved in sorting of newly synthesized lysosomal hydrolyses from the TGN (Neufeld, 1991) although CI-MPR may be more efficient than CD-MPR (Johnson and Kornfeld, 1992a)). Second, CD-MPR is involved in the secretion of newly synthesized lysosomal hydrolyses (Chao *et al.*, 1990). Third, CI-MPR is necessary for the internalization of exogenous lysosomal hydrolyses from the cell surface (Stein *et al.*, 1987). In general, CI-MPR may recapture endogenous newly synthesized proteins that have escaped endosome sorting or been actively exported by CD-MPR (Chao *et al.*, 1990; O'Brien *et al.*, 1992).

To fulfill their functions, both MPRs distribute into different subcellular compartments. They are primarily concentrated in the TGN with certain populations on the cell surface and the endosomal compartments (Klumperman *et al.*, 1993), and cycle among these compartments. The cds of both MPRs are necessary and sufficient for the intracellular transport. Several sorting determinants have been identified on the MPRs that mediate transport of the receptors (Table 2). From the cell surface, internalization is mediated by the tyrosine-based motifs of both MPRs and a phenylalanine-containing sequence of CD-MPR (Canfield *et al.*, 1991; Jadot *et al.*, 1992; Johnson *et al.*, 1990; Lobel *et al.*, 1989). These signals likely interact directly with AP-2 complex (Glickman *et al.*, 1989; Sosa *et al.*, 1993). However, the signals have not been characterized for TGN budding. Functional assays show that the di-leucine-like motifs of both MPRs and the tyrosine-

based motif of CI-MPR are important for efficient lysosomal hydrolase delivery (Johnson and Kornfeld, 1992a; Johnson and Kornfeld, 1992b). These sequences may represent the binding sites for AP-1 complex (Le Borgne *et al.*, 1996).

Additional factors have been shown to contribute to the trafficking of the MPRs. In both MPRs, the di-leucine-like motifs are flanked by casein kinase 2 (CK2) sites that are phosphorylated *in vivo* (Hemer *et al.*, 1993; Meresse and Hoflack, 1993; Meresse *et al.*, 1990). Mutational analyses on the CI-MPR have shown the CK2 site (Chen *et al.*, 1993) and an aspartic acid residue adjacent to the phosphorylatable serine (Chen *et al.*, 1997a) contribute to efficient delivery of lysosomal hydrolyses. Recently, a novel 47kD protein named TIP47 has also been identified as a binding partner for both MPRs (Diaz and Pfeffer, 1998). A phenylalanine/tryptophan motif on the CD-MPR cd is recognized by TIP47 and mediates the transport of the receptor from the late endosomes to the TGN (Diaz and Pfeffer, 1998). In addition, CD-MPR is transiently and reversibly palmitoylated on cysteine residues on its cd (Schweizer *et al.*, 1996), which is also important for sorting lysosome enzymes (e.g. cathepsin D) from the TGN (Schweizer *et al.*, 1996). An 11 amino acids of juxtamembrane domain is necessary for basolateral sorting of CD-MPR in polarized MDCK cells, which is distinct from the basolateral sorting mediated by the tyrosine-based or the di-leucine-like motifs (Bresciani *et al.*, 1997).

Other lysosomal proteins, such as lysosome-associated membrane proteins (LAMPs) (Honing and Hunziker, 1995; Ogata and Fukuda, 1994; Schweizer *et al.*, 1990), lysosomal integral membrane proteins (LIMPs) (Vega *et al.*, 1991), and lysosomal acid phosphatase (LAP) (Braun *et al.*, 1989; Peters *et al.*, 1990), can also be sorted from the TGN to the lysosomes although the transport pathway is not clearly characterized.

Evidence in yeast show that alkaline phosphatase (ALP) is directly transported from the TGN to the yeast vacuole, a compartment similar to the lysosomes in mammalian cells (Cowles *et al.*, 1997). This sorting is mediated by di-leucine-like motifs and the yeast AP-3 complex, which is different from the vacuole protein sorting (VPS) mediated by AP-1 complex (Cowles *et al.*, 1997). Studies in mammalian cells show that both LIMPII and LAMP1 are mislocalized to the PM instead of the lysosomes by reduction of AP-3 levels with antisense techniques. Thus, a direct transport from the TGN to the lysosomes, which is mediated by AP-3, may exist in mammalian cells (Honing *et al.*, 1998; Le Borgne *et al.*, 1998). Moreover, a di-leucine-like motif has been identified on both LIMP II and tyrosinase that is necessary for their sorting from the TGN to lysosomes (Sandoval *et al.*, 1994). The di-leucine-like motifs selectively interact with AP-3 in vitro, supporting the AP-3-meidated lysosome-sorting directly from the TGN (Honing *et al.*, 1998).

1.2.3. Regulated secretory pathway

In endocrine and neuroendocrine cells, special granules termed mature secretory granules (MSGs) store peptide hormones at very high concentration in the regulated secretory pathway. The proteins in the MSGs can be mobilized for exocytosis in response to external stimulation (Mellman, 1992). Along the pathway, MSGs are originally derived from the TGN as TGN-associated condensing vacuoles. These vacuoles, which are also described as ISGs, are enriched with regulated secretory cargo molecules (Tooze and Tooze, 1986). While the granular proteins are condensed and concentrated during granule maturation, non-granular proteins are excluded. However, some TGN proteins, such as furin and MPRs but not TGN38/41, can be delivered into the intermediate compartment ISGs and retrieved out the regulated pathway (Dittie *et al.*, 1997; Klumperman *et al.*, 1998; Orci *et al.*, 1987b; von Zastrow and Castle, 1987). Despite the distinct morphological characteristics, the ISGs are engaged in a number of activities similar to

those of the TGN, such as sulfation, proteolytic processing, and H^+-ATPase activity (Barr, 1991; Blair *et al.*, 1991). Thus, the ISG might be best regarded as an extension of specialized TGN-like compartments with additional roles in processing and condensing secretory proteins for storage. However, the content of the ISGs can be stimulated to undergo regulated exocytosis (Arvan and Chang, 1987; Tooze, 1991), a feature in common with the MSGs, but not with the TGN.

Although whether the sorting of granular proteins is an active process is controversial, protein aggregation plays a major role in the regulated sorting pathway. Since different aggregation conditions have been reported for secretory granule proteins in the same cells, the process of aggregation of each hormone is actively controlled (Kuliawat and Arvan, 1994). To date, two models have been proposed for the regulated pathway sorting: sorting for entry and sorting for retention (Tooze, 1998).

The "sorting for entry" model proposes that the sorting of the secretory proteins occurs during initial granule formation with the selection of the appropriate cargo and the exclusion of non-secretory granular proteins in the TGN (Tooze, 1998). Signals have been identified on different cargo molecules for sorting into the regulated pathway. For instance, a N-terminal 26 amino acids of the prohormone pro-opiomelanocortin (POMC) are necessary for sorting POMC to the regulated secretory pathway (Cool and Loh, 1994). The tyrosine-based motif on the P-selectin cd has also been shown necessary for the appearance of the protein in the ISGs and MSGs (Blagoveshchenskaya *et al.*, 1999). Moreover, membrane-associated carboxypeptidase E (CPE) has been identified as a sorting receptor for soluble cargos in secretory granule (Cool *et al.*, 1997). CPE specifically binds the regulated secretory proteins, including prohormones, but not other secreted proteins (Cool and Loh, 1998). Deficiency of CPE function by antisense causes

the mislocalization of multiple prohormones in the secretory pathway (Normant and Loh, 1998). In addition, a group of proteins called granins were able to promote efficient sorting of prohormone, by acting as "helper" proteins in the aggregation of the secretory cargo (Chanat *et al.*, 1991; Huttner *et al.*, 1991; Natori and Huttner, 1996). Together, all these data support the selective sorting of granular proteins in the regulatory pathway.

The "sorting for retention" model proposes that protein selection may occur after secretory granule formation. Secretory granule-specific components are probably retained by aggregation while non-secretory granule molecules are selectively removed by the vesicles budded from the ISGs during granule maturation. This model is supported by studies showing that non-granule proteins (e.g. furin and MPR) are present in the ISGs but are subsequently removed from the ISGs by selective sorting (Dittie *et al.*, 1997; Kuliawat *et al.*, 1997). In agreement with these sorting, AP-1/clathrin coats have been observed on the ISG membranes and are involved in the retrieval of MPRs from the ISGs (Klumperman *et al.*, 1998; Orci *et al.*, 1987a; Tooze and Tooze, 1986). In addition, the AP-1/clathrin is also thought to play a role in the ISG constitutive-like secretion with very slow kinetics comparing to the normal constitutive secretion in fibroblast cells (Kuliawat and Arvan, 1992; Tooze, 1991). Together, the sorting activities in the ISGs support the "sorting for retention" model.

1.3 Endocytosis and endosomal sorting

1.3.1 Endocytic pathway

Along the endocytic pathway, proteins are internalized from the PM into the early sorting endosomes, which are vesicular and tubular structures located at peripheral region of cells (Figure 1). In the early endosomes, some receptors (e.g. Tfr) uncouple from their ligands upon acidification in the vesicular lumen, and recycle back to the cell surface via

the early recycling endosomes (Dautry-Varsat, 1986; Mellman, 1992). Other proteins, including some receptor/ligand complexes (e.g. epidermal growth factor (EGF) and EGF receptor), are transported to the late endosomes, which are multi-vesicular structures located near perinuclear region. From there, the ligand/receptor complexes are delivered to lysosomes for degradation (Gill *et al.*, 1991; Wells, 1999).

Proteins can also be transported from the endosomes to the TGN (Figure 1). MPRs direct delivery of lysosomal hydrolyses from the cell surface. After the receptor/ligand complexes are transported from the early endosomes to the late endosomes, MPRs are uncoupled from the ligands and delivered to the TGN while the ligands are transported to the lysosomes (Le Borgne and Hoflack, 1998b). Furin, a TGN endoprotease that recycles between the TGN and the cell surface has also been shown to follow the same pathway from the cell surface to the TGN (Mallard *et al.*, 1998; Mallet and Maxfield, 1999). However, the TGN marker protein TGN38/41 takes a different pathway than those of furin and MPRs. After internalization, TGN38/41 is delivered from the early endosomes directly to the TGN (Mallard *et al.*, 1998; Mallet and Maxfield, 1999).

1.3.2 Endocytic sorting machinery

The endocytosis of proteins from the cell surface is mediated by the internalization signals on their cds and the AP-2 dependent clathrin-coated pits (CCP) (Le Borgne and Hoflack, 1998b; Marks *et al.*, 1997; Robinson *et al.*, 1996). For instance, mutation analyses on the Tfr and low-density-lipoprotein receptor (LDLr) have revealed the first type of internalization motifs: tyrosine-based motifs YXXΦ and NPXY (where X could be any residue and Φ is a hydrophobic residue), which are critical for the endocytosis of Tfr and LDLr respectively (Trowbridge and Collawn, 1993). Since then, the tyrosine-based motifs have been identified on many other proteins such as EGF receptor,

TGN38/41, furin, and MPRs (Marks *et al.*, 1997). A second type of internalization signals are the di-leucine-like motifs (Table 2), which have been identified on the CD3γ receptor and other cell surface proteins (Le Borgne and Hoflack, 1998b; Letourneur and Klausner, 1992). However, many proteins, such as furin and MPRs, contain multiple internalization signals, though the tyrosine-based motif is usually the major contributor for endocytosis (Marks *et al.*, 1997).

The internalization mediated by either the tyrosine-based or the di-leucine-like motifs are distinct saturable processes (Warren and Enns, 1997; Warren *et al.*, 1997). Consistent with these observations, overexpression of one to the level of saturation does not affect the incorporation into the CCPs of the other (Marks *et al.*, 1996). These data suggests that the internalization mediated by different sorting motifs use distinct sorting machinery. In addition, the internalized vesicles containing Tfr represent distinctly different populations from those containing β2-adrenergic receptor (Cao *et al.*, 1998). Thus, different populations of CCPs and/or vesicles may exist for the internalization of different cargo proteins.

The transport from the early sorting endosomes to early recycling endosomes, such as the recycling of Tfr and many other proteins, is believed to be a default pathway. However, studies show that the recycling of furin to the cell surface is mediated by an acidic cluster on the cd of the protein, suggesting an active sorting event (Molloy *et al.*, 1998). The transport from the early sorting endosomes to late endosomes requires the COPI-coat proteins although other necessary components for this sorting are not clear (Aniento *et al.*, 1996; Daro *et al.*, 1997; Gu *et al.*, 1997; Whitney *et al.*, 1995). A vesicular portion of the early endosomes is believed to give rise to multi-vesicular structures, which will act as intermediate compartments that transport materials to the late endosomes (Gruenberg

and Kreis, 1995). Moreover, the fact that only proteins destined for degradation or delivery to the TGN are transported to the late endosomes suggests that an active sorting mechanism is involved in this process.

The delivery of contents from the late endosomes to the lysosomes is not well understood. This event is proposed to occur by fusion of the late endosomes with the lysosomes to produce a 'hybrid' organelle from where the lysosomes are derived (Mullock *et al.*, 1998; Mullock *et al.*, 1994). *In vitro* studies show that the sucrose in lysosomes is accessible to immobilized invertase on the late endosomes, suggesting a retrograde traffic of soluble material between the late endosomes and the lysosomes (Jahraus *et al.*, 1994).

1.4 TGN/endosome sorting signals

1.4.1 Tyrosine based motifs and Di-leucine-like motifs

Tyrosine-based motifs are involved in many protein sorting events (Table 2), including internalization from the cell surface (Section 1.3.2), the sorting from the TGN to endosomes (Section 1.2.2), and the basolateral targeting in polarized cells (Section 1.2.1). Key residues in a tyrosine-based motifs (YXXΦ) are the tyrosine and the hydrophobic amino acid. The critical tyrosine must be nonphosphorylated for the signal to be active (Boll *et al.*, 1996; Marks *et al.*, 1997; Ohno *et al.*, 1996; Stephens and Banting, 1997). Meanwhile, the flanking residues can be important for the function of the signals, i.e. PGYRHV on the LAP cd (Lehmann *et al.*, 1992). In addition, the position of a motif on the cytoplasmic sequence is also important for its function (Boge *et al.*, 1998; Ohno *et al.*, 1997; Rohrer *et al.*, 1996). In general, most tyrosine-based motifs are capable of mediating rapid internalization from the PM into endosomes, whereas only a subset of these signals (i.e. YRSV of MPR) have been shown to direct TGN sorting.

Tyrosine-based motifs bind to μ chains of AP complexes, which recruit the membrane proteins into the AP/clathrin sorting machinery. The first interaction has been shown between the TGN38 tyrosine-based motif containing sequences -SDYQRL- and μ2 subunit of AP-2 complex with the yeast two-hybrid system (Ohno *et al.*, 1995). Subsequently, most identified tyrosine-based motifs have been shown to bind to one or multiple of identified μ chains of AP complexes. The tyrosine residues are essential for binding and can not be substituted even by the structurally related phenyalalanine (Canfield *et al.*, 1991; Ohno *et al.*, 1996; Shiratori *et al.*, 1997). Leucine is the preferred residue at the hydrophobic position, although isoleucine, phenylalanine, methionine and to a lesser extent, valine, are all tolerated (Ohno *et al.*, 1996; Ohno *et al.*, 1995). Specific residues between the tyrosine and the hydrophobic residue contribute to additional contact points. For instance, structural studies show that the side chain of arginine is preferred on the second X position in YXXΦ for hydrophobic interaction with μ2 (Owen and Evans, 1998). Moreover, non-polar and acidic residues are preferred by μ1 and μ3 respectively on the residues by the tyrosine residue. Although the functional significance of the μ1 preference is not clear, the preference by μ3 suggests a role in lysosomal targeting since the tyrosine-based signals of several proteins (CD63, lamp-2a and GMP-17) targeted to lysosomes and lysosome-related organelles contain acidic residues at position adjacent to the tyrosine residue (Bonifacino and Dell'Angelica, 1999).

Another major sorting signal in the TGN/endosomal system is di-leucine-like motifs (Table 2). Di-leucine-like motifs have been shown to mediate internalization from the cell surface and lysosomal sorting from the TGN (Letourneur and Klausner, 1992; Ogata and Fukuda, 1994; Sandoval *et al.*, 1994). In addition, di-leucine-like motif is also

involved in basolateral sorting of the macrophage Fc receptor in the polarized MDCK cells (Hunziker and Fumey, 1994). In some cases, one of the two leucines in a motif may be replaced by isoleucine, valine or methionine (Le Borgne and Hoflack, 1998a). In addition, many di-leucine-like motifs require upstream acidic residues and phosphorylatable residues for their function (Le Borgne and Hoflack, 1998a). For example, CD3γ receptor uses SDXXXLI as an internalization signal, and the phosphorylation of a serine residue and the aspartic acid are both important for the function of the signal (Dietrich *et al.*, 1994; Dietrich *et al.*, 1997). The MHC class II-associated invariant chain contains a sequence DDXXXLI, which is similar to the AP-3 binding motifs of limp-II and tyrosinase, also functions as lysosome-sorting signal (Honing *et al.*, 1998).

In vitro binding assays show that di-leucine-like motifs can be recognized by both β1 and β2 adaptins, by which the cargo molecules are recruited into the AP/clathrin coated vesicles. In addition, using phage display, a conserved region in μ chains was found to interact weakly with the di-leucine-like motif on the invariant chain although the physiological relevance of this interaction is still not clear (Bremnes *et al.*, 1998a; Bremnes *et al.*, 1998b).

Both tyrosine-based and di-leucine-like motifs can selectively bind different adaptor complexes. The interactions can be further complexed by the local environment in cells. Incorporation of adaptors into a clathrin coat increases the affinity of AP-2 for the tyrosine-based motifs on TGN38 by approximately 10 fold, judged by cross-linking efficiency (Rapoport *et al.*, 1997). Using the same techniques, incorporation of adaptors into a clathrin coat does not influence the affinity of AP-1 for di-leucine-like motifs (Rapoport *et al.*, 1998). Meanwhile, addition of phosphatidylinositol 3,4-biphosphate

(PIP_2) resulted in a decreased affinity of AP-1 for the di-leucine-like motif containing CD3γ peptide, but an enhanced affinity for the tyrosine-based motif containing lamp-1 tail (Rapoport *et al.*, 1998). All these data suggest that local environment, such as vesicle formation and production of selective PtdIns, may thus significantly alter the sorting properties of coats.

1.4.2 Acidic clusters

Acidic cluster (AC) motifs, consisting of a cluster of acidic residues, was first defined on the furin cd (Jones *et al.*, 1995; Schafer *et al.*, 1995; Voorhees *et al.*, 1995). The furin AC motif contains two CK2 phosphorylated serines, which are necessary for the TGN localization of the proteases (Dittie *et al.*, 1997; Jones *et al.*, 1995; Takahashi *et al.*, 1995). A similar AC motif has also been recently identified on varicella-zoster virus envelope glycoprotein gE (VZV gE) and functions as a TGN localization signal as well (Molloy *et al.*, 1999; Zhu *et al.*, 1996). A literature search shows that similar AC motifs are also present on many other TGN/endosomal proteins, and most of them contain either bona fide or potential CK2 phosphorylation site(s) (Table 3). The function of many AC motifs are regulated by CK2 phosphorylation (Albini *et al.*, 1995; Dittie *et al.*, 1997; Jones *et al.*, 1995; Korner *et al.*, 1994; Meresse and Hoflack, 1993; Meresse *et al.*, 1990; Takahashi *et al.*, 1995; Zhu *et al.*, 1996). The phosphorylation probably modifies the conformation of protein structure and the accessibility of the sorting determinants for adaptor complexes (Teuchert *et al.*, 1999b). Evidence shows that many AC motifs are functionally important for protein sorting. Therefore, AC motif-mediated sorting may represent a common mechanism in the TGN/endosomal system and (Molloy *et al.*, 1999; Voorhees *et al.*, 1995).

In addition to directing TGN targeting, the acidic amino-acid containing motifs also appear to mediate other intracellular sorting such as ER export (i.e. E/DXE/D of LDL receptor) (Bannykh and Balch, 1998), basolateral targeting (i.e. EEDE of LDL receptor and EEDE of furin) (Matter *et al.*, 1994; Simmen *et al.*, 1999), lysosome targeting (i.e. DXXXLI of CD3γ) (Le Borgne and Hoflack, 1998b), early to late endosome transport (i.e. EE of HIV nef) (Piguet *et al.*, 1999). Despite the diversified roles of acidic residues in different protein transport steps, the mechanism of how these acidic residues are involved in protein intracellular sorting is unknown.

Table 3. ***Acidic clusters on membrane proteins.***

Shown are acidic clusters (AC) of various membrane proteins whose sorting might be regulated by PACS-1 and/or CK2/PP2A signaling pathways. Included are several proprotein convertases, as well as carboxpeptidase D (CPD), polymeric immunoglobulin receptor (pIgR), synaptotagmin, human cytomegalovirus (HCMV) gB, herpes simplex virus-1 (HSV-1) gE, pseudorabies virus gE, and varicella zoster virus (VZV) gE. PACS-1 binds to the phosphorylated VZV gE-cd and to the CI-MPR cd. Trafficking of HCMV gB is sensitive to tautamycine, suggesting a potential role for PP2A in herpes virus assembly (Fish *et al.*, 1998). Known phosphorylated residues are depicted by a solid circle, whereas potential CK2 sites are depicted by an open circle. Reference: [a] (Jones *et al.*, 1995); [b] (Nakagawa *et al.*, 1993b); [c] (Bruzzaniti *et al.*, 1996b; Meerabux *et al.*, 1996; Seidah *et al.*, 1996b); [d] (Xin *et al.*, 1997); [e] (Stoffers *et al.*, 1991); [f] (Petersen *et al.*, 1997); [g] (Meresse and Hoflack, 1993); [h] (Korner *et al.*, 1994); [i] (Okamoto *et al.*, 1994); [j] (Bennett *et al.*, 1993); [k] (Norais *et al.*, 1996); [l] (Yao *et al.*, 1993); [m] (Edson *et al.*, 1987); [n] (Edson, 1993) (This table is adapted from the review of (Molloy *et al.*, 1999).

1.5 AP/clathrin mediated TGN/endosomes sorting

1.5.1 AP-2

The AP-2 complex (Table 1) recruits cell surface cargo molecules into CCPs by directly binding the sorting motifs on the cds of the proteins. The μ2 subunit of AP-2, for example, recognizes the tyrosine-based motif (YXXΦ) (Le Borgne and Hoflack, 1998a).

Table 3. Acidic clusters on membrane proteins

Protein	AC Sequence	Ref.
Furin	...Q$_{768}$EECPSDSEEDEGRG...	a
PC6B	...D$_{1830}$RDYDEDDEDD.....D$_{1860}$EAEDDELEYDDE...	b
PC7/8	...S$_{727}$SKDPDEVETESRG...	c
CPD	...S$_{1359}$HEFQDETDTEEET...	d
PAM	...Q$_{951}$EKEDDGTESEEEYSAP...	e
Sortilin	...H$_{824}$DDSDEDLLE...	f
CI-MPR	...G$_{2416}$DEQDSEDEVLT.....H$_{2489}$DDSDEDLL...	g
CD-MPR	...Y$_{256}$RGVGDDQLGEESEERDDHL...	h
PIgR	...K$_{723}$RSSKEEADEAFT...	i
Synptgm.	...D$_{120}$DDAETGLTDGEEK...	j
HCMV gB	...L$_{897}$KDSDEEENV	k
VZV gE	...F$_{590}$EDSESTDTEEEF...	l
HSV-1 gE	...D$_{474}$WSSDSEGERDQ...	m
PrV gE	...D$_{489}$GDDDDEEAGD.....E$_{523}$DEFSSDEDDGL...	n

Mutations on μ2, which cannot bind tyrosine-based motifs, block the Tfr internalization (Nesterov *et al.*, 1999). Structural analyses have established that the C-terminal two-thirds of μ2 are involved in the interaction with the tyrosine-based signal (Aguilar *et al.*, 1997). A YXXΦ motif recognizes a region of μ2 containing a binding pocket for both Y and Φ residues (Owen and Evans, 1998). Sequence alignments indicate that most of the residues directly involved in the interaction with the Y and Φ residues are conserved in all μ chains. Indeed, μ1, μ3 and μ4, as well as μ chains from non-mammalian organisms, have all been shown to interact with the YXXΦ signals by the yeast two-hybrid system, albeit with lower affinity compared with μ2 (Dell'Angelica *et al.*, 1999a; Dell'Angelica *et al.*, 1997a; Ohno *et al.*, 1997; Ohno *et al.*, 1996; Ohno *et al.*, 1995; Stephens and Banting, 1998). One exception is the tyrosine-based motif of Tfr, which interacts with μ2, but not with μ1 and μ3 (Ohno *et al.*, 1997; Ohno *et al.*, 1996), suggesting selectivity for a tyrosine-based motif in specific sorting steps. Alternatively, proteins can also be recruited to the clathrin coat through a low affinity interaction between di-leucine-like motifs and β2 adaptin although the significance of such weak interactions is not yet clear (Rapoport *et al.*, 1998).

The ear domains of α-adaptin binds to many other clathrin-associated proteins such Eps15, Epsin, amphiphysin, dynamin, AP-180 and auxilin (Owen *et al.*, 1998; Traub *et al.*, 1999; Wang *et al.*, 1995). All these AP-2 binding partners have been shown to form a larger complex required for clathrin-coat assembly on the PM (Slepnev *et al.*, 1998). The structural analyses have shown that the ear domain of α-adaptin has a solvent-exposed hydrophobic pocket on the C-terminal platform subdomain (Traub *et al.*, 1999). A single residue, W840, is critical for interaction with Eps15 and other AP-2 binding partners (Owen *et al.*, 1998).

Eps15 has been originally identified as a substrate for the kinase activity of the epidermal growth factor receptor (EGFR) (Fazioli *et al.*, 1993). Eps15 has a tripartite structure comprising a N-terminal portion, which contains three EH domains, a central putative coiled-coil region, and C-terminal domain containing multiple copies of the amino acid triplet DPF (Fazioli *et al.*, 1993; Mayer, 1999; Wong *et al.*, 1995). A pool of Eps15 is localized at CCPs where the C-terminal DPF domain interacts with the AP-2 complex and its N-terminal EH domain interacts with another AP-2 binding protein, Epsin (Chen *et al.*, 1999). Perturbation of Eps15 and Epsin function inhibits receptor-mediated endocytosis of EGF and transferrin (Benmerah *et al.*, 1998), demonstrating that both proteins are components of the endocytic machinery. In addition, the Eps15 EH domain binds to many proteins implicated in endocytosis, including CALM and nonneuronal isoforms of synaptojanin (Chen *et al.*, 1999; Marsh and McMahon, 1999). Homologues of the Eps15 EH domain have also been identified on many proteins involved in endocytosis (Kay *et al.*, 1999). Therefore, all these proteins could interact with each other to form protein networks, indicating the complexity of clathrin-cage assembly.

AP-180 was identified from the isolated clathrin-coated vesicles from brain (Ahle and Ungewickell, 1986). The C-terminal of AP-180 is required to bind to the β-propeller of the clathrin heavy chain (Owen *et al.*, 1999; Traub *et al.*, 1999). Moreover, *in vitro* assays show that AP-2 and AP-180 associate with each other with high affinity and promote clathrin-coat assembly (Hao *et al.*, 1999). Recent *in vivo* studies have demonstrated that AP-180 is required not only for clathrin assembly but also for determining the vesicle size (Morgan *et al.*, 1999; Zhang *et al.*, 1998).

After the assembly of clathrin-coated vesicles, a cytoplasmic GTPase, dynamin, must be recruited for the scission of the CCPs from the donor membrane (Sweitzer and Hinshaw,

1998). Amphiphysin, a cytosolic protein that can simultaneously bind α-adaptin and dynamin (Wigge *et al.*, 1997a; Wigge *et al.*, 1997b), has been implicated in the recruitment of dynamin to the CCPs. Amphiphysin also recruits synaptojanin, a phosphatase that hydrolyzes inositol phospholipids such as PIP2, to the CCPs on the PM (de Heuvel *et al.*, 1997). Together, these proteins coordinate with each other to regulate the clathrin-coat formation.

Once formed, the clathrin coat is relatively stable and the disassembly of the clathrin coat required an uncoating ATPase, Hsc70. The ATPase has been shown to release clathrin from the coated vesicles and presumably act through clathrin light chain (Schmid *et al.*, 1984). Auxilin, a 100kD DNA-J related protein, acts as co-factor for Hsc70 by targeting the Hsc70 to the clathrin coat and promotes the ATPase activity of (Ungewickell *et al.*, 1997). The uncoated vesicles are allowed to fuse with early endosomes.

In summary, many cytosolic factors are involved in the AP-2/clathrin-mediated internalization. These proteins can interact with each other to form a big complex, which are regulated by phosphorylation (Chen *et al.*, 1999; Slepnev *et al.*, 1998). Although the mechanism is still not clear, the protein network by the protein-protein interactions is critical in regulating the clathrin-coat vesicle formation.

1.5.2 AP-1

AP-1 is involved in endosome/lysosome sorting from the TGN (Table 1). *In vitro* studies show that AP-1 recognizes both the tyrosine-based motif of CI-MPR and the di-leucine-like motifs of invariant chain Ii and MPRs, which are functional endosome-sorting signals (Bakke and Dobberstein, 1990; Johnson and Kornfeld, 1992a; Johnson and Kornfeld, 1992b; Marks *et al.*, 1995; Salamero *et al.*, 1990). *In vivo* studies show that

mice with mutant γ-adaptin in AP-1 complex die during the embryonic development, revealing that AP-1 is essential for viability (Zizioli *et al.*, 1999). The missorting of some functionally important proteins by AP-1 may be the cause of developmental abnormalities resulting in death.

A pool of AP-1 associates with the TGN membrane, and the membrane recruitment of AP-1 is regulated by the GTPase ARF1. Therefore, the AP-1-mediated sorting can be blocked by BFA. Electron microscopy studies show that the TGN AP-1/clathrin-coated vesicles contain MPRs, the best characterized cargo following the transport from the TGN to endosomes. In the MPR-deficient cells, the association of AP-1 with membrane is reduced three times compared to that in normal cells, and can be restored by transfection of MPR cDNA (Le Borgne and Hoflack, 1997; Zhu *et al.*, 1999). Moreover, two other cargo molecules, VZV gE and class II major histocompatibility complexes, could also increase the membrane association of AP-1 when overexpressed (Alconada *et al.*, 1996; Salamero *et al.*, 1996). Together, these data demonstrate the importance of cargo in clathrin/AP-1 coat assembly.

A γ-adaptin homologue, γ2-adaptin, has been identified recently. This adaptin has been found on different populations of clathrin coated vesicles on the TGN membrane than γ adaptin, suggesting different roles of γ– and γ2-adaptins in the TGN sorting (Lewin *et al.*, 1998; Takatsu *et al.*, 1998). Moreover, an epithelial specific μ1B has been identified through the EST database search and subsequently isolated from a library screen (Ohno *et al.*, 1999). This μ chain shares 50% identity with the previously reported μ1 chain (hereafter μ1A) and recognizes tyrosine motifs on cargo proteins (Ohno *et al.*, 1999). Kidney epithelial LLC-PK1 cells that lacks the μ1B chain missort many basolateral

proteins onto the apical surface. These defects can be recovered by introducing the μ1B into the LLC-PK1 cells (Folsch *et al.*, 1999).

Like α-adaptin in the AP-2 complex, γ-adaptin is considered to interact with many clathrin-associated proteins. Two novel proteins, γ-synergin and p34, have been identified that interact with the ear domain of γ-adaptin. Immunofluoresence staining shows that γ-synergin displays an extended perinuclear localization similar to that of γ-adaptin (Page *et al.*, 1999). Moreover, γ-synergin also bears EH domains homologous to those identified on Eps15 and related proteins. Therefore, γ-synergin may play a similar role in the AP-1/clathrin-dependent coat assembly on the TGN as those EH-domain containing proteins on the AP-2/clathrin-mediated endocytosis.

1.5.3 AP-3

Two mammalian AP-3 complexes have been isolated (Table 1), including an ubiquitously expressed AP-3 (δ, β3A, μ3A, σ3A) and a neuronal specific AP-3 (δ, β3B or βNAP, μ3B, σ3B) (Dell'Angelica *et al.*, 1997b; Simpson *et al.*, 1997). Both AP-3 complexes display perinuclear and endosomal staining in cells (Dell'Angelica *et al.*, 1997b; Simpson *et al.*, 1997). A conserved clathrin-binding site on β3 adaptins has been shown to interact with clathrin heavy chain *in vitro* and AP-3 display overlapping with clathrin on some endosomal membranes. However, whether or not AP-3 functional associates with clathrin coat *in vivo* is controversial (Dell'Angelica *et al.*, 1998; Dell'Angelica *et al.*, 1997a; Simpson *et al.*, 1997). Similar to AP-1, the AP-3-mediated sorting is also regulated by ARF1, and it is sensitive to BFA treatment (Ooi *et al.*, 1998).

Genetic evidence from yeast and metazoan cells suggest a role of AP-3 in biogenesis of lysosomes and lysosome-related compartments (i.e. mammalian melanosomes and the

yeast vacuoles) (Cowles *et al.*, 1997; Ooi *et al.*, 1997; Stepp *et al.*, 1997). In yeast, AP-3 deletion results in the mislocalization of the vacuolar proteins alkaline phosphatase and the t-SNARE Vamp3p, but not carboxypeptidase Y, which is transported by the classic VPS pathway (Golgi-endosomes-vacuoles) (Cowles *et al.*, 1997; Stepp *et al.*, 1997). This observation suggests that AP-3 maybe involved in a direct pathway from the post Golgi compartment to the vacuole. Whether the same pathway exists in mammalian cells is not yet clear.

The *Drosophila* mutant *garnet* lacks a gene highly homologous to the mammalian δ adaptins. The *garnet* flies have reduced pigment granules or lower levels of pigment in their eyes, suggesting AP-3 is involved in the melanosomal sorting (Ooi *et al.*, 1997). Mutations in mammalian AP-3 genes has also been isolated (Dell'Angelica *et al.*, 1999b; Feng *et al.*, 1999; Kantheti *et al.*, 1998; Zhen *et al.*, 1999). These animals show abnormal hair, eyes color, prolonged bleeding and reduced secretion of lysosomal enzymes in urine, presumably due to a defect in the biogenesis of lysosomes and related organelles such as melanosomes and platelet dense granules (Odorizzi *et al.*, 1998). In biochemical studies, AP-3 but not AP-1 and AP-2 recognizes the di-leucine-like motifs on LIMP-II and tyrosinase (Honing *et al.*, 1998). Moreover, inhibiting AP-3 function by antisense techniques results in targeting both Lamp1 and LIMP-II to the cell surface instead of the lysosomes (Le Borgne *et al.*, 1998). AP-3 therefore may mediate the same pathway in mammalian cells as in yeast, a direct transport from the TGN to lysosomes. However, biochemical studies also show that μ1 and μ2 weakly recognize the tyrosine-based signals on the lysosomal proteins, suggesting that lysosomal/melanosomal proteins can be correctly targeted with limited efficiency by alternative AP-1 and AP-2 dependent pathway (Honing *et al.*, 1996; Ohno *et al.*, 1996). Further studies are necessary to elucidate the different roles of AP-1 and AP-3 in the TGN to lysosomes transport.

In a cell-free assay, AP-3 has been involved in the synaptic vesicles budding from the endosomal compartments (Faundez *et al.*, 1998), suggesting that AP-3 might be involved in different sorting pathways in neuronal cells. However, the lack of AP-3 in mice shows normal vesicle populations in the nerve terminus but a loss of zinc in synaptic-like vesicles, suggesting that AP-3 is not essential for all synaptic vesicle formation but is involved in delivery of contents in the synaptic-like vesicles (Kantheti *et al.*, 1998). The loss of zinc is probably due to defect in the correct targeting the zinc transporters to the vesicles.

1.5.4 Connectors

A large number of proteins have been identified that associate with the clathrin coat pits through binding to clathrin or adaptor complexes (Marsh and McMahon, 1999; Molloy *et al.*, 1999). Some of these proteins comprise a small group of functionally related cytosolic proteins, including the HIV/SIV Nefs (Vincent *et al.*, 1997) and G-protein coupled receptor (GPCR) binding proteins, β-arrestins (Ferguson *et al.*, 1996). These proteins can function as connectors to recruit membrane proteins to the AP/clathrin sorting machinery.

GPCRs, a group of plasma membrane receptors, bind to extracellular signaling molecules to trigger the intracellular signaling pathways. β-arrestin has been initially identified GPCR-associated cytosolic factors. These cytosolic proteins play a dual role in regulating GPCR responsiveness by contributing to both receptor desensitization and internalization (Figure 3A and (Ferguson *et al.*, 1998)). In cell cultures, β-arrestin and the GPCR are co-localized with clathrin coats in response to agonist stimulation (Goodman *et al.*, 1996). Moreover, β-arrestin has a clathrin-binding motif similar to these on β-adaptins of AP

complexes, and binds to the β-propeller domain of the clathrin heavy chain (Dell'Angelica *et al.*, 1998; Goodman *et al.*, 1997; Krupnick *et al.*, 1997a; ter Haar *et al.*, 1998). Recently, β-arrestin has also been shown to interact with AP-2 complex (Laporte *et al.*, 1999). Functional assays show that overexpressed β-arrestin promotes the internalization of the receptors by acting as a monomeric adaptor to couple the cell surface GPCRs to the clathrin-coated vesicles (Ferguson *et al.*, 1998; Goodman *et al.*, 1996). Mutations disrupting the interactions between β-arrestins and GPCRs or clathrin functionally behave in a dominant negative manner. Overexpression of these mutants can block the internalization of the receptors (Krupnick *et al.*, 1997b; Orsini and Benovic, 1998; Zhang *et al.*, 1997). Mutational analyses show that the interaction between β-arrestin and AP-2 is also necessary for the internalization of GPCRs (Laporte *et al.*, 1999). Together, β-arrestin functions as a connector necessary to couple the GPCRs to the AP-2/clathrin coat for internalization.

The nef protein of HIV is a crucial factor in viral pathogenesis. This protein can promote the downregulation of the cell surface CD4, a host cell receptor for virus infection, and the cell surface MHC class I molecules (Figure 3B and (Garcia and Miller, 1991; Guy *et al.*, 1987)). Without nef expression, the CD4 uses a di-leucine-like motif in the cd to associate with the AP-2 clathrin-coat pits for internalization after activation by antigens (Weiss and Littman, 1994). However, upon nef expression, the nef N-terminal domain recognizes the CD4 di-leucine-like motif (Aiken *et al.*, 1994; Grzesiek *et al.*, 1996). Then, a di-leucine-like motif on the nef C-terminus mediates the CD4/nef complex association with β2 adaptin of AP-2 clathrin coat (Bresnahan *et al.*, 1998; Craig *et al.*, 1998; Foti *et al.*, 1997; Greenberg *et al.*, 1998). This Nef-dependent CD4 recruitment in AP-2/clathrin coat promotes a 5-10 fold increase in the rate of endocytosis of the receptor (Foti *et al.*, 1997; Piguet *et al.*, 1998).

After directing the internalization of CD4 into early endosomes, nef is involved in the subsequent targeting of CD4 from the early endosomes to the late endosomes/lysosomes. A diacidic motif on nef could be recognized by the β-COP subunit of the endosomal COPI coatomer. Thus nef acts as a connector between CD4 and the COPI coatomer complex, which is critical for transporting the CD4/nef complex from the early to the late endosomes. This diacidic motif mediated sorting also represents the first example of early to late endosome transport (Piguet *et al.*, 1999). Together with nef's role in the CD4 endocytosis, the protein functions as connector to couple CD4 to AP-2/clathrin and COPI coats for sequential transporting the receptor from the cell surface into lysosomes.

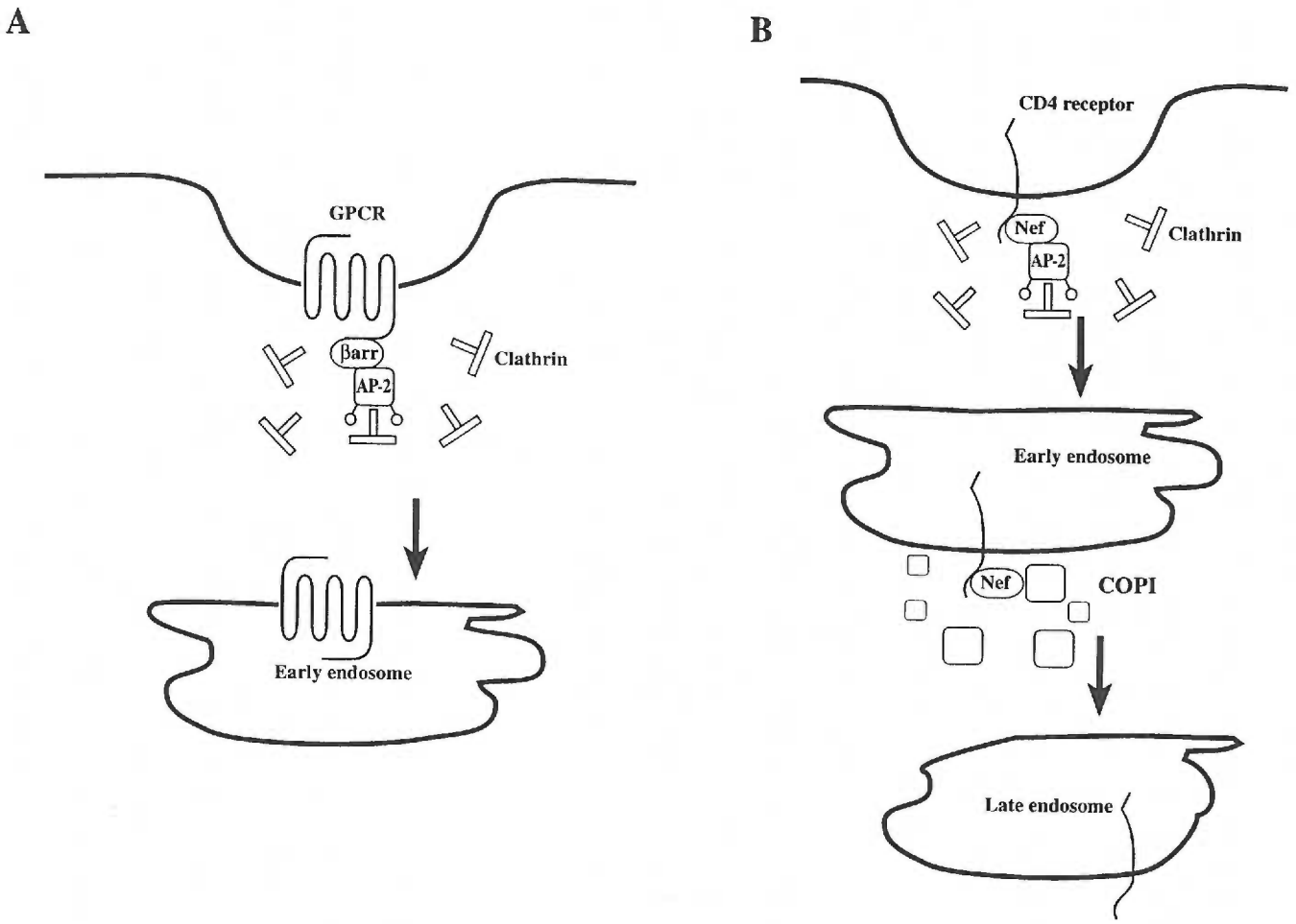
Figure 3.*Mechansim of Nef- and β-arrestin induced CD4 downregulation and GPCR internalization* (A) At the plasma membrane, β-arrestin acts as a connector between GPCR, and adaptor complexes and clathrin, thereby triggering the formation of GPCR-specific clathrin-coated pits for internalization. (B) At the plasma membrane, nef acts as a connector between the CD4 cytoplasmic domain and adaptor complexes, thereby triggering the formation of CD4-specific clathrin-coated pits. In the endosome, nef interacts instead with β-COP, diverting nef-bound CD4 molecules from a recycling to a degradation pathway.

1.6 Proprotein convertases

1.6.1 Members and structures of proprotein convertases

Proprotein convertases are a family of endoproteases that reside in the TGN, endosomes, and secretory granules, and that process proproteins to yield bioactive molecules (Figure 4). PCs are calcium-dependent serine endoproteases related to the bacterial subtilisins and the yeast processing protease Kex2p (Nakayama, 1997; Seidah *et al.*, 1998). Seven

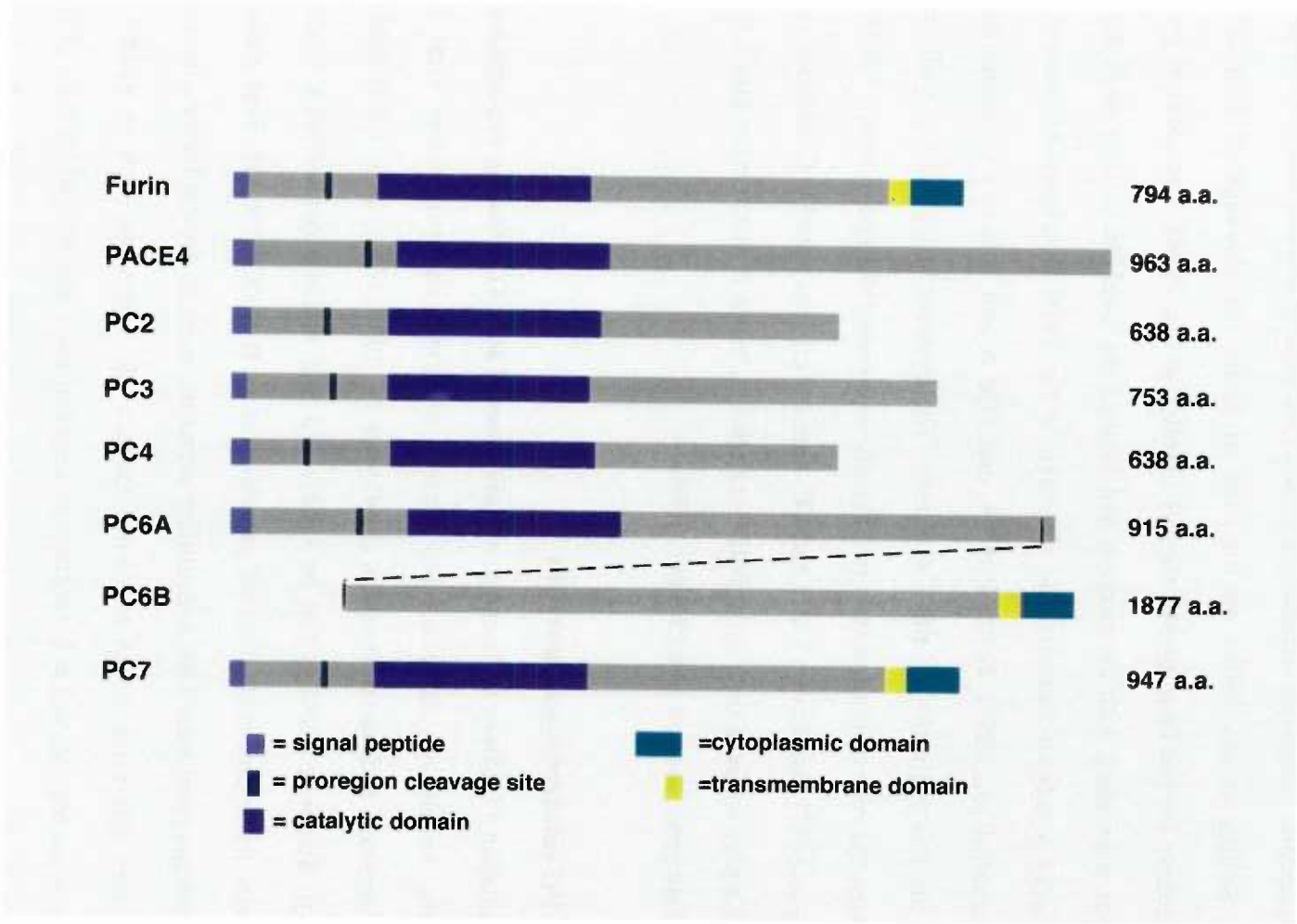
Figure 3 Mechanism of β-arrestin- and Nef-indeuced GPCR internalization and CD4 downredulation



members have been identified in mammalian cells: furin (Fuller *et al.*, 1989; Roebroek *et al.*, 1986a; Roebroek *et al.*, 1986b), PC1/3 (Seidah *et al.*, 1990; Seidah *et al.*, 1991; Smeekens *et al.*, 1991), PC2 (Smeekens and Steiner, 1990), PC4 (Nakayama *et al.*, 1992b; Seidah *et al.*, 1992b), PACE4 (Kiefer *et al.*, 1991), PC5/6 (then PC6) (Lusson *et al.*, 1993; Nakagawa *et al.*, 1993a; Nakagawa *et al.*, 1993b), and PC7/LPC/PC8 (then PC7) (Bruzzaniti *et al.*, 1996b; Meerabux *et al.*, 1996; Seidah *et al.*, 1996b). Some of these proteases exist in multiple isoforms due to alternative splicing, e.g. PC4A, B and C, (Seidah *et al.*, 1992b), seven PACE4 isoforms (Zhong *et al.*, 1996), and PC6A and B (De Bie *et al.*, 1996a). Mammalian PCs are synthesized on the ER-associated ribosomes, transported through the early secretory pathway, and become active in the TGN. Based on their topological structures in the secretory pathway, the PCs can be divided into two categories: secretory compartment lumenal proteins and type-I membrane proteins. The members in the latter group have TMDs and short C-terminal cds, which contain information for directing the localization and intracellular trafficking of these proteases (Le Borgne and Hoflack, 1998b; Molloy *et al.*, 1999). These proteases therefore represent model proteins for intracellular sorting in both the biosynthetic and the endocytic pathways.

Mammalian PCs are conserved in the N-terminal regions including propeptides, catalytic domains and P-domains, but differ in their C-terminal regions (Figure 4). The most conserved domain is the catalytic domain, which extends over 330 amino acids. In particular, the active-site residues of the aspartic acid, histidine and serine catalytic triad are tightly conserved (Bryan *et al.*, 1986). A downstream region following the catalytic domain, the P-domain, is required for folding and activity of the proteases (Seidah *et al.*, 1994). This domain influences both calcium and pH optima of the enzyme activity (Zhou *et al.*, 1998). Another conserved region is the propeptide, which is autocatalytically

Figure 4. Schematic Structure of the Proprotein Convertases



cleaved during maturation of the convertases (Jean *et al.*, 1993; Leduc *et al.*, 1992; Moehring *et al.*, 1993; Zhou and Lindberg, 1993). The propeptide is also proposed as an intramolecular chaperone required for proenzyme folding and activation (Creemers *et al.*, 1998; Seidah *et al.*, 1998). In the case of furin, the cleavage of propeptide is a prerequisite for the furin to exit the ER (Molloy *et al.*, 1994). The cleaved propeptide remains associated with the enzyme and inhibits the catalytic activity until the protein undergoes a second autocatalytic cleavage in the TGN to release the active enzyme (Anderson *et al.*, 1997). Furin, PACE4, and PC6 A and B have a cysteine-rich region from the P-domain toward the C-terminus. The cysteine topography is well conserved although the role of the residues is currently unknown (Nakagawa *et al.*, 1993b). Furin, PC6B and PC7 also have a TMD and a C-terminal cd. The variable C-terminal regions of the PCs play a role in their subcellular localization, which is proposed to play a role in the substrate processing *in vivo* (Steiner, 1998).

1.6.2 PC substrates and functions

Mammalian PCs have wide range of substrates (Table 4) including endogenous growth factors, hormones, receptors and plasma proteins, and exogenous viral envelope glycoproteins and bacterial toxins (Molloy *et al.*, 1999; Nakayama, 1997; Seidah *et al.*, 1998)). Substrate proteins can be subdivided into at least four different types. Type-I includes those precursors that are processed at an R-X-(K/R)-R site. This class includes proproteins synthesized for constitutive secretion, such as growth factors, neurotrophins, receptors, bacterial toxins and viral surface glycoproteins such as gp160. Type-II proproteins are cleaved at C-terminal to a pair of basic residues of the type (K/R)-(R/R). However, often we can find a basic amino residue (R or K) at P4 or P6 positions N-terminal to the cleavage site. The precursors in this group include the majority of processed polypeptide hormone precursors such as POMC and proinsulin. Type-III

consists of precursors cleaved at a monobasic site, usually occupied by a single R residue. The cleavage of these proproteins requires the presence of a basic residue at P4, P6, or P8. Precursors in this class include polypeptide hormones such as prodynorphin, prosomatostatin, and growth factors such as pro-EGF. Type-IV includes proteins cleaved at either single or paired basic residues. These proteins differ from type-I-III precursors by the characteristic presence of either an R or K at two residues C-terminal of the cleavage site. This group include some hormone precursors such as promullerian inhibiting substance (MIS) and proglucagon, and some growth factor such as pro-IGF II (Seidah *et al.*, 1998).

The catalytic domain of the PCs is responsible for their ability to process precursors and defines their catalytic preference. Cellular co-expression of the PCs with proproteins together revealed that each enzyme is capable of cleavage at pairs and single basic residue in the general consensus sequences (K/R)-Xn-R where n=0, 2,4 or 6 (Bresnahan *et al.*, 1993; Dupuy *et al.*, 1994; Hatsuzawa *et al.*, 1992; Jean *et al.*, 1998; Molloy *et al.*, 1992; Seidah *et al.*, 1994). Furthermore, *in vitro* data revealed that the X and the C-terminal P' residues define the fine specificity of each enzymes. In general, PC1/3 and PC2 recognize the dibasic-residue precursors in type-II group, whereas furin, PACE4, PC7 and PC6 recognize different types of substrates with overlapping specificity (Seidah *et al.*, 1998).

In vivo studies show that gene deficiencies of PCs in mouse display different phenotypes. The furin-deficient mice by gene knockout are embryonic lethal (Roebroek *et al.*, 1998); the PC4-deficient male mice have severely impaired fertility (Mbikay *et al.*, 1997); and the PC2-deficient mice have defective of prohormone processing (Furuta *et al.*, 1997; Westphal *et al.*, 1999). Moreover, *in vitro* studies show that PC6B and furin are critical in

multiple substrate processing during the embryonic development (Constam and Robertson, 1999; Cui *et al.*, 1998). Together, these data show that each PC has different function *in vivo*.

Table 4. ***Substrates of proprotein convertases***

The P6-P2' cleavage site sequences for a selected list of proposed PC substrates are shown above. References, 1) (Brennan and Nakayama, 1994; Misumi *et al.*, 1991); 2)(van de Ven *et al.*, 1990; Wise *et al.*, 1990); 3) (Andreasson *et al.*, 1989; Benjannet *et al.*, 1995); 4) (Andreasson *et al.*, 1989; Benjannet *et al.*, 1995); 5) (Alarcon *et al.*, 1994; Lane *et al.*, 1985; Smeekens *et al.*, 1992); 6) (Rothenberg *et al.*, 1995); 7) (Dupuy *et al.*, 1994); 8) (Gluschankof *et al.*, 1984; Xu and Shields, 1994); 9) (Bresnahan *et al.*, 1990); 10) (Cui *et al.*, 1998); 11) (Sawada *et al.*, 1997); 12) (Dubois *et al.*, 1995); 13) (Nachtigal and Ingraham, 1996); 14) (Chaudhuri *et al.*, 1992); 15) (Duguay *et al.*, 1998); 16) (Lane *et al.*, 1985); 17) k) (Bravo *et al.*, 1994; Mondino *et al.*, 1991); 18) (Logeat *et al.*, 1998); 19) (Paquet *et al.*, 1994); 20) (Kessler and Safrin, 1994); 21) (Sato *et al.*, 1996); 22) (Lehmann *et al.*, 1996); 23) (Milewicz *et al.*, 1995); 24) (Pei and Weiss, 1995); 25) (Klimpel *et al.*, 1992; Molloy *et al.*, 1992); 26) (Tsuneoka *et al.*, 1993); 27) (Moehring *et al.*, 1993); 28) (Garred *et al.*, 1995); 29) (Subbarao *et al.*, 1998); 30) (Spaete *et al.*, 1988); 31) (Volchkov *et al.*, 1998); 32) (Decroly *et al.*, 1994; Hallenberger *et al.*, 1992); 33) (Richardson *et al.*, 1986).

1.6.3 Distribution of proprotein convertases

Mammalian PCs have different tissue distributions, which also support their different functions *in vivo*. Northern blot analyses show that PC1/3, PC2, PC4 and PC6A are expressed in endocrine and neuroendocrine cells, whereas furin, PC6B, PC7 and PACE4 are ubiquitously expressed (Table 5). During embryonic development, mammalian PCs also display differential expression patterns. For instance, the expression of furin is distinct from those of the neuroendocrine-specific PC1/3 and PC2 (Marcinkiewicz *et al.*, 1994; Marcinkiewicz *et al.*, 1993). The expression of furin also has distinct patterns compared to those of the other ubiquitously expressed PACE4 and PC6 although they are overlapped in many tissues (Constam *et al.*, 1996; Zheng *et al.*, 1997). These

Table 4. Substrates of proprotein convertases

	P6		P4		P2	P1 ↓	P1′	P2′	Ref.
Serum proteins									
Proalbumin	R	G	V	F	R	R	D	A	1
Pro-von Willebrand Factor	S	H	R	S	K	R	S	L	2
Hormones									
POMC(αMSH/CLIP)	P	V	G	K	K	R	R	P	3
(γLPH/βEnd)	P	P	K	D	K	R	Y	G	4
Proinsulin	T	P	K	T	R	R	E	A	5
ProGlucagon	L	M	N	T	K	R	N	R	6
Prodynorphin	F	K	V	V	T	R	S	Q	7
Prosomatostatin	R	L	E	L	Q	R	S	A	8
Growth factors									
Pro-β-nerve growth factor	T	H	R	S	K	R	S	S	9
BMP-4 precursor	R	R	R	A	K	R	S	P	10
Pro-BNP	T	L	R	A	P	R	S	P	11
Pro-TGF β1	S	S	R	H	R	R	A	L	12
Pro-MIS	R	G	R	A	G	R	S	K	13
Pro-IGF-I	P	T	K	A	A	R	S	I	14
Pro-IGF-II	P	A	K	S	E	R	D	V	15
Pro-EGF	G	H	H	L	D	R	N	S	16
Cell surface receptors									
Insulin pro-receptor	P	S	R	K	R	R	S	L	17
Notch1 receptor	G	G	R	Q	R	R	E	L	18
"Helper" protein/chaperone									
Pro-7B2	Q	R	R	K	R	R	S	V	19
Extracellular matrix proteins									
BMP-1	R	S	R	S	R	R	A	A	20
Human MT-MMP1	N	V	R	R	K	R	Y	A	21
Integrin α3-chain	P	Q	R	R	R	R	Q	L	22
Profibrillin	R	G	R	K	R	R	S	T	23
Stromelysin-3	R	N	R	Q	K	R	F	V	24
Bacterial toxins									
Anthrax toxin PA	N	S	R	K	K	R	S	T	25
Diphtheria toxin	G	N	R	V	R	R	S	V	26
Pseudomonas exotoxin A	R	H	R	Q	P	R	G	W	27
Shiga toxin	A	S	R	V	A	R	M	A	28
Viral coat proteins									
Avian influenza HA (H5N1)	R	R	R	K	K	R	G	L	29
Cytomegalovirus gB	T	H	R	T	R	R	S	T	30
Ebola Zaire GP	G	R	R	T	R	R	E	A	31
HIV-1 gp160	V	Q	R	E	K	R	A	V	32
Measles virus F_O	S	R	R	H	K	R	F	A	33

observations suggest that the endoproteases have different roles necessary for embryonic development.

Table 5. ***Tissue and subcellular distributions of proprotein convertases***

Reference, a) (Schafer *et al.*, 1993; Takumi *et al.*, 1998; Tanaka *et al.*, 1996), b) (Hornby *et al.*, 1993; Muller *et al.*, 1998), c) (Schafer *et al.*, 1993; Takumi *et al.*, 1998; Tanaka *et al.*, 1996), d) (Hornby *et al.*, 1993; Muller *et al.*, 1998), e) (Dong *et al.*, 1997; Nakagawa *et al.*, 1993a; Nakagawa *et al.*, 1993b; Villeneuve *et al.*, 1999), f) (De Bie *et al.*, 1996b), g) (Nakayama *et al.*, 1992a; Seidah *et al.*, 1992a; Torii *et al.*, 1993), h) (Day *et al.*, 1993; Schafer *et al.*, 1993) i) (Molloy *et al.*, 1994; Shapiro *et al.*, 1997), j) (Nakagawa *et al.*, 1993b), k) (De Bie *et al.*, 1996b), l) (Bruzzaniti *et al.*, 1996a; Bruzzaniti *et al.*, 1996b), m) (S *et al.*,), n) (Johnson *et al.*, 1994; Tsuji *et al.*, 1994), o) (Dong *et al.*, 1997; Viale *et al.*, 1999)

PCs can be categorized into two major groups based on their subcellular localization. The first group (Class I) includes PC1/3, PC2, and PC6A (Table 5). These enzymes function in the regulated secretory pathway in endocrine and neuroendocrine cells, where they act on prohormone and neuropeptide precursors within dense core vesicles (De Bie *et al.*, 1996a; Rouille *et al.*, 1995; Seidah *et al.*, 1998). PC4, expressed only in testis, can also be classified in this group due to its potential role in endocrine gland (Table 5). The other major mammalian PC branch (Class II) includes furin, PACE4, PC6B, and PC7 (Bruzzaniti *et al.*, 1996a; Bruzzaniti *et al.*, 1996b; Meerabux *et al.*, 1996; Nakagawa *et al.*, 1993a; Nakagawa *et al.*, 1993b; Seidah *et al.*, 1996b). The active forms of these enzymes are present in the TGN and small secretory vesicles of the constitutive secretory and the endocytic pathways (Table 5). This strategic localization provides access to many precursors in different subcellular compartments (Nakayama, 1997). Furin and PC7 are concentrated in the TGN by their cds. PC6B is localized in the Golgi region although the precise compartment is unknown (De Bie *et al.*, 1996a). PACE4 differs from the other convertases by lacking a transmembrane anchor and having different cleavage specificity

Table 5. Tissue and Cellular Distribution of the Proprotein Convertases

Convertase (PC)	Tissue	Cellular
Class I		
PC1	Neural and endocrine cells, rich in periphery tissues and CNS[a]	TGN, secretory granules[b]
PC2	Neural and endocrine cells, rich in periphery tissues and very rich in CNS[c]	Secreoty granules[d]
PC5/6A	Endocrine & non-endocrine, wide-spread, very rich in adrenal cortex, endothelial & sertoli cells and digestive system[e]	secretory granules TGN[f]
PC4	Testicular and ovarian germ cells[g]	?
Class II		
Furin	Ubiquitously, rich in liver, kidney[h]	TGN,endosomes, PM[i]
PC5/6B	Ubiquitously, rich in digestive system and adrenal cortex[j]	TGN?[k]
PC7	Ubiquitously, rich in lymphoid-associated tissues[l]	TGN[m]
PACE4	Ubiquitously, rich in pituitary and chondrocytes[n]	TGN, secretory granule?[o]

(Mains *et al.*, 1997). Although PC7 is localized in the TGN, its cytosolic tail differs significantly from those of furin and PC6B in that it undergoes palmitoylation (van de Loo *et al.*, 1997) and lacks a tyrosine-based motif.

1.6.4 Intracellular trafficking of furin of PC6B

Furin is the most extensively studied mammalian PC and its intracellular localization and trafficking of the protein have been well characterized. The furin cd is necessary and sufficient for the intracellular localization and trafficking of the protease. The enzyme contains information that mediates the TGN concentration and recycling between the TGN and the cell surface (Molloy *et al.*, 1999; Nakayama, 1997). The current data on furin trafficking shows that the CK2 phosphorylated acidic cluster (AC motif) together with the tyrosine-based motifs (YGKL), maintain the TGN concentration and cycling between the TGN and the cell surface. At the TGN, the tyrosine-based motif (YGKL) directs the efficient TGN budding, apparently by direct binding to AP-1 complex (Teuchert *et al.*, 1999b). From the cell surface, furin uses primarily the tyrosine-based motif for internalization, which is mediated by the AP-2/clathrin-coated vesicles via the interactions between these motifs and the AP-2 complex (Teuchert *et al.*, 1999a; Voorhees *et al.*, 1995). After internalization, furin uses the AC motif for the TGN targeting (Molloy *et al.*, 1994; Schafer *et al.*, 1995; Voorhees *et al.*, 1995). Analyses of trafficking from early (sorting/recycling) endosomes indicate that the dephosphorylated furin is delivered to the TGN, probably via the late endosomes (Jones *et al.*, 1995; Mallard *et al.*, 1998).

Furin cd also contains information for protein trafficking in some specialized cells. In polarized MDCK cells, the sequence of EEDE within the AC acts in conjunction with the phenylalanine-isoleucine motif (and independent of the AC phsophorylation) to direct the

basolateral sorting of the enzyme (Simmen *et al.*, 1999). In neuroendocrine PC12 cells, furin is found in the ISGs in addition to the TGN and endosomes, but not in the MSGs. Thus, furin can be delivered into the ISGs but retrieved out of the regulated secretory pathway probably with clathrin/AP-1 coated vesicles (Section 1.2.2). Consistent with this routing, the ISG furin interacts with AP-1 in a CK2 phosphorylated-AC motif dependent manner (Dittie *et al.*, 1997). Moreover, furin with mutations on the CK2 phosphorylation sites accumulates in the MSGs in neuroendocrine AtT20 cells, demonstrating that the CK2 phosphorylation is necessary for removal of furin from the regulated secretory pathway (Dittie *et al.*, 1997).

The overall similarity of the *in vivo* processing sites in proproteins has raised the question of functional overlap between the PCs. Biochemical and cell biological analyses have provided some insight into this issue (Jean *et al.*, 1998; Seidah *et al.*, 1994; Seidah *et al.*, 1998; Steiner, 1998). PC6B and furin, the most closely related enzymes with similar protein structures, are ubiquitously expressed in many tissues with coexpression in many cells (Seidah *et al.*, 1994). In addition, they share similar enzymatic characteristics including both pH and Ca^{2+} optima, and are selectively targeted by the inhibitor α1-PDX (Jean *et al.*, 1998). When coexpressed with substrates, they can process most known substrates (Seidah *et al.*, 1998; Steiner, 1998). Together, these data suggest that furin and PC6B appear to be redundant to each other. In contrast, genetic studies show that they have distinct function *in vivo* since the deficiency of furin is not complemented by endogenous PC6B. The compelling question here is how these two redundant enzymes could function differently *in vivo*. The selective expression patterns of furin and PC6B during embryonic development (Constam *et al.*, 1996; Zheng *et al.*, 1997) partially answer the question. Since both enzymes are coexpressed in many cells, another possible explanation is that they are functionally active in different subcellular compartments.

Although the intracellular trafficking of furin has been extensively studied, little information is known about the intracellular localization of PC6B. The only data have showed that PC6A was in the regulatory pathway and co-localized with a granule marker, ACTH, in the MSGs in AtT20 cells. The C-terminal PC6A specific sequences contain about 30 amino acids necessary for sorting into the regulated secretory pathway (De Bie *et al.*, 1996a). In contrast, the ubiquitously expressed PC6B is primarily located in the perinuclear region and overlaps with the TGN marker, TGN38 (De Bie *et al.*, 1996a). The characterization of the transport and subcellular localization of the proteases is still incomplete. However, the sequence analyses show that PC6B cd contains putative acidic clusters, and tyrosine-based and di-leucine-like motifs, suggesting that, like furin, the intracellular trafficking of the protease is complex (De Bie *et al.*, 1996a).

1.7 Objective of the thesis

The aim of the thesis is to characterize the intracellular localization of PC6B in the secretory pathway and the molecular mechanism involved the trafficking of PC6B and furin in the TGN/endosomal system.

PCs have been shown to process many critical bioactive molecules for their cellular and physiological functions. PC6B and furin are coexpressed in many mammalian cells and share indistinguishable substrate specificities (Jean *et al.*, 1998; Molloy *et al.*, 1999; Seidah *et al.*, 1994; Steiner, 1998). The studies on intracellular localization of these enzymes will help us to understand their function *in vivo*. While the localization of furin is well known, the information of PC6B localization is still incomplete (De Bie *et al.*, 1996a). Therefore, the intracellular distribution of PC6B was examined. The studies show that PC6B is localized to the post TGN/endosomal compartments or a subregion of the TGN which is regulated by two AC motifs (AC1 and AC2) on the PC6B-cd. Meanwhile,

our lab has identified a novel cytosolic factor PACS-1 obtained from a rat cDNA library (Appendix), which interacts with the CK2 phosphorylated furin AC motif and is necessary for the furin's TGN localization. Since AC1 functions as a TGN localization signal, similar to the CK2 phosphorylated furin AC, I tested the interaction between AC1 and PACS-1. The results show that AC1 interacts with PACS-1, and the interaction is necessary for the function of AC1 as well as the PC6B localization. In addition, I have shown that PACS-1 associates with clathrin adaptor complex AP-1 in cytosol, suggesting that PACS-1 act as a connector to couple the cargo molecules to clathrin-coated vesicles.

In order to study the function of PACS genes, database analyses have been performed to search for homologues of rat PACS-1. Subsequently, two human PACS genes have been isolated through the cDNA library screen. Both genetic and biochemical features of both human PACS genes have been characterized, which suggests that they may play similar roles in the membrane protein trafficking in tissue and development stage specific manners. In order to understand the molecular mechanism of PACS involved protein sorting, a detailed characterization on the sequences of human PACS-1 required for binding to AP-1 complex has been carried out. This information has been used to determine the role of PACS-1 in the localization and sorting of cargo proteins in the TGN/endosomal system.

In this thesis, I have characterized the intracellular localization and sorting signals of PC6B, and the interaction between PC6B and PACS-1, which is summarized in chapter 2. The identification and characterization of human PACS-1 and PACS-2 genes are summarized in Chapter 3. Chapter 4 is the characterization of the interaction between human PACS-1 and AP-1 complex. The characterization on the human PACS-1 sequences required for interaction with AP-1, and generation of dominant negative

human PACS-1 based the AP-1 binding site are summarized in chapter 4. Together, I have characterized a group of sorting signals directing the intracellular localization and sorting of furin and PC6B, and a new gene family involved in the sorting of these two proprotein convertases.

Chapter 2

THE PC6B CYTOPLASMIC DOMAIN CONTAINS TWO ACIDIC CLUSTERS THAT DIRECT SORTING TO DISTINCT TGN/ENDOSOMAL COMPARTMENTS

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The chapter has been accepted for publication on *Molecular Biology of the Cell.* In this publication, I performed all the experiments except Figure 1E and Figure 6B, which were done by Laurel Thomas and Sean Molloy, respectively.

ABSTRACT

The mammalian proprotein convertases (PCs) are a family of secretory pathway enzymes that catalyze the endoproteolytic maturation of peptide hormones and many bioactive proteins. Two PCs, furin and PC6B, are broadly expressed and share very similar cleavage site specificities suggesting they may be functionally redundant. However, germline knockout studies show they are not. Here we report the distinct subcellular localization of PC6B and we identify the sorting information within its cytoplasmic domain (cd). We show that, in neuroendocrine cells, PC6B is localized to a perinuclear, BFA-dispersible, $BaCl_2$-responsive post-TGN compartment distinct from furin and TGN38. The 88-amino acid PC6B-cd contains sorting information sufficient to direct reporter proteins to the same compartment as full-length PC6B. Mutational analysis indicates that endocytosis is predominantly directed by a canonical tyrosine-based motif (-Tyr_{1802}GluLysLeu-). Truncation and sufficiency studies reveal that two clusters of acidic amino acids (ACs) within the PC6B-cd contain differential sorting information. The membrane proximal AC (AC1) directs TGN localization and interacts with the TGN sorting protein PACS-1. The membrane-distal AC (AC2) promotes a localization characteristic of the full length PC6B-cd. Our results demonstrate that AC motifs can target proteins to distinct TGN/endosomal compartments and indicate that the AC-mediated localization of PC6B and furin contribute to their distinct roles *in vivo*.

INTRODUCTION

The identification of members of the mammalian family of proprotein convertases (PCs[1]) has enhanced our understanding of how numerous proproteins and prohormones become proteolytically activated in the biosynthetic and endocytic pathways (Nakayama, 1997; Seidah *et al.*, 1994; Steiner, 1998; Steiner *et al.*, 1992). In addition, characterization of these enzymes has provided insight into areas as diverse as mechanisms of intracellular protein targeting, embryogenesis and pathogen activation (Molloy *et al.*, 1999). However, such studies have also raised several compelling questions concerning the relative roles of the PCs *in vivo*.

Structurally, the PCs share a similar domain organization including an amino-terminal signal peptide followed by a proregion necessary for enzyme transport and activation and a highly conserved catalytic domain characteristic of the subtilisin family of serine endoproteases (Molloy *et al.*, 1999; Nakayama, 1997; Seidah *et al.*, 1994; Steiner, 1998). Consistent with their shared structural similarity, all the PCs have conserved enzymatic properties including the cleavage of proproteins at sites containing multiple basic amino acids (e.g. -Lys/Arg-Arg$^{\downarrow}$- or -Arg-X-Lys/Arg-Arg$^{\downarrow}$-).

The overall similarity of the *in vivo* processing sites in proproteins has raised the question of functional overlap between the PCs. Biochemical and cell biological analyses have provided some insight into this issue. The restricted tissue expression, acidic pH optima and regulated pathway localization of the neuroendocrine specific PC1/3 (hereafter termed PC3) and PC2 convertases reflect their specialized function in processing of prohormone substrates (Nakayama, 1997; Seidah *et al.*, 1994; Steiner, 1998). Similarly, the unique expression of PC4 in the testes, together with impaired fertility in PC4-

knockout [Mbikay, 1997 #2231], indicates a highly specialized function for this convertase. Several other PCs, however, have broader, overlapping expression patterns in a large number of tissues and cell types (Seidah *et al.*, 1994; Steiner, 1998). These include the type I transmembrane proteins furin, LPC/PC7 (hereafter termed PC7) and PC5/6B (hereafter termed PC6B), all of which reportedly traffic in the constitutive secretory pathway.

Immunocytochemical studies show furin, PC7 and PC6B each concentrate in Golgi-associated compartments (De Bie *et al.*, 1996a; Molloy *et al.*, 1994; van de Loo *et al.*, 1997). Although furin and PC7 both localize to the *trans*-Golgi network (TGN), recent studies showing their distinct cleavage site preferences suggest that they may process complementary sets of proproteins *in vivo* (van de Loo *et al.*, 1997) and J. Christian, personal communication). By contrast, furin and PC6B display overlapping substrate specificities. Both enzymes have been implicated in the proteolytic activation of BMP-4 (Cui *et al.*, 1998) and are the only PCs potently targeted by the selective inhibitor α_1-PDX (Jean *et al.*, 1998). Thus, furin and PC6B appear to share several structural, biochemical and cell biological characteristics which suggest that they could have overlapping roles *in vivo*. Their activities, however, are not fully redundant since furin knockout mice are embryonic lethal despite expressing PC6B (Roebroek *et al.*, 1998).

The properties of furin, including its activation and intracellular trafficking, are well characterized (Molloy *et al.*, 1999). While furin is concentrated in the TGN, the enzyme cycles between this compartment and the cell surface through the endocytic pathway. The dynamic localization of furin within the TGN/endosomal system allows the protease to activate a large number of proproteins in multiple compartments. The cytoplasmic domain (cd) of furin is both necessary and sufficient to direct the TGN localization and

recycling of the enzyme (Bosshart *et al.*, 1994; Chapman and Munro, 1994; Jones *et al.*, 1995; Molloy *et al.*, 1994; Schafer *et al.*, 1995; Voorhees *et al.*, 1995). A single cluster of acidic residues, which contains a pair of serines phosphorylated by casein kinase 2 (CK2) *in vivo*, is a key determinant for directing furin localization (Dittie *et al.*, 1997; Jones *et al.*, 1995; Molloy *et al.*, 1998; Takahashi *et al.*, 1995; Wan *et al.*, 1998). Furin is localized to the TGN by the interaction of the phosphorylated furin-cd with the connector protein PACS-1 (Wan *et al.*, 1998).

Relative to furin, little is known regarding the intracellular trafficking of PC6B. When expressed in AtT-20 cells, PC6B has been shown to concentrate in a perinuclear compartment where its staining pattern overlaps with TGN38 (De Bie *et al.*, 1996a). The shorter, lumenally-restricted PC6A splice variant, however, is localized to regulated secretory granules (De Bie *et al.*, 1996a). This indicates that PC6B contains sorting information within its extended cysteine-rich lumenal, transmembrane and/or cytoplasmic domains. The 88 amino acid PC6B-cd represents a candidate for TGN/endosomal sorting information since it contains numerous sequences which resemble sorting motifs identified in other itinerant type I transmembrane proteins localized within TGN/endosomal compartments (Marks *et al.*, 1997). These potential targeting signals include two tyrosine- and one di-leucine based clathrin coated pit (CCP) recruitment motifs, as well as clusters of acidic residues. Similar acidic clusters (ACs) have been shown to direct several distinct sorting steps in the TGN/endocytic pathway, including localization to the TGN (furin, carboxypeptidase D and VZV-gE, (Alconada *et al.*, 1996; Eng *et al.*, 1999b; Schafer *et al.*, 1995; Voorhees *et al.*, 1995; Zhu *et al.*, 1996), basolateral sorting (LDL receptor and furin, (Matter *et al.*, 1994; Simmen *et al.*, 1999), and delivery of lysosomal hydrolases (both the CI- and CD-MPRs, (Boker *et al.*, 1997; Chen and Okayama, 1988; Mauxion *et al.*, 1996; Schweizer *et al.*, 1997).

As a first step toward determining the PC6B sorting itinerary, we report here a comparison of the localization of furin and PC6B in AtT-20 cells, and an analysis of sorting motifs within the PC6B-cd. We show that, in contrast to furin, PC6B is localized to a perinuclear, post-TGN compartment which is brefeldin A (BFA)-dispersible, $BaCl_2$-responsive, and in communication with early endosomes. Furthermore, the PC6B-cd is sufficient to direct sorting to the same compartments as the full length protein. We also identified multiple active sorting signals, including a tyrosine-based internalization signal and two functional AC motifs. The membrane proximal AC (AC1)interacts with the connector protein PACS-1 to direct TGN localization while the second AC (AC2) promotes a distribution resembling that of the full-length PC6B-cd. These results identify unique targeting information in the PC6B-cd and further indicate that differential localization contributes to distinct roles for furin and PC6B *in vivo*.

Recombinant DNA constructs

The DNA sequence of the rat PC6B-cd was obtained from intestine by RT-PCR (Boehringer Mannheim) with two primers [5'-GGTGAATTCCTGCAGCTGCG-GAAGTCTCGAAGCAGA (oligo 1) and 5'-GGAGTCGACTTATTGGTAAGAG-TAGCT (oligo 2)] containing sequences identical to the N- and C-termini of the mouse PC6B-cd (Nakagawa *et al.*, 1993b). Sequence analysis of the PCR product revealed a single amino acid difference compared to that reported for the mouse PC6B-cd (Thr_{1861} → Ala, see Figure 1A). Metabolic labeling studies show that neither the rat nor the mouse PC6B-cds are phosphorylated in vivo, indicating that Thr_{1861} is not a functional phosphorylation site (see Results). The PCR product was digested with Pst I and Sal I and subcloned into pGEM7Z.FBct cut with the same enzymes to replace the furin-cd with that of PC6B. The insert encoding furin-PC6B-cd was excised with Bam HI and subcloned into pZVneofur/f cut with the same enzyme. This in frame, C-terminal "swap" generated a fur/f-PC6B chimera in which the furin-cd was replaced with that of PC6B. Point mutants and C-terminal truncations of the PC6B-cd were generated in the fur/f-PC6B chimera by either standard PCR or single-primer (Promega) mutagenesis techniques. The mutagenic primers used for the PC6B-cd truncation constructs (stop codons are underlined) were oligo 3 (Δ1, 5'-GCGGGATCCTTATTACTCATCATCTTCTGCCTCATC), oligo 4 (Δ2, 5'-GCGGGATCCTTATTACAGCAGCCCGTACTTGAACTT), oligo 5 (Δ3, 5'-GCGGGATCCTTATTAAACAGTGCCATCTTGGCCCAT), oligo 6 (Δ4, 5'-GCGGGATCCTTATTAGTCATCCTCATCATCTTCATC), oligo 7 (Δ5, 5'-GCGGGATCCTTATTACCTGTACTCAATCACCTGGTC), oligo 8 (Δ6, 5'-GCGGGATCCTTATTAATAGCTGCTCCTGTAGGAGGA), oligo 9 (Δ7, 5'-

GCGGGATCCTTATTAGGTGGGTTCTGCCAGCTTTTC) and oligo 10 (Δ8, 5'-GCGGGATCCTTATTACTTTGCCACAGGTCTGCTTCG). Alanine substitutions (Ala codons underlined) of potential PC6B-cd internalization motifs were created using either oligo 11 (L1858A, 5'-AAGTTCAAGTACGGGGCCCTGGATGAGGCAGAA), oligo 12 (Y1804A, 5'-GTGGCAAAGGGGCGCGCGGAAAAGCTGGCAGAA), oligo 13 (Y1856A, 5'-TACCGGAAGTTCAAGGCCGGGCTGCTGGATGAGGCA) or oligo 14 (Y G L L / A G A A , 5 ' - TACCGGAAGTTCAAGGCCGGGGCCGCCGATGAGGCAGAAGAT) in the appropriate combinations (see Figure 1A).

Standard PCR was also used to amplify specific fragments of PC6B-cd from pZVneo-based plasmids for subcloning into pGEX-3X (Pharmacia) and expression of GST fusion proteins. For each construct the 5' primer was oligo 15 (5'-GTGGGATCCGGAAGTCTCGAAGCAGA) whereas oligos 2, 4, 16 (5'-CGCGTCGACTTACATATAGACGATGTCATC) and 17 (5'-CGGTGAGTCGACCACCTGGTCCTCGTCGAG) were used as the 3' primers to amplify PC6B-cd, Δ2, Δ4 and Δ5, respectively. To amplify AC1, oligo 18 (5'-CAGCGGATCCTGATTGAGTACAGGGACCGA) and oligo 16 were used as primers whereas to amplify AC2 oligos 19 (5'-AGCGGATCCAGTATGGGCTGCTGGATG) and 2 were used. The PCR amplified inserts were then subcloned into pGEX-3X. pET16b (Novagen) expressing HtPACS-1fbr is described elsewhere (Wan *et al.*, 1998).

The lumenal domain of Tac cDNA was amplified by PCR of pBSSK(+)/Tac (provided by S. Milgram) with oligo 20 (5'-GCCGGATCCGGTCCCAAGGGTCAGGAA) and oligo 21 (5'-GCGTCGACTTACTGCCAGGTTAACCCACTCAGGAGGAG), and then subcloned into pBSSK(+) cut with BamHI and SalI. The Δ7 fragment was amplified with

oligo 22 (5'-GCGTCGACTTAGGTGGGTTCTGCTAGCCTTTTCGTAGCG) and oligo 23 (5'-GTGGGTTAACCTGGCAGCGGAAGTCTCGAAGCAGA). The fragment was cut with HpaI and SalI and subcloned into pBSSK(+) with Tac lumenal domain to generate pBSSK(+)/Tac-Δ7. PCR was also used to amplify AC1 with oligo 18 and 24 (5'-TCAGGCTAGCAGAACCCACCATTGAGTACAGGGACCGA), and AC2 with oligo 2 and 25 (5'-TCAGGCTAGCAGAACCCACCTACGGGCTGCTGGATGAG). The fragments were subcloned into pBSSK(+)/Tac-Δ7 cut with NheI and SalI. Oligos 22 and 2 were used to amplify rat PC6B-cd and the product was subcloned into pBSSK(+)/Tac-Δ7 cut with HpaI and SalI to generate pBSSK(+)/Tac-PC6B.

Cell culture and vaccinia virus expression

AtT-20 cells (provided by B. Eipper) were cultured in complete DMEM containing 5% FCS. BSC-40 cells as well as the PACS-1 control (C12) and antisense (AS19) cell lines were maintained as previously described (Wan *et al.*, 1998). Vaccinia recombinants expressing fur/f, furin, fur/f$S_{773,775}$D, PC6B/f and PC3/f have been described (Bresnahan *et al.*, 1990; Jean *et al.*, 1998; Jones *et al.*, 1995; Molloy *et al.*, 1994). PC6B-cd-containing expression vectors were generated by subcloning the DNA inserts into pZVneo (Hayflick *et al.*, 1992) and recombinant viruses were generated as described (VanSlyke *et al.*, 1995). Expression of each protein using vaccinia recombinants was performed as described previously (Molloy *et al.*, 1994).

For pulse-chase analysis, AtT-20 cells were grown to 80% confluence, then infected with recombinant viruses as described above. At 4 hr post-infection, the media was removed and the cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS (PBS-D), then incubated with methionine and cysteine-free DMEM (ICN) supplemented with Express label (Met/Cys, 100 μCi/ml, NEN) at 37°C for 30 min. Cells were then washed with PBS and harvested

in mRIPA (50mM Tris pH 8.0, 150mM NaCl, 1% NP-40, 1% DOC), or were refed with DMEM containing 10% serum and incubated for varying times prior to cell harvest. FLAG-tagged proteins were immunoprecipitated using mAb M1 followed by isolation of the immune complexes with Protein G Sepharose. Digestion with neuraminidase (2 x 10^{-4} U, Boehringer Mannheim) was performed in 50mM sodium citrate pH 6.0, 0.1% SDS (16 hrs at 37°C). Samples were resolved by SDS-PAGE (8% gels) and visualized by fluorography (Kodak X-OMAT film).

Immunofluorescence microscopy and antibodies

Immunofluorescence analyses were performed as previously described (Molloy *et al.*, 1994) using a Leica DMRB digital capture fluorescence microscope. Confocal images were acquired with a Bio-Rad MRC 1024 ES laser scanning confocal imaging system. mAbs M1 (IgG2b) and M2 (IgG1) were from IBI. Anti-TGN38 was provided by S. Milgram. Anti-ACTH AS29 antibody was provided by S. Tooze. Anti-Tac lumenal domain antibody 2A3A1H (IgG1) was provided by F. Maxfield. Anti-Tac lumenal domain antibody 7G7 (IgG2a) was obtained from ATCC. Anti-furin-cd PA1-062 antibody was from Affinity Bioreagents (Golden, CO). All FITC and TXR secondary antibodies were from Fisher. In double label experiments, species- and subtype-specific secondary antisera were used to distinguish the primary antibodies. Fluorescently conjugated human transferrin (Tf) was from Molecular Probes.

Stimulated ACTH release

AtT-20 cells were rinsed 3 times with PBS and then incubated for 1 hr at 37°C in 1 ml Ca^{2+}-free HBBS (125 mM NaCl, 4.75 mM KCl, 1.4 mM $MgCl_2$, 10 mM glucose, 0.07% BSA, 25 mM HEPES pH 7.35) in the absence or presence of 3 mM $BaCl_2$. Released β-

endorphin immunoreactive material was quantified by RIA as described previously (Thorne *et al.*, 1989).

Quantitative internalization

mAb M1 was radioiodinated using the chloramine T method and purified as described previously [Liu, 1997 #1934]. For uptake assays, AtT-20 cells were infected with either wild-type vaccinia virus or vaccinia recombinants expressing FLAG-tagged constructs. At 4 hr post-infection, cells were detached by incubation with 10 mM EDTA in PBS. The suspended cells were then washed with DMEM containing 10 mM HEPES (pH 7.4), and incubated with 1 x 10^7 cpm/ml of ^{125}I-mAb M1 for 30 min on ice. Cells were washed twice with ice-cold medium and once with ice-cold complete medium. Cells were warmed to 37°C for increasing times to permit internalization of bound ^{125}I-mAb M1. At each time point, replicate aliquots were removed and added to 500 µl ice-cold DMEM/HEPES with or without 1 mg/ml protease K (Boehringer Mannheim). The suspensions were incubated for 30 min at 4°C and pelleted by centrifugation through a serum pad. Cell pellets were counted in a gamma counter. The activity of each sample was corrected by subtraction of activity values obtained for VV:WT-infected cells.

Bacterial fusion proteins and binding assay.

GST fusion proteins were made from the PC6B-cd constructs cloned into the pGEX-3X expression vector as described above. The constructs were transformed into E.coli BL21 lysS and recombinant GST fusion proteins were purified using glutathione-agarose beads. HtPACS-1fbr was made as described (Wan *et al.*, 1998). For binding assays, 3 µg Ht-PACS-1fbr was incubated for 45 min. at room temperature with 5 µg of each respective GST protein in binding buffer (150 mM NaCl, 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 2% NP-40). Protein complexes were isolated using glutathione-agarose beads, resolved by

SDS-PAGE, and visualized by western blotting using an anti-PACS-1fbr antiserum (678, (Wan *et al.*, 1998) and chemiluminescent detection (NEN). Binding was quantified using NIH image software. Western blot analysis was performed as described (Molloy *et al.*, 1994).

RESULTS

Differential targeting of PC6B and furin.

To compare the localization of PC6B with that of furin, an epitope (FLAG) tag was introduced into the mouse full-length PC6B sequence (see Fig. 1A). The FLAG tag was inserted following the PC6B pro-region cleavage site which allows for specific detection of the proteolytically mature form of the enzyme (Jean *et al.*, 1998; Molloy *et al.*, 1994). Analysis by immunofluorescence showed furin and PC6B localized to distinct compartments in AtT-20 cells (Fig. 1B).

Characteristic of its localization to the TGN (Molloy *et al.*, 1994), furin concentrated in a perinuclear compartment which condensed upon treatment of cells with BFA prior to fixation. While PC6B also showed a perinuclear concentration (consistent with a previous study, (De Bie *et al.*, 1996a), this staining was dispersed by BFA treatment, indicating a non-TGN localization. In addition, PC6B displayed significant staining at the tips of AtT-20 cells which was not observed for furin. This differential distribution of furin and PC6B was verified in double-labeling experiments (Fig. 1B) in which the proteases were visualized independently using antibodies directed against the cytoplasmic domain of non-tagged furin (antiserum PA1-062) and the FLAG tag in PC6B/f (mAb M1). As seen with single-labeling, both proteins were predominantly localized in the perinuclear region but the PC6B staining pattern was selectively dispersed by BFA treatment.

PC6B cytoplasmic domain mediates sorting.

The furin-cd contains sorting information which directs its trafficking within the TGN endosomal system. Therefore, to determine the sorting capacity of the PC6B-cd, chimeric proteins were constructed consisting of the lumenal and transmembrane domains of either

FLAG-tagged furin (fur/fR_{739}t) or Tac, and the PC6B-cd (fur/f-PC6B and Tac-PC6B respectively, see Figs. 1A and 5A). The Tac lumenal and transmembrane domains have been used previously to assess the sorting capacity of cytoplasmic sequences (Voorhees *et al.*, 1995), while the use of fur/fR_{739}t as an additional reporter provided a means to monitor the efficacy of transport of the proteins through early secretory pathway compartments. FLAG-directed antibody reactivity was used to monitor ER-localized propeptide cleavage (Molloy *et al.*, 1994) as evidence of correct folding of the structurally similar lumenal domains (see legend to Fig. 1A) and the presence of N-linked carbohydrate was used to monitor transport of the chimera through the Golgi complex.

Metabolic labeling analyses showed that the PC6B-cd had no marked effect on maturation and transit of reporter protein to late secretory pathway compartments (Fig. 1C). Parallel plates of cells expressing either fur/f or fur/f-PC6B, were pulse-labeled with [^{35}S]-Met/Cys for 30 min. and either harvested immediately or chased in the absence of label for various times. Immunoprecipitation of the cell lysates with mAb M1 (requiring a free amino terminal FLAG tag) showed that the pulse-labeled proteins underwent propeptide cleavage at the correct site (C-terminal to Arg_{107}) to generate the mature fur/f (98 kD) and fur/f-PC6B (100 kD) forms (double arrowhead). During the initial chase periods (30 min.), a larger, neuraminidase-sensitive M_r form (single arrowhead) appeared for both the fur/f (104 kD) and fur/f-PC6B (106 kA) constructs, demonstrating transport of the mature fur/f and fur/f-PC6B molecules to late secretory pathway compartments. Upon extended chase periods, both fur/f and fur/f-PC6B were released into the medium via a proteolytic shedding event (Fig. 1C). Pulse-chase analysis of the full length PC6B/f also showed proteolytic shedding with a time course indistinguishable from that of fur/f and the fur/f-PC6B chimera (data not shown). The level of shed enzyme recovered in the

medium (approx. 70%) indicated that neither construct underwent appreciable intracellular degradation.

Immunofluorescence analysis of the steady-state distribution of the Tac-PC6B (Fig. 5A) and fur/f-PC6B chimeras in AtT-20 cells showed a staining pattern matching that of full length PC6B, including a concentration in tips as well as the perinuclear region (Fig. 1D). As seen for the full length PC6B, the perinuclear staining observed with the chimeric proteins was dispersed upon treatment with BFA, indicating a non-TGN localization. Analysis using additional markers further indicated a distinct localization of PC6B. The perinuclear PC6B compartment appeared distinct from recycling endosomes since Tac-PC6B failed to co-localize with internalized fluorescently labeled transferrin (Tf, loaded at high concentrations to detect the recycling compartment, Fig. 1E). In addition, despite the similar staining patterns of Tac-PC6B and the CI-MPR, the two proteins appear to localize to distinct compartments since treatment of cells with BFA causes dispersal of Tac-PC6B but a clustering of the CI-MPR (Fig. 1E). These observations demonstrate that the PC6B cytoplasmic domain contains sufficient sorting information to target reporter proteins to intracellular compartments indistinguishable from the full length enzyme. Further, this analysis indicates a distinct localization of PC6B compared to other TGN/endosomal markers.

Antibody (mAb M1) uptake analyses in AtT-20 cells (Fig. 2A) further showed that fur/f, PC6B/f and the chimeric proteins were all delivered to, and internalized from, the cell surface. However, in agreement with steady-state staining patterns, mAb M1 internalization indicated that the furin and PC6B-cds localized proteins to distinct compartments within the TGN/endosomal system. As described previously (Jones *et al.*, 1995; Molloy *et al.*, 1994), the native furin-cd directs efficient retrieval to the TGN

where the internalized mAb M1 and post-fix mAb M2 staining patterns overlap. By contrast, PC6B and the PC6B-cd-containing chimeric proteins localized to both a perinuclear BFA-dispersible compartment (data not shown and Fig. 1D) as well as a population of punctate structures concentrated in tips. These data demonstrate that the PC6B-cd can direct internalized proteins to compartments distinct from that of furin. Furthermore, the accessibility of the BFA-sensitive PC6B perinuclear compartment to endocytosed protein suggests that it represents a post-TGN localization.

To assess the routing of internalized protein containing the PC6B-cd, cells expressing fur/f-PC6B or Tac-PC6B (not shown) were incubated with both mAb M1 and fluorescently-labeled Tf, which labels early endosomes at low concentrations. Following short times of uptake, an overlap of the internalized Tf and mAb M1 was observed and was concentrated in tips (Fig. 2B). If, following an initial 10 min. uptake of mAb M1, cells were chased for 50 min., substantial punctate staining remained in the tips. However, this tip-localized mAb M1 staining no longer co-localized with Tf (data not shown). Double-labeling experiments (Fig. 2B) using mAb M1 and an antibody directed against ACTH, a granule marker, further showed that the vesicular PC6B-cd directed tip staining of AtT-20s was not localized to mature secretory granules (MSGs). The absence of PC6B from MSGs contrasted with the co-localization of ACTH and a mislocalized, non-phosphorylatable form of furin (fur/f$S_{773,775}$A) observed under the same conditions (Fig. 2B and (Dittie *et al.*, 1997).

Together these results demonstrate that the PC6B-cd i) is sufficient for directing intracellular localization and cell surface internalization, and ii) localizes protein to late secretory pathway compartments distinct from the TGN and MSGs, and which are in

communication with early endosomes. The latter observation indicates that the PC6B-cd directs a steady-state distribution unique from that of the related endoprotease furin.

Truncation analysis of PC6B cytoplasmic domain.

A series of incremental C-terminal truncations of fur/f-PC6B were constructed (see Fig. 1A) to begin to identify the PC6B-cd trafficking signals. The internalization and localization of each construct in AtT-20 cells was determined by mAb M1 uptake combined with post-fixation mAb M2 staining either immediately following a short uptake period or after an additional chase (Fig. 3). This protocol provided an assessment of internalization of the constructs into early endosomes as well as their sorting to later compartments. The analysis revealed three distinct localization patterns represented by Δ1-Δ4, Δ5-Δ7, and Δ8, as described below. Deletion of the C-terminal eleven residues (Δ1, Leu_{1867} - Gln_{1877}) containing a cluster of acidic amino acids (AC2) resulted in a reduction in the vesicular tip staining (following a 50 min. chase), coupled with a concomitant increase in the perinuclear immunofluorescent signal (not shown). This redistribution was more pronounced upon removal of an additional seven amino acids, including the remainder of the distal acidic cluster (Δ2, see Fig. 3). Further deletions of Ile_{1841} - Leu_{1859} (Δ3 and Δ4) produced staining patterns indistinguishable from Δ2 (not shown). Truncation of the next 10 residues (Δ5, Asp_{1830} - Asp_{1840}) including a second, membrane proximal acidic cluster (AC1) resulted in a more dispersed, endosome-like punctate pattern following uptake and chase. Similar results were observed upon deletion of Val_{1810} - Arg_{1829} (Δ6 and Δ7, not shown). Deletion of the ten amino acids from Gly_{1800} to Thr_{1809} (Δ8), which contain a canonical tyrosine-based CCP recruitment motif (-Tyr_{1802}GluLysLeu-), blocked internalization of the chimera and led to its accumulation at the cell surface.

Treatment of cells expressing the various PC6B-cd truncation constructs with BFA revealed a further distinction in their localization patterns. In contrast to the results with the full length PC6B-cd, the perinuclear concentration observed with Δ2 condensed with BFA treatment. This result indicates that removal of the distal 18 amino acids from the PC6B-cd leads to a redistribution of the chimeric protein to the TGN. Further truncation (e.g. Δ5), however, resulted in the loss of this TGN pattern. Together, these results indicate that there are at least three prominent sorting signals within the PC6B-cd; a membrane proximal segment (Gly_{1800} - Thr_{1809}) containing a potential tyrosine-based endocytosis motif, a centrally located acidic cluster, AC1, promoting localization to the TGN, and a distal acidic cluster, AC2, which is required for the characteristic PC6B distribution (i.e. tips and a BFA sensitive perinuclear compartment).

Identification of the PC6B-cd internalization motif.

The pronounced accumulation of the Δ8 construct at the cell surface suggested that the tyrosine-based consensus internalization sequence -Tyr_{1802}GluLysLeu- directs endocytosis of PC6B. However, two other consensus internalization sequences are also present in the PC6B-cd (see Fig. 1A); a second tyrosine-based motif (-Tyr_{1856}GlyLeuLeu_{1859}-) and a contiguous di-leucine motif (-LeuLeu_{1859}-). To determine the relative contributions of these motifs to PC6B endocytosis, a series of point mutation was constructed. As expected, a fur/f-PC6B chimera in which all three potential internalization signals were disrupted by substitution with alanines (IS-) showed prominent cell surface staining with no discernible uptake of extracellularly applied mAb M1 (Fig. 4A). Mutations in which combinations of two of the three signals were disrupted by alanine substitutions showed differential effects. The IS1 construct, with an

intact membrane proximal tyrosine-based motif (-Tyr_{1802}GluLysLeu-), displayed a staining pattern of internalized mAb M1 indistinguishable from that of fur/f-PC6B. By contrast, the IS2 and IS3 constructs, containing only intact C-terminal tyrosine- or di-leucine motifs respectively, showed significant cell surface staining with reduced internalization.

Quantitative uptake studies were performed to verify the differences in the relative effectiveness of the hydrophobic internalization motifs (Fig. 4B). The endocytosis of each construct was determined by sequestration of ^{125}I-labeled antibody as described in Materials and Methods. The internalization data replicated the results of the immunofluorescence analysis, showing that IS1 alone was sufficient to support endocytosis at a level indistinguishable from the native PC6B-cd. By contrast, IS2 and IS3 supported only low levels of internalization. Thus, the point mutant analysis, together with truncation studies (Fig. 3), indicate that -Tyr_{1802}GluLysLeu- is the principal internalization signal in the PC6B-cd.

PC6B ACs direct compartment-specific localization.

In addition to the internalization motifs, the truncation analysis in Figure 3 indicated the presence of at least two AC containing sorting signals in the PC6B-cd, one between Leu_{1820} and Asp_{1840} (AC1) and a second between Leu_{1859} and the C-terminus (AC2) (see Fig. 1A). In order to examine the sorting potential of the two ACs, they were analyzed independently. In the case of furin and the LDL receptor, the acidic motifs alone are not sufficient to support normal localization (Matter *et al.*, 1994; Schafer *et al.*, 1995). Indeed, a CCP recruitment motif is also required to form a bipartite localization signal and allow efficient internalization from the cell surface (Jones *et al.*, 1995; Matter *et al.*, 1994; Schafer *et al.*, 1995). Our results with the IS mutations indicate that the localization

of PC6B also requires an intact internalization motif. Therefore, each PC6B AC (see Fig. 1A) was fused to a Tac chimera, Tac-Δ7 (see Fig. 5A), consisting of residues Arg_{1790}-Thr_{1809} of the PC6B-cd, that includes the PC6B-cd internalization signal (-Tyr_{1802}GluLysLeu-).

Analysis of the AC1 construct, Tac-AC1, by antibody uptake (mAb 2A3A1H, see Materials and Methods) showed a concentrated perinuclear staining pattern which overlapped completely with post-fix (mAb 7G7) staining (Fig. 5B). Upon treatment with BFA, the perinuclear staining of Tac-AC1 concentrated in a manner analogous to that seen for TGN-localized furin (compare with Fig. 2). Double-label experiments (Fig. 5C) further demonstrated that the Tac-AC1 chimera co-localized with the TGN marker TGN38 both in the presence and absence of BFA, indicating that AC1 constitutes a functional TGN targeting motif.

By contrast, antibody uptake by Tac-AC2 resulted in a pattern resembling that of PC6B/f and full-length PC6B-cd chimeras, with pronounced labeling of punctate structures in tips as well as a less concentrated perinuclear component overlapping the post-fix staining (Fig. 5B). As seen for constructs containing the full length PC6B-cd (see also Fig. 1D), BFA treatment caused the perinuclear component of the Tac-AC2 staining pattern to disperse, indicating a non-TGN localization. Furthermore, the peripheral vesicular staining observed for Tac-AC2 with antibody uptake overlapped with internalized transferrin (Fig. 5C), indicating transport through early endosomes. Thus, unlike AC1, the AC2 region confers a distribution similar to that of the full PC6B-cd, including both endosomal and BFA-dispersible perinuclear compartments.

PC6B AC1 binds to the TGN sorting protein PACS-1.

The AC-directed TGN localization of furin requires interaction with the cytosolic sorting protein PACS-1 (Wan *et al.*, 1998). Because phosphorylation of the furin-cd AC by CK2 is required for binding to PACS-1, the ability of PC6B to be phosphorylated was first examined. Both metabolic labeling with $^{32}P_i$ as well as incubation of the PC6B-cd with CK2 in vitro showed no detectable phosphorylation of PC6B (data not shown). Next, a protein-protein binding assay was used to test the ability of the PC6B-cd to interact with PACS-1 (Fig. 6A). A His-tagged construct composed of the 141 amino acid fragment of PACS-1 including the furin-cd binding region, HtPACS-1fbr, was incubated with GST or GST fusion proteins containing either the entire PC6B-cd, the Δ2, Δ4 or Δ5 truncated cds (see Fig. 1A) or the individual AC1 and AC2 regions. As controls, two GST furin-cd constructs, GST-F_{DDD} a positive control and GST-F_{ADA}, a negative control were also tested (Wan *et al.*, 1998). In analyses of test constructs, binding of HtPACS-1fbr to the entire PC6B-cd was readily detected. Analysis of the PC6B-cd truncations showed that constructs containing the AC1 sequence (Δ2cd and Δ4cd) bound HtPACS-1fbr whereas Δ5cd, a PC6B-cd truncation lacking the AC1 sequence, did not. Furthermore, and consistent with the localization of Tac-AC1 to the TGN, the GST construct containing only the 19 residue AC1 bound HtPACS-1fbr. By contrast, the analogous AC2 construct did not support binding in these assays. This differential PACS-1 binding by AC motifs is similar to that reported previously for the CI-MPR (Wan *et al.*, 1998).

PACS-1 is required for AC1-directed TGN localization.

The results of the binding assays suggested that the ability of AC1 to function as a TGN localization signal is dependent on its interaction with PACS-1. To examine this possibility, Tac-AC1 was expressed in either PACS-1 antisense or control (empty vector) cells (see Materials and Methods). The antisense cells were previously used to establish the requirement of PACS-1 for the localization of furin (Wan *et al.*, 1998). In agreement

with our studies of PC6B trafficking in AtT-20 cells, antibody uptake in control cells showed that Tac-AC1 cycles to the cell surface and is retrieved to the TGN where the staining pattern overlaps with that of post-fixation staining. By contrast, in PACS-1 antisense cells antibody uptake failed to concentrate in the perinuclear region and resulted in a dispersed punctate staining pattern. Furthermore, the post-fix staining pattern was distended from the perinuclear region and, unlike Tac-AC1 in control cells, dispersed following treatment with BFA. Together these results demonstrate that AC1-directed sorting to the TGN requires PACS-1, and further suggest that AC1 functions in a manner analogous to the phosphorylated form of the furin-cd acidic cluster.

The interaction of AC1 with PACS-1 suggested that localization of PC6B was dependent, in part, on PACS-1. To examine this possibility, the localization of fur/f-PC6B and fur/f was examined in parallel plates of PACS-1 control and antisense cells (Fig. 6C). Antibody uptake studies showed that in control cells fur/f-PC6B was localized to a perinuclear compartment, whereas in PACS-1 antisense cells this construct was mislocalized and exhibited a diffuse staining pattern. Similarly, and in agreement with previous studies (Molloy *et al.*, 1998; Wan *et al.*, 1998), TGN localization of fur/f required expression of PACS-1. These results suggest that steady-state localization of PC6B requires cycling through the TGN, or perhaps some other PACS-1 dependent step.

The PC6B compartment is mobilized by stimulated secretion.

To help define the perinuclear, BFA-dispersible compartment in which PC6B localizes at steady-state, the localization of several markers of the late secretory pathway were compared in AtT-20 cells (Fig. 7A). Both ACTH and the epitope (FLAG)-tagged form of the neuroendocrine-specific endoprotease PC3 (PC3/f, visualized with mAb M1) show staining patterns resembling that of PC6B at steady-state, including both perinuclear and

tip staining. This distribution is consistent with the presence of ACTH and PC3 in multiple compartments of the regulated secretory pathway (i.e. immature secretory granules (ISGs) and MSGs). Interestingly, like PC6B, the perinuclear staining for both of these regulated pathway markers dispersed upon treatment with BFA. This similarity prompted an analysis of protein localization after treatment with $BaCl_2$ to stimulate secretory granule exocytosis. When AtT-20 cells were treated with $BaCl_2$ the perinuclear pools of ACTH, PC3/f and fur/f-PC6B were depleted. This $BaCl_2$-effect correlated with the enhanced release of β-endorphin immunoreactivity into the culture medium (Fig. 7B). However, in agreement with a lack of PC6B in MSGs (Fig. 2B), the tip-localized fur/f-PC6B was unaffected by the seretagogue. Furthermore, this effect was specific for the BFA-dispersible markers since the perinuclear staining for TGN-localized furin was unaffected by the secretagogue. Together, these data indicate that the perinuclear component of the PC6B staining pattern shares some characteristics with those of known regulated secretory pathway markers.

PC6B AC2 imparts sensitivity to secretagogue.

To examine the ability of the PC6B acidic clusters to replicate the $BaCl_2$ sensitivity imparted by the full-length PC6B-cd, the Tac-AC1 and Tac-AC2 constructs were analyzed by immunofluorescence microscopy as described above. Consistent with its similarity to furin and localization to the TGN, the steady-state distribution of Tac-AC1 was unaffected by $BaCl_2$ treatment (Fig. 7C). By contrast, the perinuclear staining observed for Tac-AC2 was depleted. This indicates that AC2, in conjunction with the IS1 CCP recruitment motif (-Tyr_{1802}GluLysLeu-), can replicate several characteristics of PC6B trafficking, including localization to a BFA-dispersible, $BaCl_2$-responsive perinuclear compartment that is in communication with the endocytic pathway.

DISCUSSION

These studies show that the mammalian PCs furin and PC6B have distinct distributions within the TGN/endosomal pathway. While furin localizes to the TGN, PC6B accumulates in a BFA- dispersible and $BaCl_2$-responsive perinuclear compartment and in the tips of AtT-20 cells (Fig. 1B and 7A). Furthermore, we demonstrated that the cytoplasmic domain of PC6B contains targeting information which directs endocytosis and promotes localization of reporter proteins to compartments characteristic of full-length PC6B (Figs. 1 and 2). A combination of truncation, point mutation and chimeric constructs further showed that the trafficking information contained within the PC6B-cd is distributed between multiple sorting motifs. These include a tyrosine-based motif for internalization (-Tyr_{1802}GluLysLeu-) as well as two clusters of acidic residues, or ACs, each of which imparts a distinct intracellular localization (Figs. 3 - 5). The function of the membrane proximal PC6B AC, AC1, is dependent upon a previously identified sorting protein, PACS-1, which directs the TGN localization of multiple AC containing proteins (including the related endoprotease furin, (Wan *et al.*, 1998). By contrast, the distal acidic cluster, AC2, which does not efficiently bind PACS-1, promotes a localization reminiscent of that of PC6B itself.

Intracellular localization may determine distinct roles for PCs in vivo.

Furin and PC6B have several common characteristics in addition to their structural similarity. Both are delivered to the plasma membrane and recycled via transferrin-containing early endosomes (Fig. 2 and (Jones *et al.*, 1995; Molloy *et al.*, 1998) and also share enzymatic features such as overlapping cleavage site specificities and sensitivity to the selective inhibitor α_1-PDX (Jean *et al.*, 1998). Furthermore, both furin and PC6B have been implicated in the processing of common substrates, including neurotrophins

and BMP-4 (Cui *et al.*, 1998; Seidah *et al.*, 1996a). Despite these similarities, our data show that, at steady state, PC6B and furin are concentrated in different parts of the TGN/endosomal system. Such differential localization may facilitate the activation of substrates within distinct cellular processing compartments *in vivo* and also help explain the inability of embryonic PC6B to compensate for the absence of furin in knockout mice (Roebroek *et al.*, 1998). This would contrast with furin and PC7 which co-localize within the TGN but have distinct substrate specificities (Jean *et al.*, 1998; van de Loo *et al.*, 1997) and J. Christian, personal communication). Thus, a combination of differential cleavage site specificity and subcellular localization may contribute to the requirement for multiple PCs within TGN/endosomal compartments. Indeed, whereas some furin (or PC6B) substrates are reported to be cleaved in the TGN, others are apparently cleaved in post-TGN compartments within the biosynthetic pathway (Sariola *et al.*, 1995).

Communication between multiple post-TGN processing compartments.

While the accumulation of PC6B in a perinuclear compartment is consistent with a previous description of Golgi-like staining (De Bie *et al.*, 1996a), our data showing dispersal by BFA (Figs. 1B and 7A) indicate that this pattern is not indicative of TGN-localization. Similarly, the lack of overlap between PC6B and a marker for MSGs indicates that the enzyme does not concentrate in late compartments of the regulated secretory pathway (Figs. 2B and 7A, also in agreement with (De Bie *et al.*, 1996a). However, the redistribution of PC6B upon treatment of cells with secretagogue (Fig. 7A) suggests that the perinuclear pool of PC6B is in communication with the regulated pathway. One possibility is that like furin and the CI-MPR, PC6B enters early compartments of the regulated pathway only to be removed during the granule maturation process. While MPR and furin are likely to be retrieved ultimately to endosomes and TGN respectively, PC6B may be transported to a distinct endosomal compartment. Upon

stimulation of secretion, the enhanced membrane flux may shift the equilibrium of the PC6B retrieval pathway, leading to a depletion of the perinuclear pool (as seen for the bona fide regulated pathway proteins ACTH and PC3, Fig. 7A). This is in contrast to furin which still appears to be effectively localized to the TGN (Fig. 7A).

This link with regulated secretion suggests several similarities between PC6B and another secretory pathway protein, peptidylglycine α-amidating monooxygenase (PAM), which catalyzes essential modifications in the biosynthesis of neuroendocrine peptides (Milgram *et al.*, 1997). Like PC6A and B, this bi-functional protein is expressed in multiple isoforms, including one, PAM1, with transmembrane and cytosolic domains. As described here for PC6B, the PAM1-cd contains trafficking information which directs retrieval from the cell surface and concentration in a perinuclear region showing partial overlap with TGN markers. In addition, the truncated, soluble isoforms of both PAM and PC6 (PC6A) are localized to MSGs. Further, the PAM1-cd is thought to direct its retrieval from the maturing granule. While the exact roles of the transmembrane isoforms of PC6 and PAM have not been elucidated, they could represent key components of a post-TGN, endosomal protein processing system. The observation that PC6B and furin maintain their distinct distributions in epithelial cells (our unpublished data) indicates that any such specialized role for PC6B may not be limited to neuroendocrine cell types.

Protein sorting by bipartite AC motifs.

The steady-state localization of furin to the TGN is dependent upon phosphorylation of a pair of serine residues which form CK2 phosphorylation sites within its single AC (Jones *et al.*, 1995; Takahashi *et al.*, 1995). Phosphorylation of the AC regulates the binding of PACS-1 to both the furin and VZV-gE cds (Wan *et al.*, 1998). While the PC6B AC1 also functions as a PACS-1 dependent TGN localization signal (Figs. 5-7), its binding is not

phosphorylation dependent (Fig. 6). This result is consistent with the absence of detectable phosphorylation of the PC6B-cd, and indicates that PACS-1 can participate in the sorting of additional proteins with non-phosphorylatable ACs. While it is not yet known how the interaction between PC6B-cd and PACS-1 may be regulated *in vivo*, these results indicate that it is independent of AC1 modification. The observation that PACS-1 is a phosphoprotein which can be recruited to membranes (Wan *et al.*, 1998) suggests that its phosphorylation state and/or subcellular localization could modulate binding.

While the PC6B AC1 can function as a TGN localization signal, the distal acidic cluster (AC2) appears to mediate a different sorting step, producing an accumulation in the BFA dispersed perinuclear compartment and tips of AtT-20 cells. The distinct localization pattern obtained for AC2-directed sorting suggests that this AC may interact with a different member of the PACS family or perhaps an unrelated binding partner. Regardless of its effector, the ability of AC2 to localize a reporter protein to the same compartments as the full length PC6B-cd (compare Figs. 2 and 5) suggests that it is the dominant signal for directing steady-state localization of PC6B. While our data indicate that AC2 is the dominant sorting signal, the mislocalization of fur/f-PC6B in the PACS-1 antisense cells (Fig. 6C) indicates a role for AC1 in the steady state localization of PC6B. It is possible that PC6B cycles through the TGN in an AC1-dependent manner to maintain its steady state localization. Further, it seems likely that under some conditions AC1 may play a more prominent role, perhaps redistributing PC6B to the TGN in response to particular stimuli. This potential for dynamic interplay between multiple AC motifs could also arise for additional proteins routed through the TGN/endosomal system, such as CI-MPR.

The importance of an intact internalization signal for PC6B-cd directed sorting (Fig. 4) is consistent with previous observations showing that, while AC motifs can be dominant localization signals, they generally function in the context of additional sorting information (Molloy *et al.*, 1999). Indeed, the idea of bipartite localization signals has arisen in part from studies of AC-directed furin and LDL receptor trafficking (Jones *et al.*, 1995; Matter *et al.*, 1994; Schafer *et al.*, 1995; Simmen *et al.*, 1999). Although the phosphorylated furin AC is a TGN localization signal, it requires the presence of an independent internalization motif for efficient targeting (Jones *et al.*, 1995; Matter *et al.*, 1994; Schafer *et al.*, 1995; Simmen *et al.*, 1999). Similarly, clusters of acidic residues with nearby tyrosine and phenylalanine-based motifs regulate the basolateral sorting of LDL receptor and furin in polarized epithelial cells (Matter *et al.*, 1994; Simmen *et al.*, 1999). One explanation for these observations is that the CCP recruitment motifs are essential for directing the proteins into vesicles that bud from the cell surface and/or TGN. Once in endosomal compartments, however, the AC motifs act to localize the protein within the TGN/endosomal system. While the point mutation analysis shown here indicates that the -Tyr_{1802}GluLysLeu- motif is the most efficient internalization signal in the PC6B-cd, it does not rule out an important role for the -Tyr_{1856}GlyLeuLeu- sequence in modulating the action of either one or both of the ACs, or in a separate routing step.

Together, the results discussed above highlight several interesting aspects of protein sorting by cytosolic targeting motifs including i) the importance of ACs in regulating multiple steps in the TGN/endocytic trafficking pathway, ii) the ability of structurally similar motifs to produce unique patterns of localization (e.g. the phosphorylatable furin AC and PC6B AC1 vs. PC6B AC2), and iii) the relationship between differential interaction with cytosolic binding proteins and AC function. In addition, the differential localization observed for furin and PC6B suggests that these enzymes have unique

properties and may activate proproteins in distinct subcellular compartments. The continued study of the proprotein convertases will help establish the importance of localization within the TGN/endosomal system in determining their functions *in vivo*, as well as provide insight into the mechanics and regulation of protein sorting.

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FOOTNOTES

[1]Non-standard abbreviations: PC, prohormone convertase; cd, cytoplasmic domain; BFA, brefeldin A; AC, acidic cluster; α_1-PDX, alpha-1-antitrypsin Portland; CK2, casein kinase 2; CCP, clathrin coated pit; VZV-gE, varicellar zoster virus glycoprotein E; LDL, low density lipoprotein; CI-MPR, cation independent mannose-6-phosphate receptor; CD-MPR, cation dependent mannose-6-phosphate receptor; RT-PCR, reverse transcription - polymerase chain reaction; GST, glutathione-S-transferase; Tac, T cell receptor alpha chain; HBBS, Hank's balanced salt saline; RIA, radioimmunoassay; mAb, monoclonal antibody; Tf, transferrin; MSG, mature secretory granule; ISG, immature secretory granule; ACTH, adrenocorticotrophic hormone.

FIGURE LEGENDS

Figure 1. **(A)** Epitope-tagged furin and PC6B constructs. Furin (white) and PC6B (gray) are shown with transmembrane domains (stippled and striped, respectively) and the FLAG epitope (black). The differential reactivities of the FLAG directed antibodies are also represented. mAb M1 recognizes only the N-terminal exposed FLAG sequence following excision of the proregion while mAb M2 recognizes the epitope in both the pro and mature forms of the enzymes. The amino acid sequence of the PC6B-cd and its derivatives are shown. Membrane-proximal (AC1) and membrane-distal (AC2) acidic clusters as well as the potential tyrosine-based and di-leucine like CCP recruitment motifs are highlighted in shaded boxes. Sites of Ala substitutions within these motifs are indicated with darker shading. **(B)** Comparison of intracellular localization between furin and PC6B. Panels A - D; AtT-20 cells expressing either fur/f (A and B) or PC6B/f (C and D) were fixed either directly or following exposure to 10 μg/ml BFA for 20 min. The cells were post-fix stained with mAb M1. Panels E - H; AtT-20 cells co-expressing non-epitope-tagged furin (E and F) and PC6B/f (G and H) were fixed either directly or following exposure to 10 μg/ml BFA for 20 min. then incubated with PA1-062 (anti-furin-cd) and mAb M1 (PC6B/f). The primary antibodies were distinguished using fluorescently tagged species-specific secondary antisera conjugated to either FITC (PC6B/f) or TXR (furin). **(C)** Pulse-chase analysis of fur/f and fur/f-PC6B. Replicate plates of AtT-20 cells expressing either fur/f or fur/f-PC6B were pulse-labeled with [^{35}S]-Met/Cys and either harvested directly (0) or following increasing chase times (hrs.) in the absence of label. [^{35}S]-labeled proteins in both cells and media were immunoprecipitated and resolved by SDS-PAGE/fluorography. Migration positions of both non-sialylated (double arrowhead) and sialylated (based on neuraminidase sensitivity, single arrowhead) forms of the proteins are indicated. The diminution in

signal in the chase samples is due primarily to shedding of the proteins into media [Jean, 1998 #2098; Molloy, 1994 #907]. **(D)** PC6B-cd is sufficient to direct protein localization. AtT-20 cells expressing either fur/f-PC6B (A and B) or Tac-PC6B (C and D) were fixed either directly or following exposure to 10 μg/ml BFA for 20 min.. The fixed cells were stained with either mAb M1 (A and B) or anti-TAC (7G7) (C and D). **(E)** PC6B localization is distinct from CI-MPR and recycling endosomes. Panels A and B; AtT-20 cells expressing Tac-PC6B were incubated with 50 μg/ml fluorescently labeled Tf (to load recycling endosomes, panel B) for one hr, fixed and incubated with anti-TAC (7G7) (panel A). Panels D – F; AtT-20 cells expressing Tac-PC6B were fixed either directly (panels C and D) or following exposure to 10 μg/ml BFA for 20 min. (panels E and F). The fixed cells were then stained with anti-TAC (7G7) (panels C and E) or anti-CI-MPR (panels D and F). The primary antibodies were distinguished using fluorescently tagged species-specific secondary antisera conjugated to either FITC (CI-MPR) or TXR (Tac-PC6B).

Figure 2. **(A)** Comparison of sorting by PC6B and furin cds. AtT-20 cells expressing either fur/f (A, B), PC6B/f (C, D), fur/f-PC6B (E, F) or Tac-PC6B (G, H) were incubated with mAb M1 (6 μg/ml) (A,C and E) or anti-Tac (2A3A1H, 6 μg/ml) (panel G) for 1 hr. The cells were then fixed, incubated with either mAb M2 (B,D and F) or anti-Tac (7G7) (panel H) and processed for immunofluorescence. The primary mAbs were distinguished using subtype-specific secondary antisera. **(B)** Panels A and B; AtT-20 cells expressing fur/f-PC6B were incubated with mAb M1 (6 μg/ml) and fluorescently labeled Tf (40 ng/ml) for 10 min. then fixed and processed for immunofluorescence. Internalized mAb M1 was visualized using a TXR-conjugated secondary antiserum. Arrows depict co-localized fur/f-PC6B and transferrin. Panels C - F; AtT-20 cells expressing fur/f-PC6B (C and D) or fur/f$S_{773,775}$A (E and F) were fixed and double-labeled with mAb M1 (C and

E) and anti-ACTH (AS-29) (D and F). The primary antibodies were distinguished using fluorescently tagged species-specific secondary antisera conjugated to either FITC (ACTH) or TXR (fur/f-PC6B and fur/f$S_{773,775}$A). Insets show enlarged tips marked by the boxes. Note the co-localization of ACTH and fur/f$S_{773,775}$A (panel F, inset) but not fur/f-PC6B in MSGs (panel D, inset).

Figure 3. Truncation analysis of the PC6B-cd by immunofluorescence. AtT-20 cells expressing either fur/f-PC6B or one of the PC6B-cd truncation constructs (Δ2, Δ5 and Δ8, see Fig. 1A) were incubated with mAb M1 (6 μg/ml) for 10 min. and either fixed directly (A - D) or following an additional 50 min. chase (E - H). The fixed cells in panels E -H were then stained with mAb M2 (I - L). Alternatively (M - P), cells were treated with 10 μg/ml BFA for 20 min. before fixation and staining with mAb M1. The primary mAbs were distinguished using subtype-specific secondary antisera.

Figure 4. Identification of PC6B internalization motifs. (A) AtT-20 cells expressing either fur/f-PC6B, Δ8, or one of the fur/f-PC6B CCP mutants (IS-, IS1, IS2 or IS3) (see Fig. 1A) were incubated with mAb M1 (6 μg/ml) for 1 hr, then fixed and processed for immunofluorescence microscopy. **(B)** AtT-20 cells expressing either fur/f-PC6B, Δ8, IS-, IS1, IS2 or IS3 were chilled on ice and incubated with ^{125}I-mAb M1. The washed cells were either harvested directly or warmed to 37°C, incubated for the indicated times and then harvested. The internalized mAb M1 was measured by counting as described in Materials and Methods. Assays were performed in triplicate. Error bars depict standard deviation.

Figure 5. PC6B-cd acidic clusters contain distinct sorting information. (A) Tac-PC6B-cd chimeras. The Tac lumenal (white) and transmembrane (black) domains, as

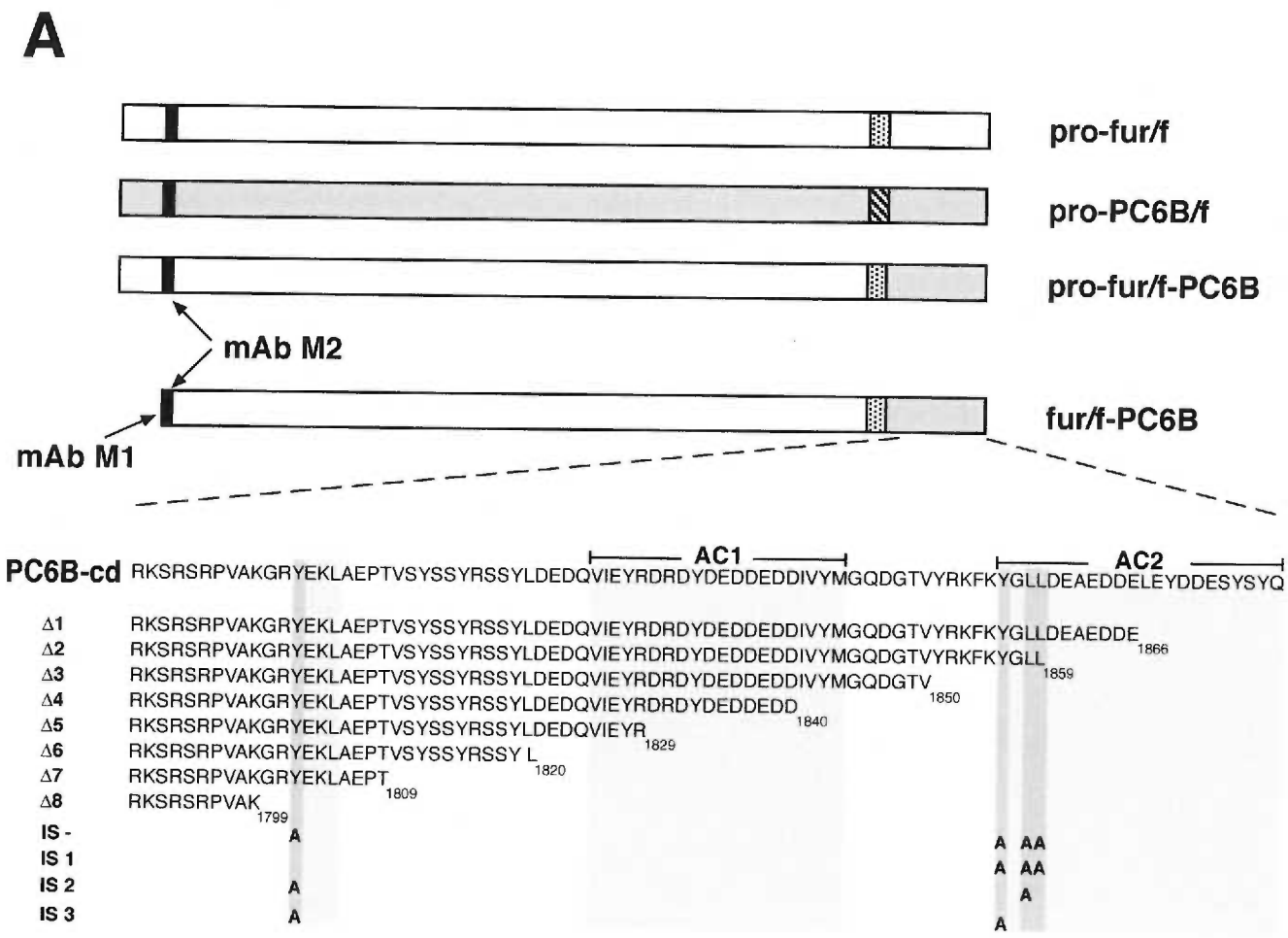
well as the PC6B-cd Δ7 (gray), AC1 (stippled) and AC2 (striped) sequences are indicated. **(B)** AtT-20 cells expressing chimeric protein Tac-Δ7 (A, E and I), Tac-AC1 (B, F and J), Tac-AC2 (C, G and K) or Tac-PC6B (D, H and L) were incubated with anti-Tac mAb 2A3A1H (6 μg/ml) for 1 hr (A - D), fixed and co-stained with anti-Tac mAb 7G7 (E - H). Alternatively, (I - L) cells were exposed to 10 μg/ml BFA for 20 min. before fixation and staining with mAb 7G7. The primary mAbs were distinguished using subtype-specific secondary antisera. **(C)** Panels A – D; Co-localization of Tac/AC chimeras with subcellular markers. Panels A - D; AtT-20 cells expressing Tac-AC1 were fixed either directly (A and B) or after a 20 min. exposure to 10 μg/ml BFA (C and D). The fixed cells were then processed for double-label immunofluorescence with anti-Tac mAb 7G7 (A and C) and anti-TGN38 (B and D). The primary antibodies were distinguished using species-specific secondary antisera conjugated to either FITC (TGN38) or TXR (TAC-AC1). Panels E and F; AtT-20 cells expressing Tac-AC2 were incubated with anti-Tac mAb 2A3A1H (E) and fluorescently labeled Tf (F) for 10 min., fixed and then processed for immunofluorescence. Internalized mAb 2A3A1H was visualized using a TXR-conjugated secondary antibody. Arrows depict co-localized TAC-AC2 and transferrin.

Figure 6. Role of PACS-1 in PC6B-AC1 targeting. **(A)** *In vitro* binding of PC6B-cd and the PACS-1 fbr. HtPACS-1fbr was incubated with either GST or GST fusion proteins containing either furin-cd constructs (GST-F_{DDD}, a phospho-mimic construct and GST-F_{ADA}, a negative control) or PC6B-cd sequences including the full length PC6B-cd, the Δ2cd, Δ4cd and Δ5cd truncation mutants or AC1 or AC2. Bound HtPACS-1fbr was separated with glutathione beads, resolved by SDS-PAGE and visualized by western blotting with the anti-PACS-1fbr antiserum 678. Binding was quantified and expressed relative to the that observed with GST-F_{DDD} (21% of input HtPACS-1fbr). Data

represent averages from three independent experiments. Error bars represent standard deviation. **(B)** PACS-1 is required for AC1-directed TGN localization. Parallel cultures of PACS-1 control (A - C) and PACS-1 antisense (D - F) cells expressing Tac/AC1 were incubated with anti-Tac mAb 2A3A1H (6 μg/ml) for 1 hr., fixed and co-stained with anti-Tac mAb 7G7. Cells in panels C and F were incubated with 10 μg/ml BFA for 20 min prior to fixation and staining with anti-Tac mAb 7G7. The primary mAbs were distinguished using subtype-specific secondary antisera. **(C)** Parallel cultures of PACS-1 control (A and B) and PACS-1 antisense (C and D) cells expressing fur/f-PC6B (A and C) or fur/f (B and D) were incubated with mAb M1 (6 μg/ml) for one hr., then fixed and processed for immunofluorescence microscopy.

Figure 7 (A) Furin and PC6B cds impart differential sensitivity to stimulated release. AtT-20 cells expressing fur/f (A, E and I), fur/f-PC6B (B, F and J) or PC3/f (C, G and K) were fixed either directly or after exposure to either 10 μg/ml BFA for 20 min. (E - H) or 3 mM $BaCl_2$ for one hr. (I - L). The cells were then labeled post-fixation with mAb M1 or antibody against ACTH (D, H and L). **(B)** Stimulated release of ACTH. AtT-20 cells were incubated for one hr. with either control media or 3 mM $BaCl_2$ and released β-endorphin immunoreactivity was quantified by RIA (Materials and Methods). Data represent the average value of three independent experiments. Error bars represent standard deviation. **(C)** PC6B AC2 is sufficient for targeting to $BaCl_2$-responsive compartment. AtT-20 cells expressing Tac-AC1 (A and B) or Tac-AC2 (C and D) were fixed either before (A and C) or after (B and D) exposure to 3 mM $BaCl_2$ then stained with anti-Tac mAb 7G7 and processed for immunofluorescence.

Figure 1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AC2
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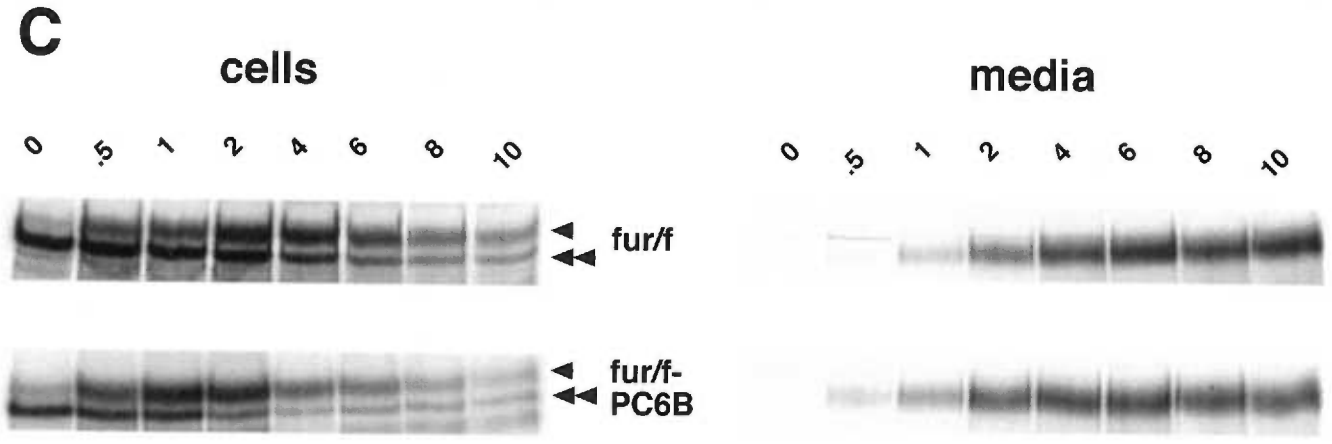
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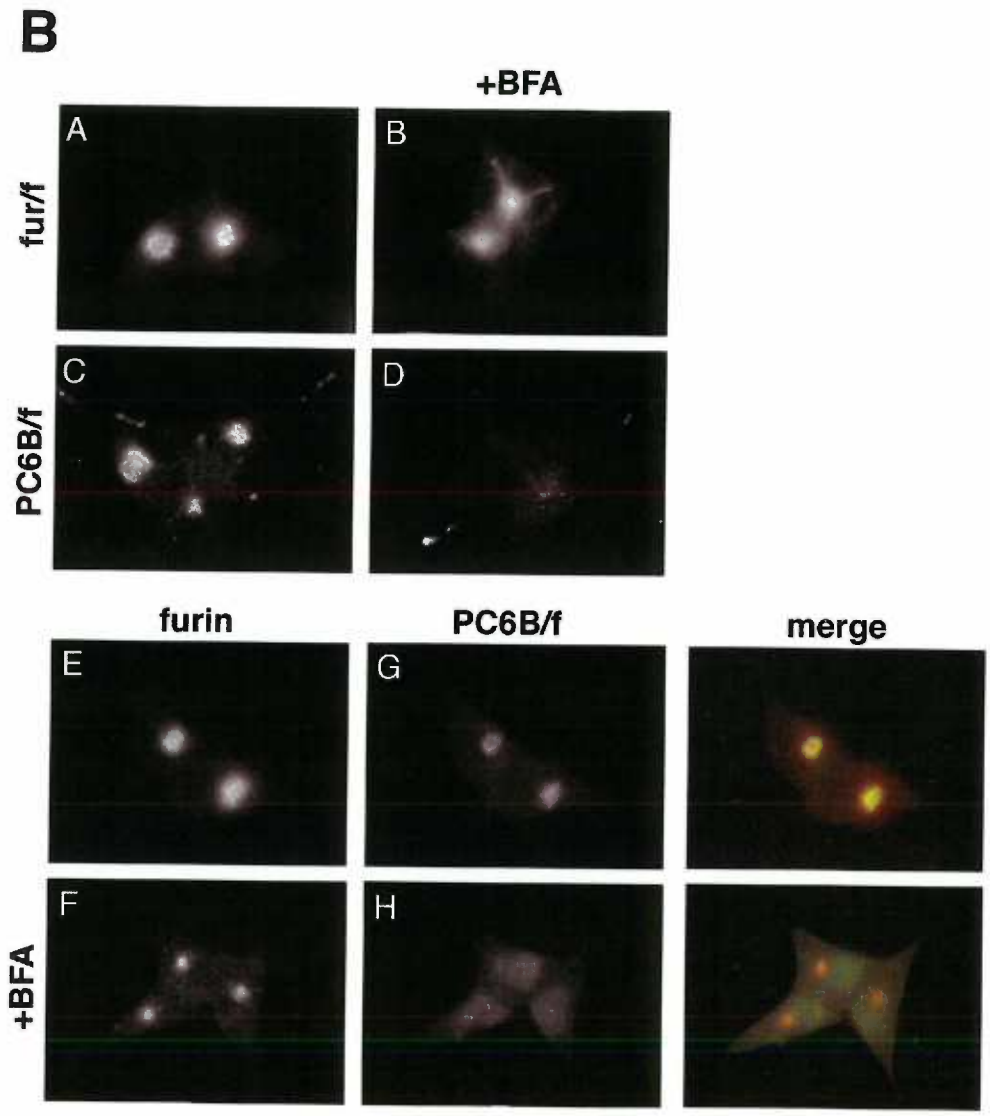
PC6B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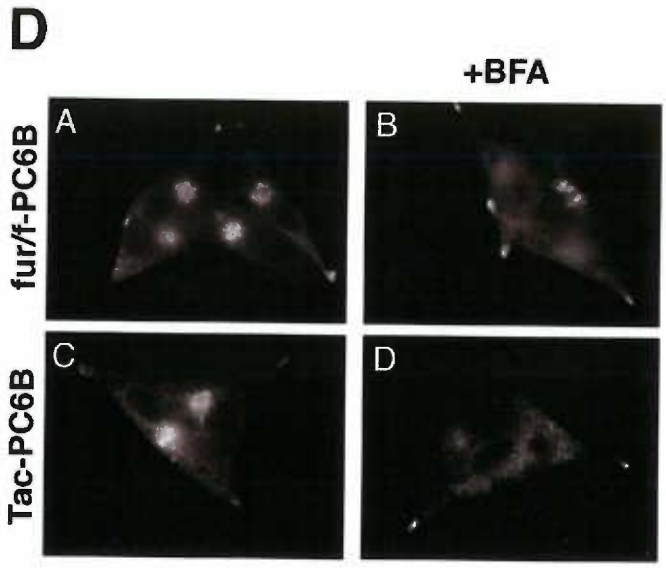
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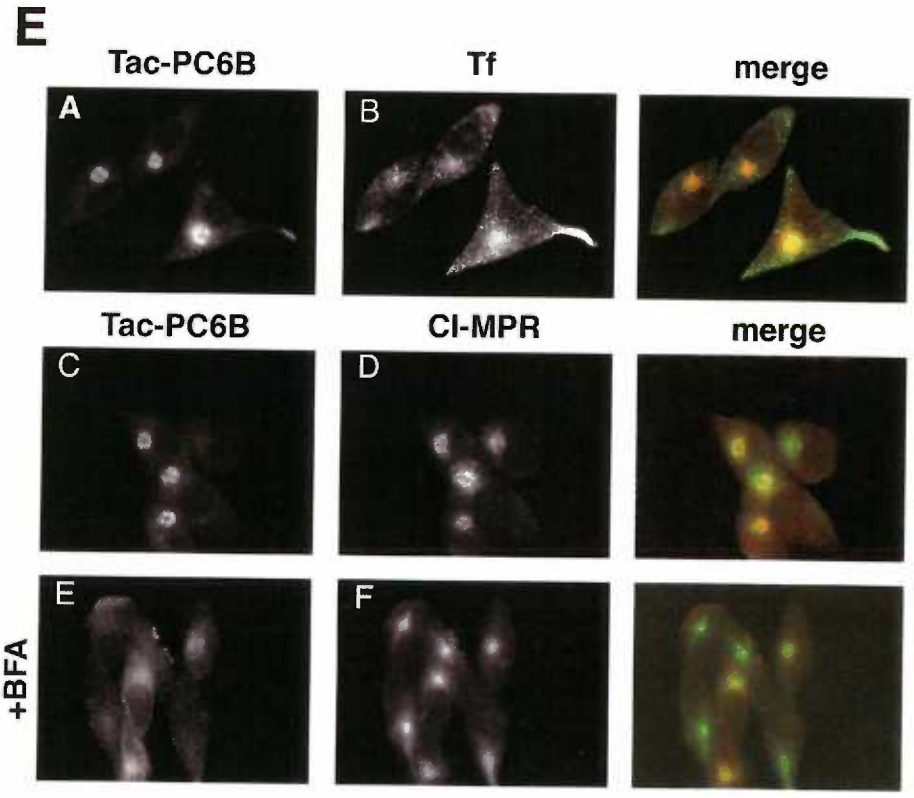
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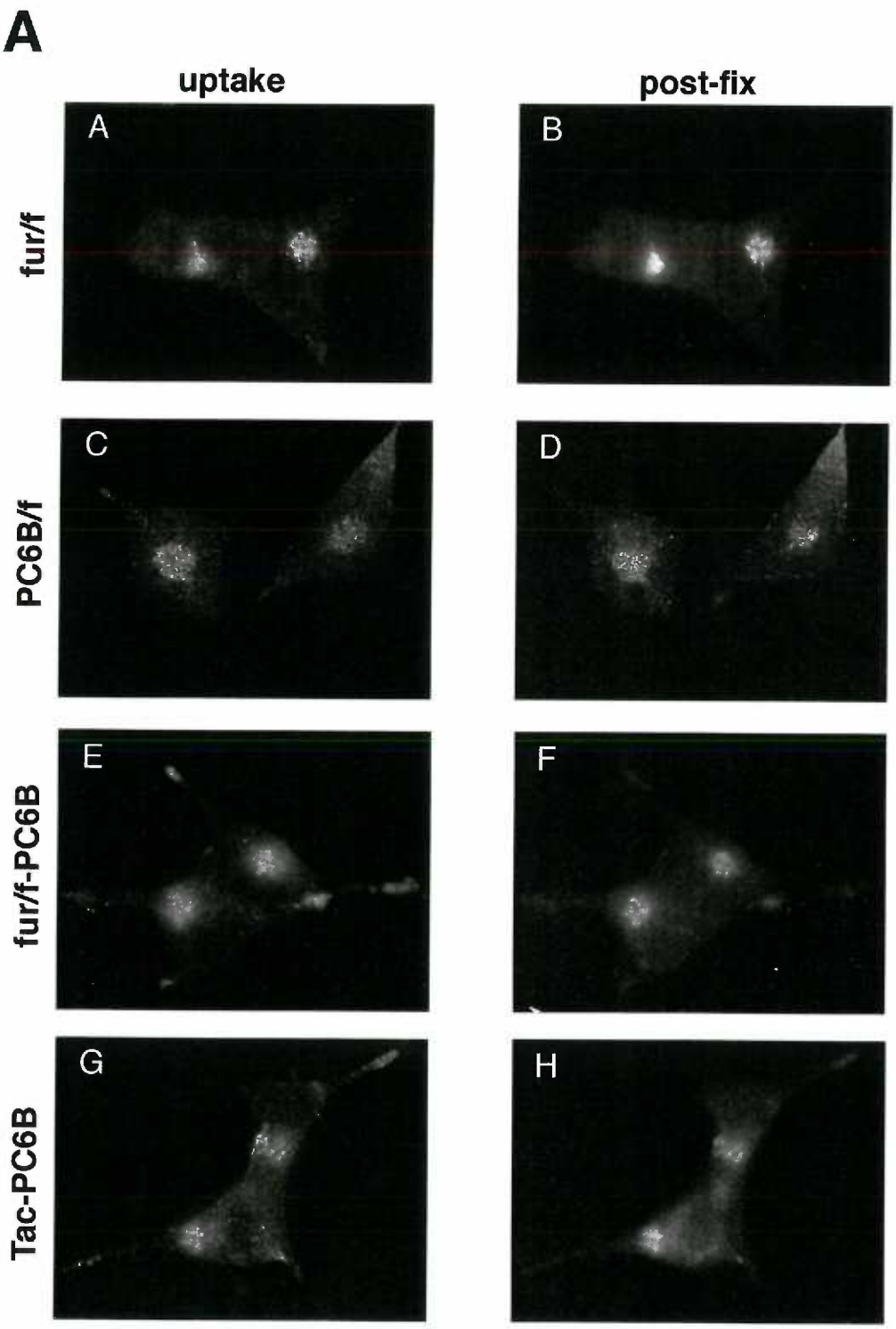
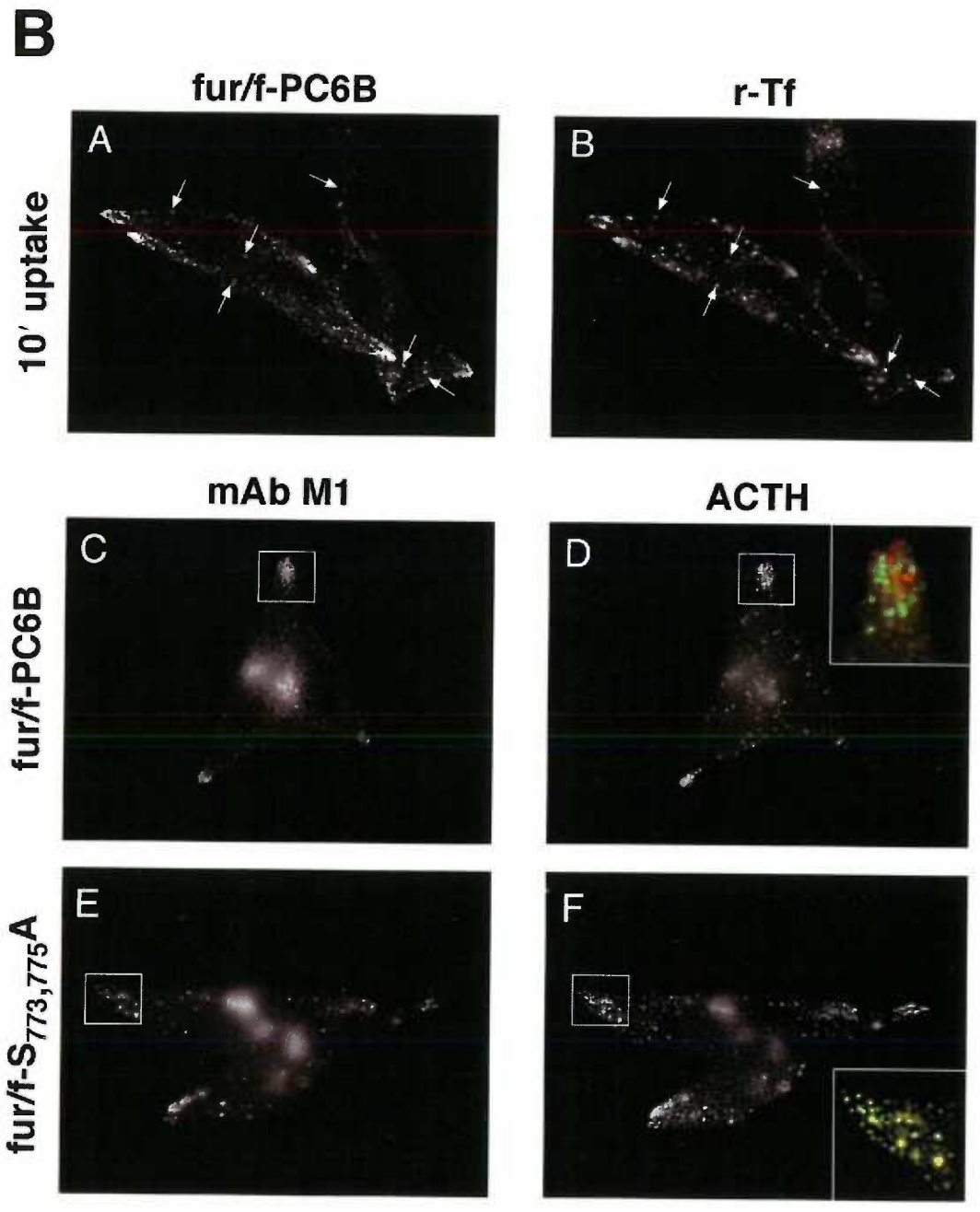
+BFA

Figure 2

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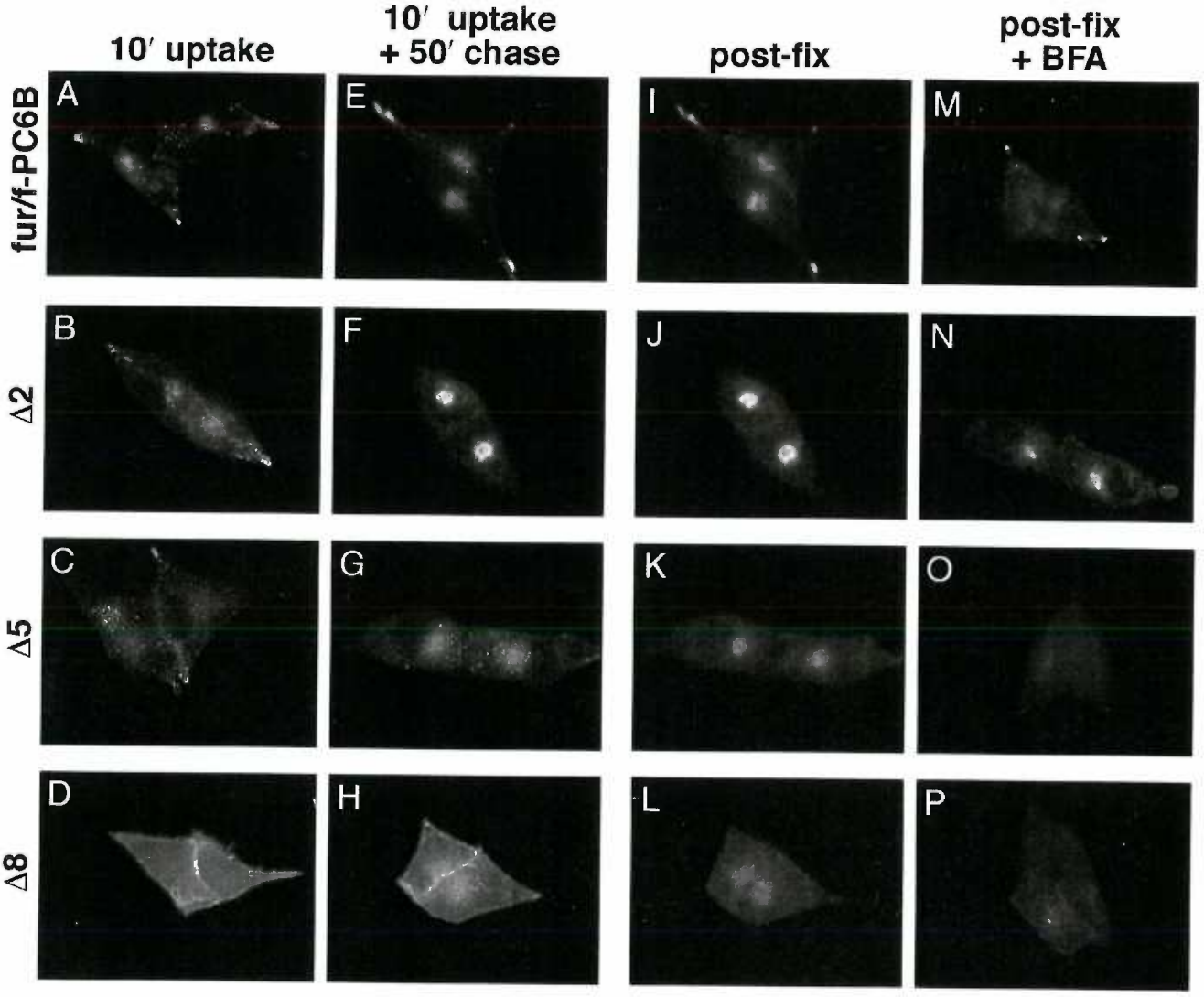
fur/f-S773,775A
E
F

Figure 3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post-fix + BFA
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Δ2
Δ5
Δ8
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J
K
L
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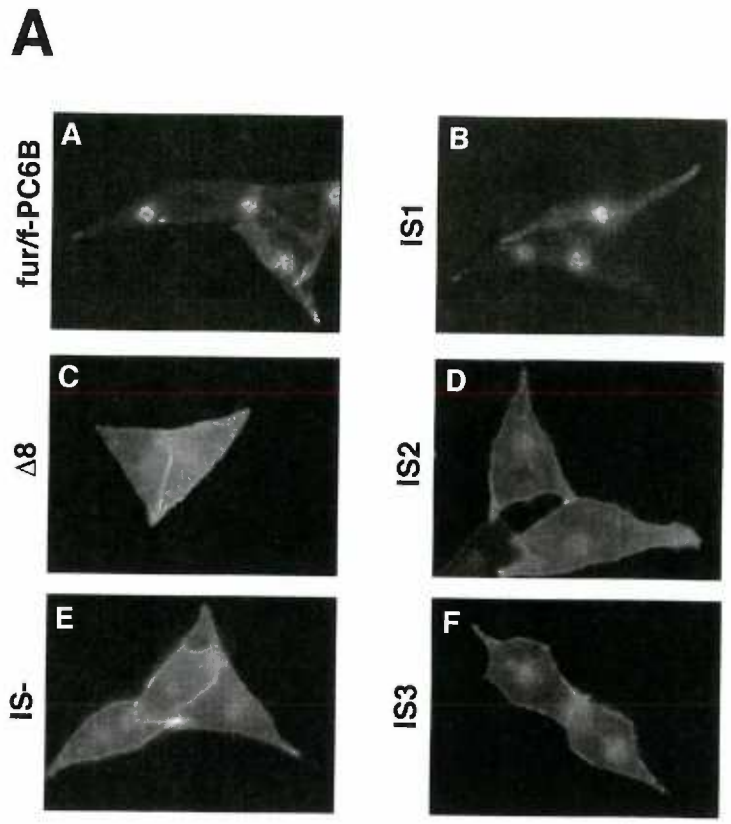
O
P

Figure 4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Δ8
IS2
IS-
IS3
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internalization (%)
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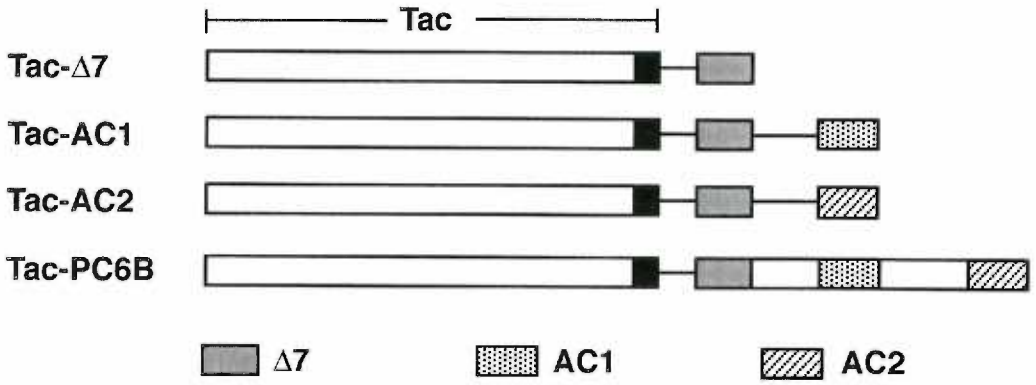
75
50
25
0
10
20
30
time (min.)

Figure 5

A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Tac-AC2
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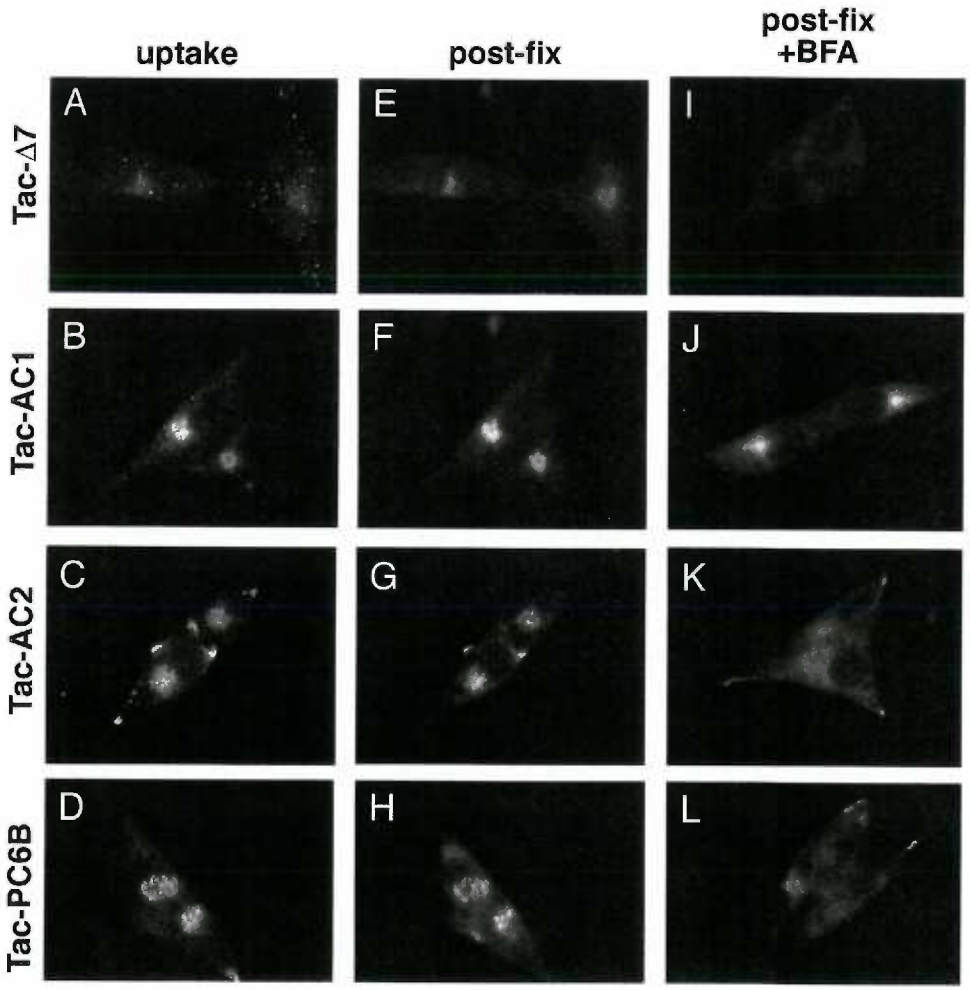
Δ7
AC1
AC2

B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post-fix +BFA
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Tac-AC1
Tac-AC2
Tac-PC6B
A
E
I
B
F
J
C
G
K
D
H
L

C

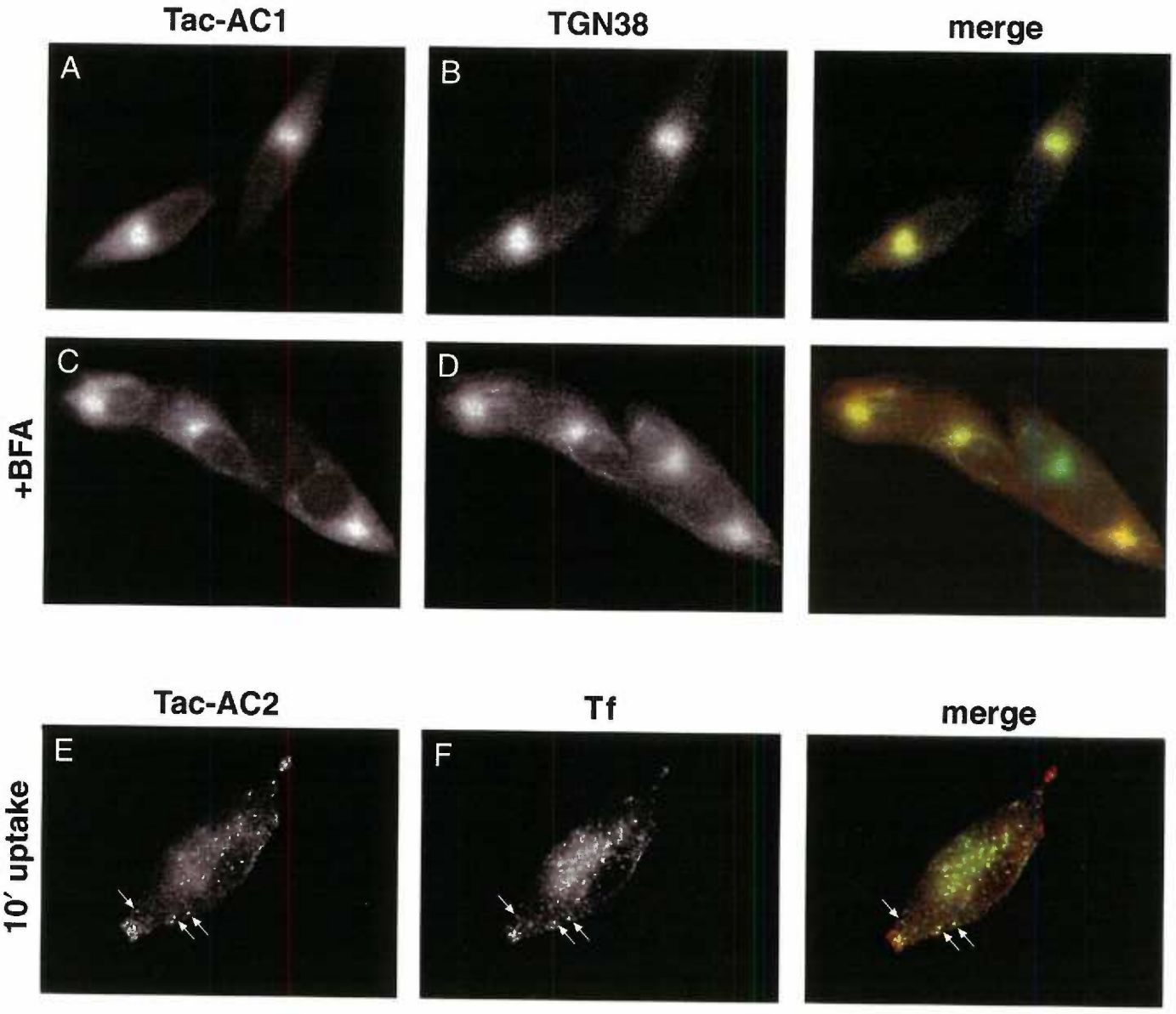
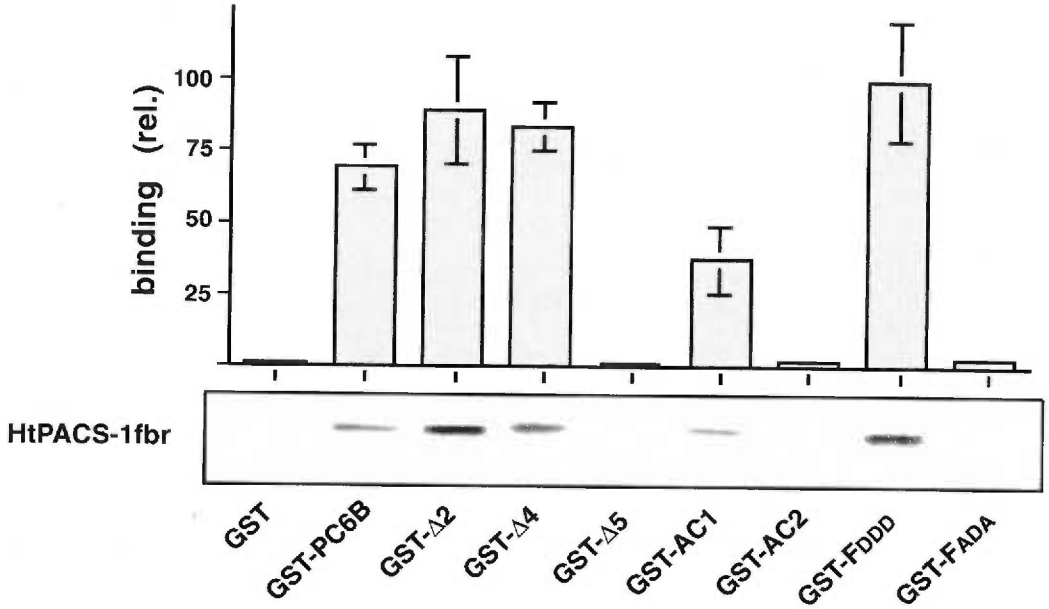


Figure 6

A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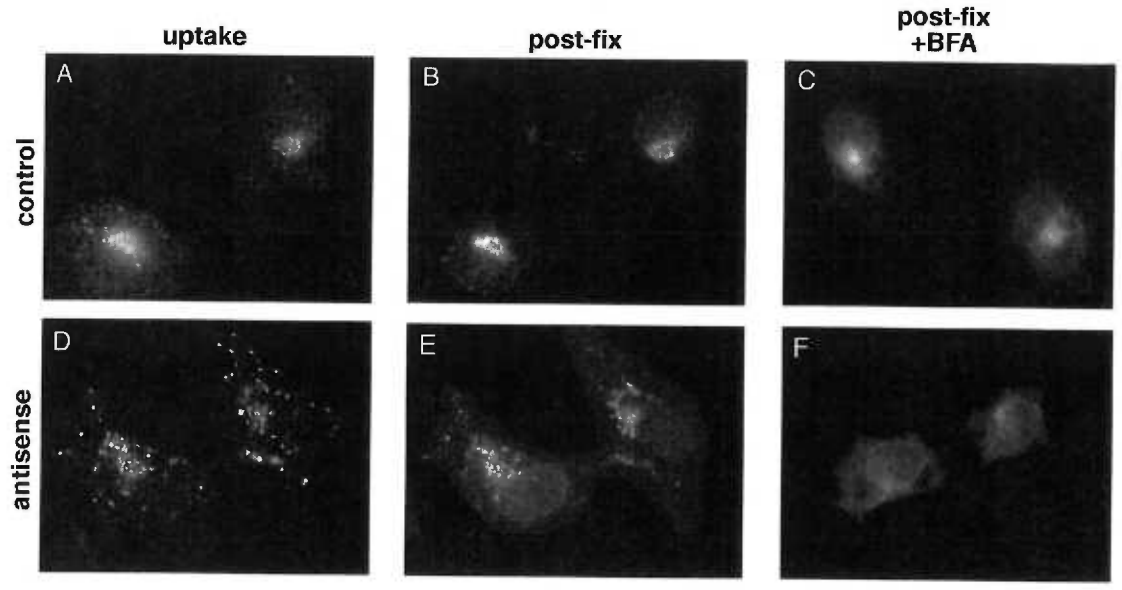
75
50
25
HtPACS-1fbr
GST
GST-PC6B
GST-Δ2
GST-Δ4
GST-Δ5
GST-AC1
GST-AC2
GST-FDDD
GST-FADA

B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post-fix
+BFA
control
antisense
A
B
C
D
E
F

C

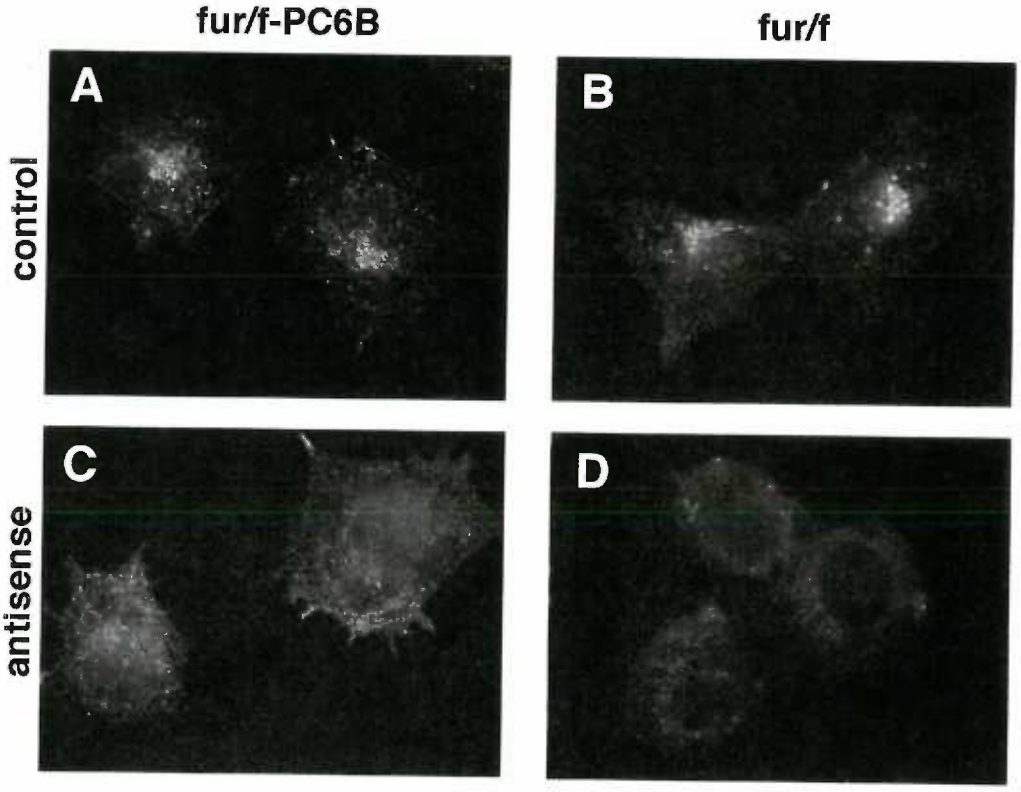
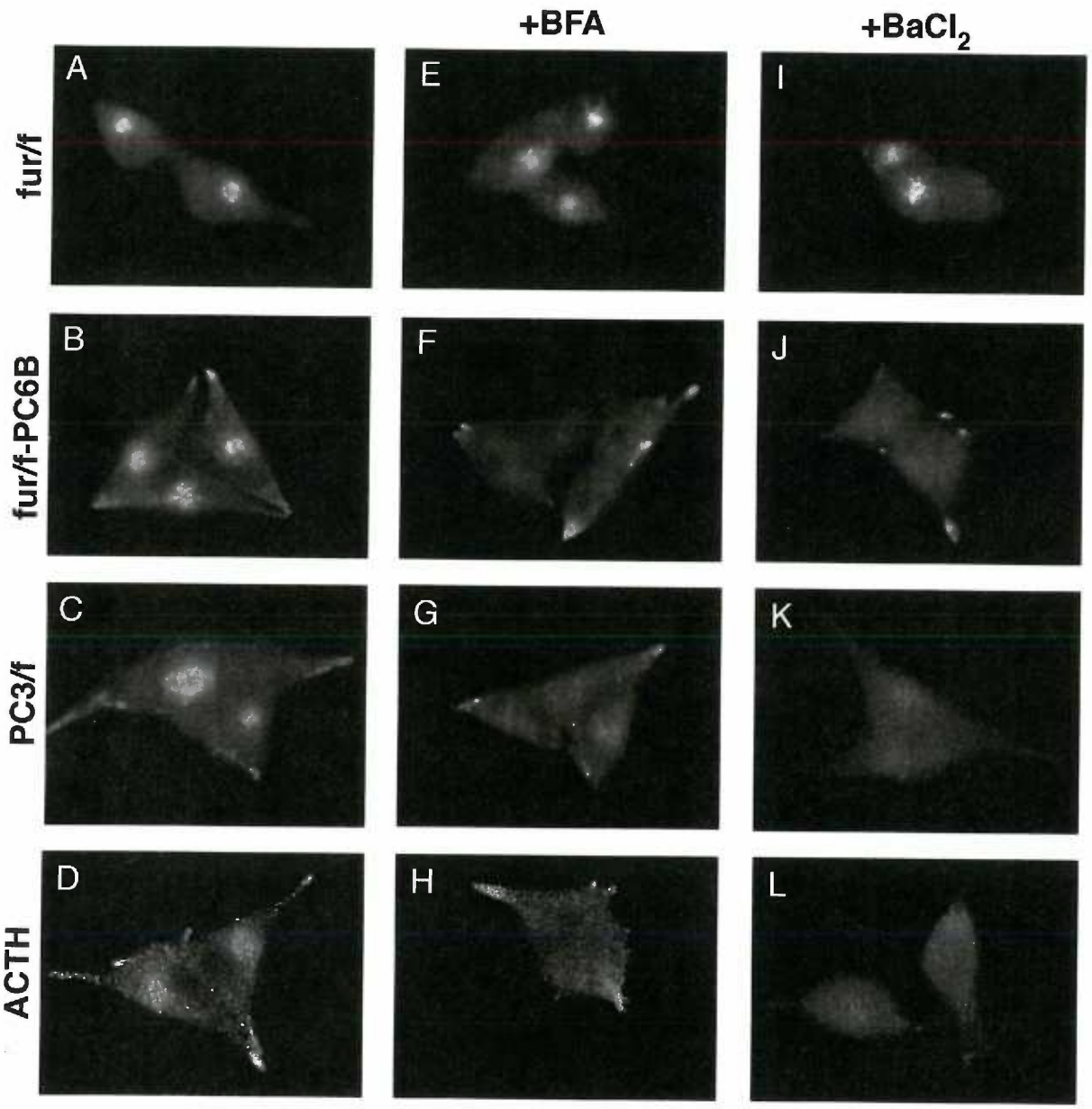
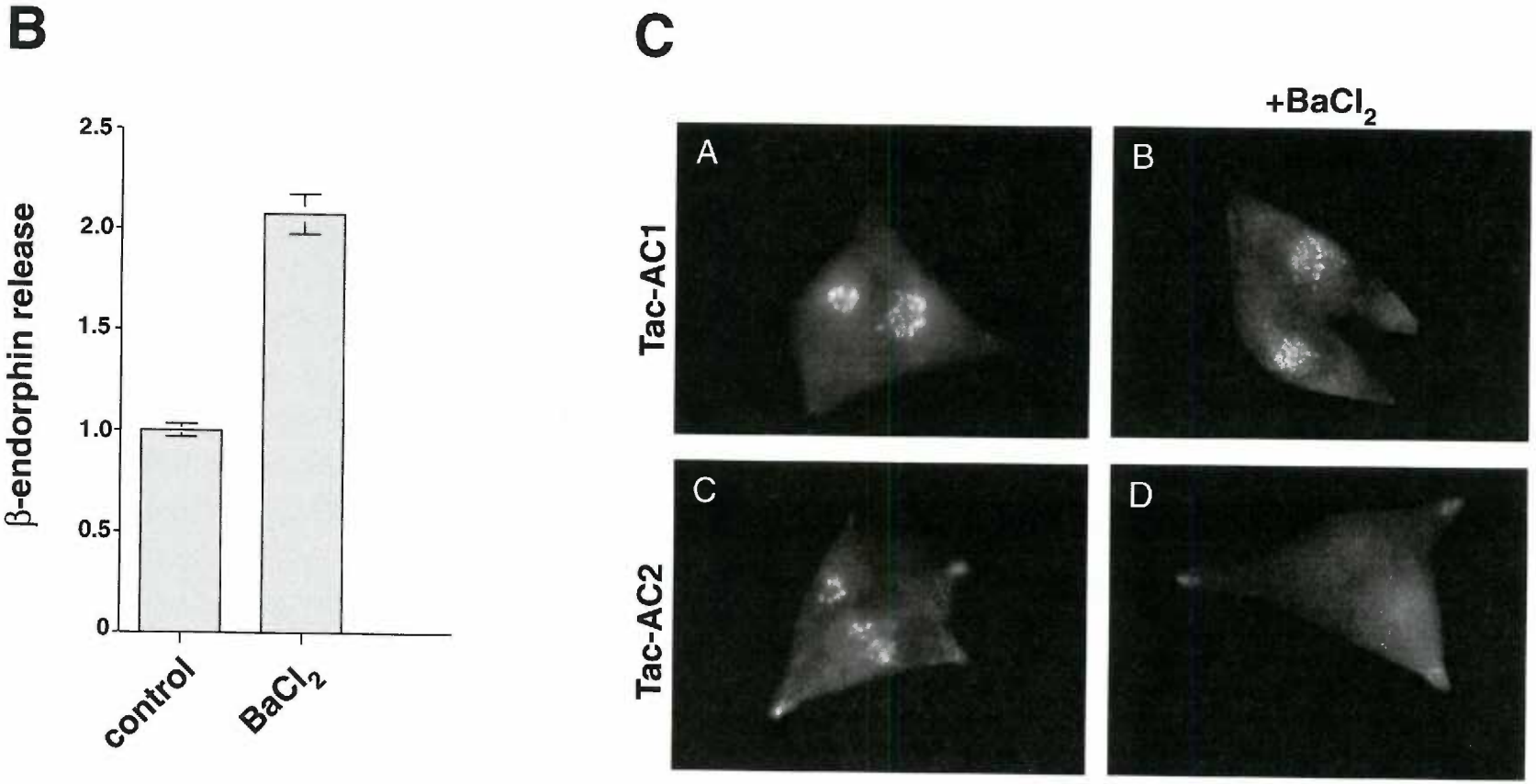


Figure 7




B
β-endorphin release
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2.0
1.5
1.0
0.5
0
control
BaCl2
C
+BaCl2
Tac-AC1
Tac-AC2
A
B
C
D

Chapter 3

Human PACS-1 and PACS-2: a Family of Cytosolic Proteins involved in Membrane Protein Sorting

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Manuscript in preparation.

In the following paper, Lei Wan and I contributed equally to the experiments in Figure 1a, and 1b, 2, and 5. I also performed the experiments in Figure 1c. The northern blot in Figure 3 was performed by Lei Wan. The in situ hybridization in Figure 4 was performed by Edith Lanone in the Department of Pharmacology, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada .

ABSTRACT

The recently identified rat PACS-1 gene encodes a cytosolic factor which binds to both membrane proteins and clathrin adaptor complex AP-1 and plays a critical role in the trans-Golgi network (TGN) localization of furin and the cation-independent mannose-6-phosphate receptor (CI-MPR) (Molloy *et al.*, 1998; Wan *et al.*, 1998). PACS-1, present in metazoans but not yeast, belongs to a novel gene family. Here, we describe the identification of human PACS-1 and PACS-2, a PACS-1 homologue. Human PACS-1 and PACS-2 are localized on chromosome 11q13 and 14q32, respectively, Both genes displayed broad tissue expressions by northern blot analyses. *In situ* hybridization revealed that PACS-1 and PACS-2 genes displayed complementary expression patterns in rat brain and endocrine glands. Biochemical analyses indicated that both PACS proteins could co-immunoprecipitate adaptor protein complexes AP-1 and AP-3, and preferentially bind to the casein kinase 2 (CK2) phosphorylated cytoplasmic domains (cds) of furin, CI-MPR and varicella-zoster virus (VZV) envelope glycoprotein gE. Together, PACS proteins may have complementary roles in tissue or developmental-selective processes by regulating the intracellular protein sorting.

INTRODUCTION

Intracellular sorting of various membrane proteins relies on the cooperation between the sorting signals within their cytoplasmic domains (cds) and the corresponding cellular sorting machinery. Vesicle coat proteins are essential for loading the correct cargo into transport vesicles. Several coat components have been identified including AP-1/clathrin, AP-2/clathrin, AP-3, AP-4, COPI, and COPII, which are involved at different steps of intracellular trafficking process (Kirchhausen, 1999; Robinson, 1997). However, apart from coat complexes, an increasing number of additional sorting factors have been found necessary to target membrane proteins. For instance, Eps15 and β-arrestin are required for the internalization of EGF receptor and β2-adrenergic receptor respectively, by connecting the receptors to AP-2/clathrin coat (Benmerah *et al.*, 1998; Goodman *et al.*, 1996; Laporte *et al.*, 1999). Tip47 was shown to direct the retrieval of both cation dependent mannose-6-phosphate receptor (CD-MPR) and CI-MPR from late endosomes to the *trans*-Golgi network (TGN) (Diaz and Pfeffer, 1998). Similarly, HIV-1 nef protein, a viral gene product, induces downregulation of CD4 receptor from cell surface into degradation pathway by sequentially connecting CD4 to AP-2/clathrin coat components on cell surface and to endosomal COPI on early endosomes (Foti *et al.*, 1997; Piguet *et al.*, 1998; Piguet *et al.*, 1999).

We have previously identified that the furin cd has two casein kinase 2 (CK2) phosphorylation serines within a cluster of acidic residues (AC motif) which is functionally critical for the TGN localization of furin (Jones *et al.*, 1995; Takahashi *et al.*, 1995). Several studies revealed that similar acidic clusters are functionally important for the intracellular sorting of other membrane proteins such as VZV gE and CI-MPR. Recently, we have isolated rat PACS-1 gene encoding two isoforms PACS-1a and PACS-

1b. They are cytosolic sorting proteins of a novel gene family, which selectively interact with the cd of the CK2 phosphorylated furin and play critical roles in the TGN localization of furin and CI-MPR (Wan *et al.*, 1998). Rat PACS-1 also associates with AP-1 complexes in cytosol. Thus, rat PACS-1 functions as a connector to recruit cargo proteins such as the phosphorylated furin into clathrin coated vesicles. This sorting serves as a retrieval step to deliver furin from endosomes to the TGN (Wan *et al.*, 1998). In addition, PACS-1 is necessary for recycling of the phosphorylated furin from early endosomes to cell surface (Molloy *et al.*, 1998). It is also involved in the Nef-induced downregulation of class I histocompatibility complex (MHC-I) by binding to the Nef/ MHC-I complex and redistributing MHC-I to the TGN (Piguet et al., 2000). These data show different functions of rat PACS-1 in membrane protein sorting. The fact that a large number of membrane proteins containing similar CK2 phosphorylation AC motifs suggests that PACS-1 may have a broader role in sorting of various membrane proteins (Molloy *et al.*, 1999; Voorhees *et al.*, 1995). However, the differential expression patterns of PACS-1, compared to those of cargo proteins in various tissues, suggest the existence of additional PACS family members involved in the AC motif-mediated intracellular sorting.

In order to identify additional PACS members, we carried out EST database searches and human cDNA library screens. We isolated cDNAs of human PACS-1 and a new PACS gene, human PACS-2. These two cDNAs encoded amino acid sequences with 54.4% identity from each other. Both proteins contained a conserved region (77% identity from each other) to rat PACS-1 furin-binding region (Fbr). Human PACS-1 and PACS-2 are located on chromosome 11q13 and 14q32 respectively, and both genes displayed broad tissue distributions. However, in rat brain and endocrine glands, PACS-1 and PACS-2 exhibited distinct but complementary distributions suggesting a specific role for each

protein. Like rat PACS-1, both human PACS-1 and PACS-2 could preferentially interact with the CK2 phosphorylated furin, CI-MPR, and VZV gE, and associate with AP-1 and AP-3 adaptor complexes, supporting their roles in regulation of membrane protein sorting. Together, the identification of both human PACS genes provides useful in understanding protein sorting in the TGN/endosomal system.

MATERIAL AND METHODS

Cloning of human PACS-1 and PACS-2 cDNA

A random-primed [^{32}P]-labeled probe made from a 950 rat PACS-1 cDNA fragment was used to screen a λ ZAPII-hMC human cortex library generated from BFO 156 human motor strip polyA+ mRNA (Stratagene, Lambda ZAP-II library, Instruction Manual). Positive clones were isolated by rapid *in vivo* excision from the pBluescript SK (-) phagemid with the ExAssist/SOLR system. The inserts in the recombinant plasmids were characterized by DNA sequencing. One clone contained a full-length open-reading frame cDNA and was identified as human PACS-1.

The EST database was searched by TBLASTN with the full-length rat PACS-1 amino acid sequences. Two EST clones from the database, gbR50031 and gbR73936 (IMAGE, USA) contained human amino acid sequences homologous to both rat and human PACS-1. A random-primed [^{32}P]-labeled probe was generated from the insert of R50031 to screen the λ ZAPII-human cortex library. Positive clones were isolated and analyzed as above. Three clones contained a full-length open reading frame cDNA showing homology to the human PACS-1, and this cDNA was named human PACS-2.

Two primers (5'-AATGGTACCATGTCGGTGGCCGGCGGC and 5'-AATGGATCCTCATGGAATGGAAGAAACGCG) were used to amplify the N-terminal region from the *Drosophila* embroynic and adult cDNA libraries kindly provided by M. Forte (Oregon Health Science University, USA). The PCR products were subcloned into pGEX.3x vector (Pharmacia, USA) and characterized by DNA sequencing.

Chromosomal mapping of human PACS-1 and PACS-2 genes

Chromosomal assignments of human PACS-1 and PACS-2 genes were determined with the Nigms Human/Rodent Somatic Cell Hybrid Mapping Panel #2. Mapping Panel #2 consists of DNA isolated from 24 human/rodent somatic cell hybrids. Each but two of these hybrids retains a single different human chromosome, respectively (Drwinga *et al.*, 1993; Hudson *et al.*, 1992; Shipley *et al.*, 1993). The chromosomal assignments were determined from PCR reactions with specific primers to either human PACS-1 or PACS-2 (Promega, USA). The designed primers specific to human PACS-1 were 5'-GCCCAGTCCCCTCAGCCGCCG and 5'-CGACGAGGTGGCCTGGGCCAGCTTG. The specific primers to human PACS-2 were 5'-TCCTGCCCCATGCTGTGA and 5'-CCAGGCTCAACTCAGGAC. The PCR products were checked on 4% NuSieve-GTG agarose gel and confirmed by southern blotting with the ^{32}P labeled primers listed above.

The same primers as described above were used in the PCR on the Whitehead Institute GeneBridge 4 Radiation Hybrid (GB4RH) mapping panel to further define the chromosomal localization of PACS-1 and PACS-2 genes (Walter *et al.*, 1994). The GeneBridge 4 Radiation Hybrid Panel contains DNAs from 93 radiation hybrid clones and two control DNA samples (HFL donor and A23 recipient). The PCR results were analyzed by the WICGR Mapping Service (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl) to generate chromosomal locations of PACS.

CAG Repeat Test

A pair of primers were designed according to the flanking sequence of the CAG rich region in human PACS-1 (5'-GGCGGTGGTCCCGGAGGCGGCG and 5'-CGACGAGGTGGCCTGGGCCAGCTTG). DNA samples (50-100 ng) from 100 donors provided by the Child Development and Research Center (CDRC, Oregon Health

Sciences University, USA) were used as templates for PCR reactions to amplify the CAG rich region. PCR products were analyzed by 4% NuSieve- GTG agarose gel.

Northern Blotting Analyses

Northern hybridization was performed on Human multiple tissue blots (Clontech, Palo Alto, CA) containing approximately 2 μg of poly A+ mRNA from different human tissues. The cDNAs of human PACS-1, human PACS-2 or 1B15 (α-tubulin) were used as templates to generate the random-prime [^{32}p]-labeled probes. The blots were hybridized with the probes following the instructions provided by manufacturer (Clontech, USA). The radioactive signals were detected with Phosphor-imager (Molecular Dynamics, USA)

In situ hybridization analyses

An N-terminal region (1- 1270bp) of rat PACS-1cDNA in pBluescript SKII plasmid (Wan et al, 1998) and a C-terminal region of rat PACS-2 from an EST clone gb AI044507 (IMAGE, USA) were used as templates to generate ^{32}P-labeled random-primed probes. The coronal sections of rat brain, pituitary, adrenal, and testis tissues were prepared as described previously (Day *et al.*, 1992). The sections were hybridized with the PACS probes and detected by autoradiography as described elsewhere (Day *et al.*, 1992).

DNA constructs for expression of human PACS-1 and PACS-2

The pGEX-3x plasmids expressing native furin-cd and VZVgE-cd were as described elsewhere (Jones *et al.*, 1995; Wan *et al.*, 1998). The GST-CI-MPR-cd plasmid was provided by Tooze S (Cambridge, UK). The expression and purification of GST-fusion proteins were performed as described in the manufacturer's manual (Pharmacia , USA).

Human PACS-1 Fbr1 was amplified by PCR using two primers (5'-CGCCCGCTCGAGGTGCCTAGGCTATTCAGC, and 5'-CGCGGATCCATGGGTTG-GCTGGACAGG); and human PACS-2 Fbr2 were amplified with two primers (5'-CGCTCGAGGTGCCCAGGTTGGTGCAGC, and 5'-CTGGATCCGGGCTGGCTG-GACAGGGA). The PCR products were subcloned into pET16 vector cut with XhoI and BamHI (Novagen, USA). The expression and purification of His-tagged proteins from the recombinant pET16b plasmids were performed according to the manufacturer's instructions (Novagen, USA).

An HA-tag was added on the C-terminus of human PACS-1 by PCR with two primers 5'-GTCTTCCGCTCAGTGCAGGT and 5'-GAGCGGATATCACTAGGCGATGTCG-GGCACGTCGTAGGGGTAGAAGGTGGCCTTGCTGCCACT, and subcloned into pBssk(-)/PACS-1a cut with BstEII and EcoRV. An HA-tag was added on the C-terminus of human PACS-2 by PCR with two primers 5'-CCGCTACAACAACTTCTTCC and 5'-GAGCGGATATCACTAGGCGTAGTCGGGCACGTCGTAGGGGTAGAAGGTGGCC TTGGAGTGTCCGAA, and subcloned into pBssk(-)/PACS-2 with AlfII and EcoRV. The HA-tagged PACS constructs were then subcloned into pZVneo plasmid for making recombinant vaccinia viruses as described elsewhere (Hayflick *et al.*, 1992).

***In vitro* binding assay**

GST-fusion proteins were phosphorylated by CK2 *in vitro* as described (Jones *et al.*, 1995), and *in vitro* binding assay between GST fusion proteins and His-tagged proteins was performed as described (Wan *et al.*, 1998). The bound His-tagged proteins were analyzed by western blotting with anti-tetra His monoclonal antibody (1:1000, Qiagen, USA) followed by chemiluminescence detection.

***In vivo* co-immunoprecipitation assay**

BSC-40 cells were grown to 100% confluence in 10cm plates, then infected with PACS recombinant viruses as described previously (Hayflick *et al.*, 1992). At 16 hr post - infection, the media was removed and the cells were washed once with Ca^{2+}/Mg^{2+}-free PBS, then harvested with 2 ml co-immunoprecipitation buffer (1X PBS with pH7.5, 1% NP-40, 1mM E64, 1mM Pepstatin, 1mM Leupeptin, 1mM PMSF and 1mM Aprotinin). The cell lysates were incubated on ice for 20 mins., then spun at 8,000 rpm for 5 mins. at 4°C. Then 7.5 µl of anti-HA antibody (HA.11, Berkeley Molecular, USA), was added to an aliquot of 500 µl supernatant and incubated at room temperature for 2 hr. Then, 25µl protein-G beads were added to each tube for additional 1.5 hr incubation at room temperature. The beads were washed four times with 1X PBS buffer without the protease inhibitors. The bound proteins were resolved with SDS-PAGE for western blotting with anti-HA antibody (HA.11, 1:3000), or antibodies against γ-adaptin (100.3, Sigma; 1:500), α-adaptin (100.2, Sigma; 1:200), δ-adaptin (generous gift from Kelly R., UCSF, USA; 1:5000).

RESULTS

Molecular cloning and sequence analyses of human PACS-1 and PACS-2

Recently identified rat PACS-1 (including two isoforms PACS-1a and PACS-1b) belongs to a gene family involved in membrane protein sorting in the TGN/endosomal system (Wan *et al.*, 1998). Rat PACS-1 encodes a cytosolic connector that recognizes membrane cargo containing AC motifs, and associates with the clathrin adaptor complex AP-1. In order to identify new genes in this family, we used rat PACS-1a cDNA as a probe to screen a human brain cDNA library. Sequence analysis of the positive cDNA clones revealed that they encoded either full-length or overlapping regions of an open reading frame of 963 amino acids. This predicted human protein shared a 95% amino acid identity to that of rat PACS-1a and was named human PACS-1a (Fig. 1a). Menawhile, we used rat PACS-1a as a probe in a TBLASTN search of EST database to search novel genes in the PACS family; and several human EST clones contained inserts encoding amino acid sequences with significant identity to rat PACS-1a. Further analyses of these clones showed some of them encoded sequences identical to human PACS-1a, whereas others encoded sequences with 50-70% identity to human PACS-1a, suggesting that these sequences represented novel protein(s) in the PACS family. We used the cDNA insert from the human EST clone R50031 in the later group as a probe to screen the same human brain cDNA library. Six positive clones were isolated and sequenced. Sequence analyses revealed that all six clones encoded overlap regions of one gene and three of them contained a full-length open reading frame of 889 amino acids. The predicted amino acids from these human cDNAs shared a 54.4% identity to human PACS-1a, and it was named human PACS-2 (Fig. 1a).

Analyses of other EST clones from the TBLASTN search indicated that both human PACS mRNAs had multiple splicing variants. Although we did not find spliced sequences equivalent to rat PACS-1b, two other alternatively spliced sites were revealed in human PACS-1 mRNA: one in the N-terminal region and the other in the C-terminus (Fig. 1b). Similar EST database analysis suggested that human PACS-2 mRNA also had several alternatively spliced variants. One splice site was located in the N-terminal region, at a similar position as the N-terminal splicing site of PACS-1. The second PACS-2 splicing site was located in the C-terminal region. In addition, a human cDNA clone (AB011174) revealed by a BLASTP search with human PACS-2 as a probe, encoded amino acid sequences identical to the human PACS-2 clone except two short insertions (Fig 1b).

As shown in Fig.1a, the most variable region between two human PACS genes is their N-termini. The human PACS-1a N-terminus contains a glycine (G)-rich region followed by proline/glutamine (P/Q) and alanine/serine (A/S) rich region, which is shared by rat PACS-1, but not by human PACS-2 (Fig. 1a and Fig. 1b). Except the PACS-1a N-termini, the homology of human PACS-1a to human PACS-2 extends over their entire amino acid sequences including two highly conserved regions. One region including about 140 amino acids (77% identity between two proteins) is near the N-terminus. It contains the furin-binding site (Fbr) originally identified on rat PACS-1a by the yeast two-hybrid screen (Wan *et al.*, 1998). Another highly conserved region is the C-terminus which contains about 100 amino acids with 78% identity between two human PACS proteins. Structural analyses revealed two conserved putative coiled-coil domains on each human PACS protein. One is located in the Fbrs, and the second one is located 100 residues downstream from the first one (Fig.1b). These two proteins also share many conserved putative phosphorylation sites throughout the whole sequences (Fig 1b).

Database searches showed that *KrT95D*, a *Drosophila* gene, also belonged to the PACS family. Our analyses suggested that the original clone deposited in the Genbank database missed one base-pair in its nucleotide acid sequence (Hartmann and Jäckle, 1997), thus the N-terminus of *KrT95D* was subcloned and sequenced. The predicted amino acid sequences of the correct *KrT95D* shared overall 28.3% and 29.5% identity with human PACS-1a and PACS-2, respectively (Fig.1c). It also displayed 35% and 45% identities with human PACS-2 gene in the conserved Fbrs and C-terminal regions, respectively.

Chromosomal localization of human PACS genes

In order to understand the genetic background of both human PACS genes, a chromosomal mapping of both human PACS genes was carried out. PCR reactions were used to amplify either PACS-1 or PACS-2 specific fragments on the genomic DNA from two different somatic cell hybrid panels: a mono-chromosomal hybrid cell panel #2 and Whitehead Institute GeneBridge 4 Radiation Hybrid (GB4RH) mapping panel. Analyses of the PCR results showed that PACS-1 had significant linkage to 11q13.1-q13.5 and was located between D11S913 and WI-2875 gene markers on human chromosome (HC) 11 (Fig.2). Meanwhile, human PACS-2 gene had significant linkage to 14q32 and is located in the telomeric region on the long arm of the HC14 (Fig.2).

Expression of PACS-1 and PACS-2 in different tissues

Northern blot analyses were performed to examine expression patterns of both human PACS genes. A 4.4 kb PACS-1 mRNA is broadly expressed in human tissues at a high level in the heart, brain, pancreas, testes and leukocytes; and at a moderate or low level in other tissues (Fig.3a). Meanwhile, a 3.8 kb PACS-2 mRNA is also broadly expressed in human tissues at a distinct high level in skeletal muscle, and at high level in the heart,

brain, pancreas and testes, and at a low level in other tissues (Fig.3a). A minor species of about 7 kb was also detected in human brain and skeletal muscle tissue using the PACS-2 probe, which could represent one of those PACS-2 mRNA spliced isoforms (Fig.3a). Furthermore, northern blotting analyses showed that both PACS genes are widely expressed in different regions of human brain. High PACS-1 mRNA levels were observed in the frontal lobe, temporal lobe, and putamen while high levels of PACS-2 mRNA were observed in the medulla, spinal cord and putamen (Fig.3b).

Further characterization of PACS gene expression was carried out by *in situ* hybridization. Figure 4.1 shows the general anatomical distributions of both PACS mRNAs in rat brain coronal sections in the rostral to caudal orientation. The analyses of autoradiogram showed that both PACS were widely distributed in the brain with large differences in regional expression levels. The expression of both mRNAs displayed complementary distribution patterns in rat brain. High levels of PACS-1 gene expression was observed in olfactory tubule, and in the hippocampus (including CA1, CA3 and dentate gyrus). High levels of PACS-1 mRNA were also observed in the piriform cortex and cerebral cortex. In the thalamus and habenula, PACS-2 mRNA were displayed high levels in thalamus and medial habenular nucleus. In pons and medulla, high levels of PACS-1 mRNA were seen in the pons, medulla, and locus coeruleus while high levels of PACS-2 mRNA were observed in the pontine nuclei. . In white matter and spinal cord regions, PACS-2 was detected at high levels in the forceps of the corpus callosum, the corpus callosum itself, the anterior commisure and the cerebral peduncle (Figure 4.1). Together, these results showed that rat PACS-1 and PACS-2 were selectively expressed in different regions in rat brain.

The expression of rat PACS genes in endocrine glands, including pituitary, adrenal and testis tissues, were also examined by *in situ* hybridization. As shown in Figure 4.2, both PACS-1 and PACS-2 mRNAs were detected in the anterior pituitary, but not in intermediate and neural lobes. In adrenal glands, PACS-2 mRNA was detected at high levels in the adrenal medulla. In testes, strong labeling for PACS-1 and PACS-2 mRNAs was observed on the out layer tubules, although at different levels of expression. Moreover, the continuous sections of testis tissue showed that the tubular expression of each PACS mRNA did not appear to be synchronous but complementary (arrows in Figure 4.3). The different intensities between adjacent tubules from serial sections strongly suggest a stage-specific expression of one of the mRNAs (arrows in Figure 4.3). These results showed that both PACS genes have selective expressions in rat endocrine tissues.

PACS proteins interaction with cargo molecules and adaptor complexes

The high sequence identity in the Fbr regions shared by PACS proteins suggests that both PACS proteins bind similarly to membrane cargo proteins. Therefore, *in vitro* binding assays were performed to test whether PACS proteins recognize the CK2 phosphorylated acidic clusters on the membrane cargo cds. GST alone, or GST fusion proteins containing either furin, CI-M6PR or VZV gE cds were phosphorylated by CK2 *in vitro*, then incubated with His-tagged fusion proteins containing the Fbrs of human PACS-1a or PACS-2, respectively. GST or the GST proteins without CK2 phosphorylation were used as controls. The bound His-tagged PACS proteins were analyzed by western blot (Fig 5a). The phosphorylated GST fusion proteins preferentially bound to the PACS Fbrs rather than to the non-phosphorylated ones. In comparison, GST-CI-M6PR bound to more PACS-1 (Fbr1) than to PACS-2 (Fbr2), whereas GST-furin and GST-VZV gE

bound more to PACS-2 (Fbr2) than to PACS-1 (Fbr1). These data showed that both PACS proteins could recognize membrane cargo molecules with different preferences.

The presence of acidic cluster motifs on membrane proteins with different intracellular sorting suggests that PACS could couple the proteins to different sorting machinery. Therefore, *in vivo* co-immunoprecipitation analyses were performed to test whether both PACS proteins are able to associate with adaptor complexes AP-1, AP-2, and AP3 (Fig 5b). An HA-epitope flag was added to the C-termini of both human PACS cDNAs to generate PACS-1a-HA and PACS-2-HA, respectively. The constructs were expressed in BSC-40 cells, and the cell lysates were immunoprecipitated with antibody against the HA-epitope flag on the PACS proteins. The bound proteins were analyzed by western blotting with antibody against the HA-epitope flag, or antibodies against AP-1 (γ-adaptin), AP-2 (α-adaptin), or AP-3 (δ-adaptin). In agreement with previous binding analyses, PACS-1a expressed in BSC-40 cells could co-immunoprecipitate AP-1 (Wan *et al.*, 1998). Meanwhile, PACS-1a could also co-immunoprecipitated AP-3. However, AP-2 failed to be co-immunoprecipitated with PACS-1a. The selective association of PACS-1a with AP-1 and AP-3 but not AP-2 are consistent with previous results showing that PACS-1 was required for sorting in the TGN/endosomal system but not for endocytosis (Molloy *et al.*, 1998; Wan *et al.*, 1998). Like PACS-1a, PACS-2 expressed in BSC-40 cells could co-immunoprecipitate AP-1 and AP-3 with lower affinities compared to PACS-1a, but could not bind to AP-2. These data showed that both PACS proteins associated with different clathrin adaptor complexes, supporting their roles in membrane protein sorting.

DISCUSSION

In this study, we described the identification of two ubiquitously expressed cytosolic sorting proteins in the PACS family, human PACS-1 and PACS-2, although additional PACS gene in mammalian cells was suggested by the EST database search. Human PACS-1 is similar to human PACS-2 not only in the primary sequence (54.4%) but also in domain organization. The highly conserved Fbrs suggest that both PACS proteins play similar functional roles in protein sorting. Indeed, *in vitro* and *in vivo* binding assays showed that both PACS proteins not only bound to cargo molecules (e.g. furin, CI-M6PR, and VZV gE) but also associated with adaptor protein complexes in clathrin sorting machinery (i.e. AP-1 and AP-3). These binding properties confirmed the connector roles of PACS proteins in coupling cargo molecules to AP/clathrin coats for protein sorting.

Human PACS-1 had a 70 amino acid N-terminal fragment, not present in human PACS-2, which could be significant for the functional differences between the two proteins. This PACS-1 specific N-terminus also shared high homology with *atrophin-1*, a gene involved in Dentatorubral pallidoluysian atrophy (DRPLA), a rare, progressive, fatal neuropsychiatric disorder similar to Huntington's disease. A poly-glutamine stretch in this region expands from three glutamines in rat PACS-1 to six glutamines in human PACS-1. The expanded glutamine repeats are involved in several neurodegenerative diseases such as spinocerebellar ataxia (SCA) (Sasaki and Tashiro, 1999). The longer CAG repeats in human PACS-1 than in rat PACS-1 suggests these repeats may be further expanded. Although a PCR screening on a small scale of samples did not yield any evidence of further expansion of the human PACS-1 glutamine repeats (data not shown), it remains to be further investigated whether human PACS-1 is related to genetic disease. In addition,

PACS-1 was located in a region on HC11q13.1, which also contains several other disease-related genes (Fig.2). HC11q13.1 is a "hot" region containing several unknown human disease related genes such as Multiple endocrine neoplasia 1 (MEN1), insulin dependent diabetes mellitus 4 (IDDM-4) (Rosier *et al.*, 1995). Thus, it is also interesting to test whether PACS-1 has any relationship with these or other unknown disease genes located in the same region on HC11.

Although both human PACS genes are broadly expressed, they display different expression patterns in human tissues (Fig 3). It is noteworthy that PACS-2 has a high expression level in human muscle, which is coincident with the *KrT95D* gene isolated from *Drosophila* muscle cells. Interestingly, the expression of a muscle specific clathrin heavy chain is increased during the myoblast differentiation (Sirotkin *et al.*, 1996), and two subunits of AP-3 complex (δ adaptin and σ3A chain) also have selectively high expressions in muscle tissue (Dell'Angelica *et al.*, 1997a; Ooi *et al.*, 1997). These information suggest specific membrane trafficking machinery could be present in muscle cells necessary for their physiological functions, and PACS-2 may have a role in these processes.

Analyses of gene expression by in situ hybridization showed that both PACS displayed distinct but complementary expression patterns in coronal sections of rat brain and endocrine glands with PACS-1 selectively expressed within neurons and PACS-2 in glia cells (Fig.4 and data not shown). These comparative regional and cellular distributions of PACS-1 and PACS-2 mRNAs may contribute to defining potential distinct and/or redundant functions of these proteins. The distributions of PACS proteins are correlated with the expressions of two membrane cargo, furin and carboxypeptidase D (CPD, which binds to PACS protein as well, data not shown) (Cullinan *et al.*, 1991; Day *et al.*, 1993;

Dong *et al.*, 1999). For instance, both PACS-1 and CPD have high expression in the locus coeruleus, a brain region where several neuropeptides, such as galanin, neuropeptide Y and corticotrophin-releasing hormones are found (Gundlach *et al.*, 1990; Holets *et al.*, 1988; Swanson *et al.*, 1983). These overlapping expression patterns between PACS genes and membrane cargo (furin and CPD) support the roles of PACS proteins in the trafficking of these proteases in the secretory pathway, which should be important for their processing function *in vivo*. However, in rat brain, neither PACS genes were detected in some regions with high furin and/or CPD expressions, suggesting that additional member(s) in PACS family may exist (Cullinan *et al.*, 1991; Day *et al.*, 1993; Dong *et al.*, 1999). This hypothesis was also supported by the fact that both PACS mRNAs were not detected in rat pituitary neuron lobe, which also has high expression of furin and CPD (Day *et al.*, 1992; Dong *et al.*, 1999). In rat testes, the distributions of PACS-1 and PACS-2 displayed notably high density in the outer layers of seminiferous tubules where spermatogonia and spermatocytes are enriched (Fig. 4.3). These tubular distributions of PACS suggest that germ cells are the primary site of expression of PACS proteins, and the different intensities between adjacent tubules from serial sections strongly support stage-specific expressions of each PACS mRNAs during spermatogenesis. Together, these data suggest that PACS proteins could play roles in protein trafficking, in tissue- or cell-dependent and/or a developmental stage-dependent manner.

Although the Fbrs of both PACS proteins are capable of binding to many cargo proteins such as furin, CI-MPR and VZV gE, each protein displayed different binding affinities to these various cargo molecules (Figure 5B). A literature search showed that similar binding selectivities were found in protein interactions necessary for membrane protein trafficking. Evidences show that different μ chains in adaptor complexes display

selectivities in recognizing tyrosine-based motifs on membrane proteins such as YXXΦ (where Y is tyrosine, X is any residue and Φ is any hydrophobic residue). This selectivity is proposed to be physiologically significant in protein sorting (Bonifacino and Dell'Angelica, 1999; Le Borgne and Hoflack, 1998a). Therefore, the selectivity between PACS and membrane proteins may play a role in determining the functions of PACS proteins in different sorting events. Both human PACS proteins associate with the clathrin adaptor complexes, including AP-1 and AP-3, but not AP-2. The interactions between PACS and AP complexes are also likely to be regulated by phosphorylation since the PACS proteins were phosphorylated *in vivo* (data not shown). The AP-1 binding to PACS proteins was in agreement with previous reports showing that PACS-1 was involved in the TGN/endosome sorting (Molloy *et al.*, 1998; Wan *et al.*, 1998). The AP-3 binding to PACS protein is interesting since human patients with deficiency of AP-3 genes display multiple disorders related to the biogenesis of lysosomes and/or lysosome-like compartments (Dell'Angelica *et al.*, 1999b; Feng *et al.*, 1999; Kantheti *et al.*, 1998; Zhen *et al.*, 1999). In addition, the recently identified novel AP-4 complex, displaying perinuclear and endosomal staining, presents a potential binding partner for PACS proteins. All together, the regulation on the selectivities of interactions among PACS, cargo molecules and AP complexes are probably critical for precise roles of PACS proteins in distinct sorting steps.

PACS proteins function as connectors between cargo molecules and clathrin coat. In this they are similar to HIV/SIV nef and β-arrestin proteins that are necessary for coupling the CD4 receptors and G-protein coupled receptors, respectively, to AP-2/clathrin coats for downregulation (Foti *et al.*, 1997; Goodman *et al.*, 1996; Piguet *et al.*, 1998). PACS, together with nef and arrestin, represents a group of functionally similar cytosolic factors having critical roles in protein sorting. The identification of PACS gene provides tools to

study the function of these proteins, which are important to illustrate the complex process of membrane protein sorting in mammalian cells.

FIGURE LEGENDS

Figure 1. Molecular cloning and sequence analyses of human PACS-1a and PACS-2. (a) Sequence alignment among, rat PACS-1a, human PACS-1a and human PACS-2. Rat PACS-1a share 95% identity with human PACS-1a whereas the human PACS-2 shares 54.4% identity to human PACS-1a. Residues identical in all three sequences are highlighted in gray. The putative phosphorylation sites are underlined with stars, Note the N-termini of human and rat PACS-1a is missing from the human PACS-2, they share high similarities with atrophin-1. (b) Schematic cartoon of human PACS-1 and PACS-2. The PACS-1 specific atrophin-related region (ARR) which share high identity with atrophin-1. It contains a poly-glutamine stretch and a poly serine/alanine stretch marked in gray, the highly conserved furin binding regions (Fbr, 77% identity) are shaded, and potential CK2 phosphorylation sites are marked in gray boxes. Two predicted coiled-coil domains are also displayed (zigzag). Several cDNA clones containing partial sequences identical to either PACS-1 or PACS-2 are also displayed, and they present the alternative splicing variants of the mRNAs of both genes. The sequences identical to PACS-1 genes are underscored with textured lines whereas the spliced sequences are underscored with wavy lines. The two insertions in AB011174 are $_{301}$CQLQ and $_{667}$RKKHFHFDFTL. (C) Sequence alignment between human PACS-2 and the *Drosophila KrT95D*. The database search with the BLASTP algorithm suggests that the originally published *KrT95D* cDNA sequence missed one base of nucleic acid in the N-terminal region. We used PCR to amplify the region from both *Drosophila* embryonic and adult cDNA libraries with two primers corresponding to cDNA sequences of *KrT95D*. The correct sequences are shown in alignment with human PACS-2. The human PACS-2 and *Drosophila KrDT95* share a 29.5% amino acid identity.

Figure 2. Chromosome localization of human PACS-1 and PACS-2. The chromosomal localizations of human PACS-1 and PACS-2 genes were determined with PCR reactions on the genomic DNA samples in the GB4RH mapping panel, and the PCR results were analyzed with the Whitehead Institute/MIT RH mapping server. Indicated by the vertical line to the right of the ideogram, PACS-1 is located in 11q13.1-q13.5 region between D11S913 marker and WI-2875 marker of chromosome 11. The genetic disease genes located in the same area are displayed: Multiple endocrine neoplasia 1 (MEN 1), Vitreoretinopathy neovascular inflammatory (VRN 1), Insulin independent diabetes mellitus 4 (IDDM 4), vitelliform macular degeneration type 2 (VMD 2), Vitrinoretinopathy exudative familial (EVR 1), and Bardet-Biedl syndrome (BBS 1). PACS-2 is located in 14q32 region on the telomeric region of the long arm on Chromosome 14.

Figure 3. Expression of human PACS-1 and PACS-2 genes by northern blot analyses. Human tissue blots were hybridized with the human PACS-1a or human PACS-2 specific ^{32}P -labeled random-primed probes respectively. To control for variability in mRNA quantity between different tissues, the blots were hybridized with 1B15 probe as control (data not shown). Positions of molecular size markers are indicated on the left. The transcripts of human PACS-1 (~4.4 kb) and PACS-2 (~3.8 kb) express broadly. Note the ubiquitous but distinct expression patterns for both human PACS-1 and PACS-2. The human PACS-1 was high in the brain, testes, pancreas and leukocytes, while the human PACS-2 showed high level expression in the brain, testes, skeleton muscle, and in the heart and pancreas.

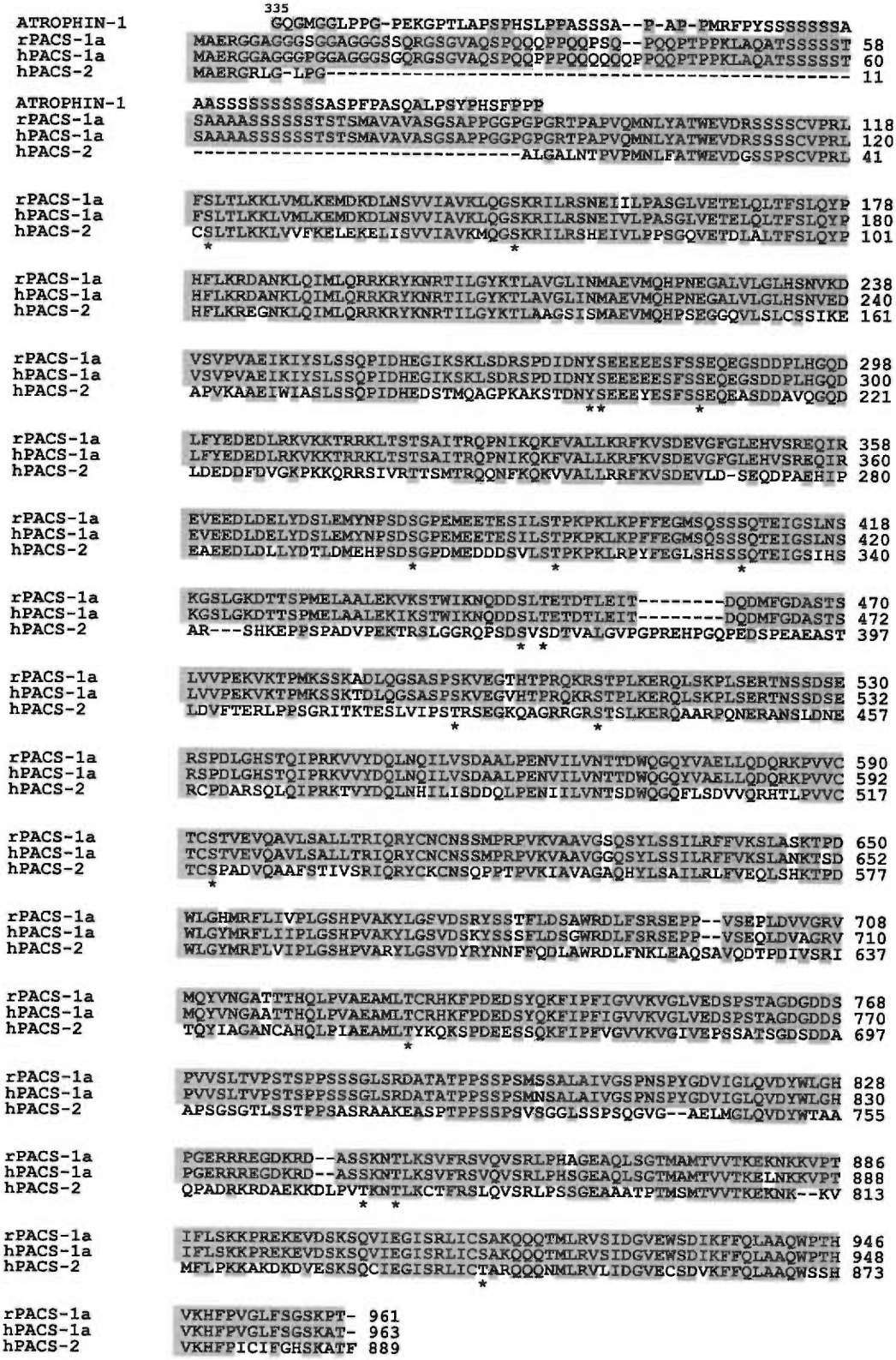
Figure 4. Autoradiographic images of PACS-1 and PACS-2 mRNA distribution in coronal sections of rat brain and glands. (1) Rat brain sections were incubated with

^{32}P-labeled sense probes specific to either rat PACS-1 or rat PACS-2. Hybridized probes were detected by autoradiography. Note the distinct and complementary distribution patterns of PACS-1 as compared to PACS-2. PACS-1 presented a very strong signal in the cortex, piriform cortex, olfactory tubule, hippocampus, hippocampal fissure, dentate gyrus, cerebellum and locus coeruleus. In contrast, PACS-2 transcript was mainly distributed to the forceps major of the corpus callosum, anterior commissure, corpus callosum, medial habenular, thalamus, cerebral peduncle and pontine nuclei. No positive labeling was observed in control sections labeled with PACS-1 or PACS-2 sense probes (not shown). Abbreviations: CBL, cerebellum; cc, corpus callosum; cp, cerebral peduncle; ctx, cerebral cortex; DG, dentate gyrus; fmj forceps major corpus callosum; Hif, hippocampal formation; Hpc, hippocampus; LC, locus coeruleus; Mhb, medial habenular nucleus; Hy, hypothalamus; Pir, prirform cortex; Po, pontine nuclei; Th, Thalamus; and Tu, olfactory tubercle. (2). mRNA distribution in pituitary, adrenal and testis tissues. In the pituitary, labeling is observed for both PACS-1 and PACS-2 in the anterior pituitary. In the adrenal gland, PACS-2 labeling was observed in the adrenal medulla. In the testes, strong labeling for PACS-1 and PACS-2 was observed within the seminiferous tubules with a stage-specific pattern that is consistent with expression within germ cells. (3). mRNA distribution of both PACS-1 and PACS-2 on consecutive rat testis tissue sections. PACS-1 and PACS-2 display complementary distributions with high density in the outer layers of the seminiferous tubules where spermatogonia and spermatocytes are enriched. The white arrows indicate the different intensities of both PACS-1 and PACS-2 in the adjacent tubules.

Figure 5. Interactions of PACS-1 and PACS-2 with acidic cluster motifs and adaptor complexes. (A) The interaction of human PACS-1 (Fbr1) and PACS-2 (Fbr2) with the cds of furin, M6PR and VZV gE were determined by *in vitro* binding. The GST

fusion proteins containing the different cds were either phosphorylated by CK2 *in vitro* or not, and incubated with the His-tagged PACS Fbrs in binding buffer. The bound proteins were detected by western blot with antibodies against the His-tag on the Fbrs. The signals from western blots were quantified using the NIH image analysis software and plotted as interaction levels relative to background signals with GST alone. (B). BSC-40 cells were infected with vaccinia viruses expressing HA-tagged PACS-1 or PACS-2, or vaccinia virus alone. Following protein expression, the cells were lysed and the PACS proteins were immunoprecipitated using monoclonal HA11 antibody. Samples were analyzed for the presence of AP-1, AP-2, and AP-3 by blotting with antibodies against γ-, α- and δ-adaptin, respectively.

Fig. 1a


335
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rPACS-1a MAERGGAGGGSGGAGGGSSQRGSGVAQSPQQQPPQQPSQ--PQQPTPPKLAQATSSSSST 58
hPACS-1a MAERGGAGGGPGGAGGGSGQRGSGVAQSPQQPPPQQQQQQPPQQPTPPKLAQATSSSSST 60
hPACS-2 MAERGRLG-LPG---------- 11
ATROPHIN-1 AASSSSSSSSSSASPFPASQALPSYPHSFPPP
rPACS-1a SAAAASSSSSSTSTSMAVAVASGSAPPGGPGPGRTPAPVQMNLYATWEVDRSSSSCVPRL 118
hPACS-1a SAAAASSSSSSTSTSMAVAVASGSAPPGGPGPGRTPAPVQMNLYATWEVDRSSSSCVPRL 120
hPACS-2 ----------ALGALNTPVPMNLFATWEVDGSSPSCVPRL 41
rPACS-1a FSLTLKKLVMLKEMDKDLNSVVIAVKLQGSKRILRSNEIILPASGLVETELQLTFSLQYP 178
hPACS-1a FSLTLKKLVMLKEMDKDLNSVVIAVKLQGSKRILRSNEIVLPASGLVETELQLTFSLQYP 180
hPACS-2 CSLTLKKLVVFKELEKELISVVIAVKMQGSKRILRSHEIVLPPSGQVETDLALTFSLQYP 101
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hPACS-1a VSVPVAEIKIYSLSSQPIDHEGIKSKLSDRSPDIDNYSEEEEESFSSEQEGSDDPLHGQD 300
hPACS-2 APVKAAEIWIASLSSQPIDHEDSTMQAGPKAKSTDNYSEEEYESFSSEQEASDDAVQGQD 221
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hPACS-1a LFYEDEDLRKVKKTRRKLTSTSAITRQPNIKQKFVALLKRFKVSDEVGFGLEHVSREQIR 360
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hPACS-2 EAEEDLDLLYDTLDMEHPSDSGPDMEDDDSVLSTPKPKLRPYFEGLSHSSSQTEIGSIHS 340
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hPACS-1a KGSLGKDTTSPMELAALEKIKSTWIKNQDDSLTETDTLEIT--------DQDMFGDASTS 472
hPACS-2 AR---SHKEPPSPADVPEKTRSLGGRQPSDSVSDTVALGVPGPREHPGQPEDSPEAEAST 397
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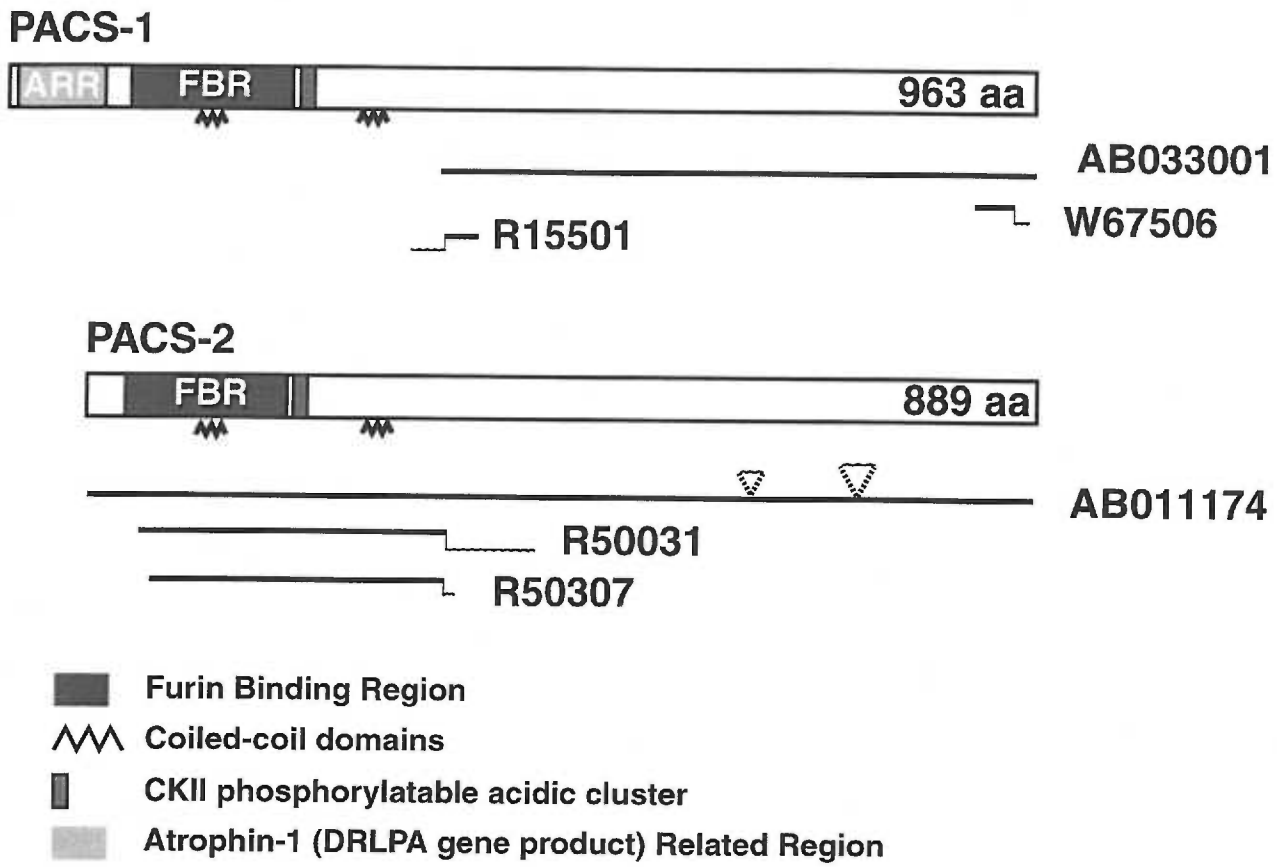
hPACS-2 LDVFTERLPPSGRITKTESLVIPSTRSEGKQAGRRGRSTSLKERQAARPQNERANSLDNE 457
rPACS-1a RSPDLGHSTQIPRKVVYDQLNQILVSDAALPENVILVNTTDWQGQYVAELLQDQRKPVVC 590
hPACS-1a RSPDLGHSTQIPRKVVYDQLNQILVSDAALPENVILVNTTDWQGQYVAELLQDQRKPVVC 592
hPACS-2 RCPDARSQLQIPRKTVYDQLNHILISDDQLPENIILVNTSDWQGQFLSDVVQRHTLPVVC 517
rPACS-1a TCSTVEVQAVLSALLTRIQRYCNCNSSMPRPVKVAAVGSQSYLSSILRFFVKSLASKTPD 650
hPACS-1a TCSTVEVQAVLSALLTRIQRYCNCNSSMPRPVKVAAVGGQSYLSSILRFFVKSLANKTSD 652
hPACS-2 TCSPADVQAAFSTIVSRIQRYCKCNSQPPTPVKIAVAGAQHYLSAILRLFVEQLSHKTPD 577
rPACS-1a WLGHMRFLIVPLGSHPVAKYLGSVDSRYSSTFLDSAWRDLFSRSEPP--VSEPLDVVGRV 708
hPACS-1a WLGYMRFLIIPLGSHPVAKYLGSVDSKYSSSFLDSGWRDLFSRSEPP--VSEQLDVAGRV 710
hPACS-2 WLGYMRFLVIPLGSHPVARYLGSVDYRYNNFFQDLAWRDLFNKLEAQSAVQDTPDIVSRI 637
rPACS-1a MQYVNGATTTHQLPVAEAMLTCRHKFPDEDSYQKFIPFIGVVKVGLVEDSPSTAGDGDDS 768
hPACS-1a MQYVNGAATTHQLPVAEAMLTCRHKFPDEDSYQKFIPFIGVVKVGLVEDSPSTAGDGDDS 770
hPACS-2 TQYIAGANCAHQLPIAEAMLTYKQKSPDEESSQKFIPFVGVVKVGIVEPSSATSGDSDDA 697
rPACS-1a PVVSLTVPSTSPPSSSGLSRDATATPPSSPSMSSALAIVGSPNSPYGDVIGLQVDYWLGH 828
hPACS-1a PVVSLTVPSTSPPSSSGLSRDATATPPSSPSMNSALAIVGSPNSPYGDVIGLQVDYWLGH 830
hPACS-2 APSGSGTLSSTPPSASRAAKEASPTPPSSPSVSGGLSSPSQGVG--AELMGLQVDYWTAA 755
rPACS-1a PGERRREGDKRD--ASSKNTLKSVFRSVQVSRLPHAGEAQLSGTMAMTVVTKEKNKKVPT 886
hPACS-1a PGERRREGDKRD--ASSKNTLKSVFRSVQVSRLPHSGEAQLSGTMAMTVVTKELNKKVPT 888
hPACS-2 QPADRKRDAEKKDLPVTKNTLKCTFRSLQVSRLPSSGEAAATPTMSMTVVTKEKNK--KV 813
rPACS-1a IFLSKKPREKEVDSKSQVIEGISRLICSAKQQQTMLRVSIDGVEWSDIKFFQLAAQWPTH 946
hPACS-1a IFLSKKPREKEVDSKSQVIEGISRLICSAKQQQTMLRVSIDGVEWSDIKFFQLAAQWPTH 948
hPACS-2 MFLPKKAKDKDVESKSQCIEGISRLICTARQQQNMLRVLIDGVECSDVKFFQLAAQWSSH 873
rPACS-1a VKHFPVGLFSGSKPT- 961
hPACS-1a VKHFPVGLFSGSKAT- 963
hPACS-2 VKHFPICIFGHSKATF 889

Fig.1b


PACS-1
ARR
FBR
963 aa
AB033001
W67506
R15501
PACS-2
FBR
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AB011174
R50031
R50307
Furin Binding Region
Coiled-coil domains
CKII phosphorylatable acidic cluster
Atrophin-1 (DRLPA gene product) Related Region

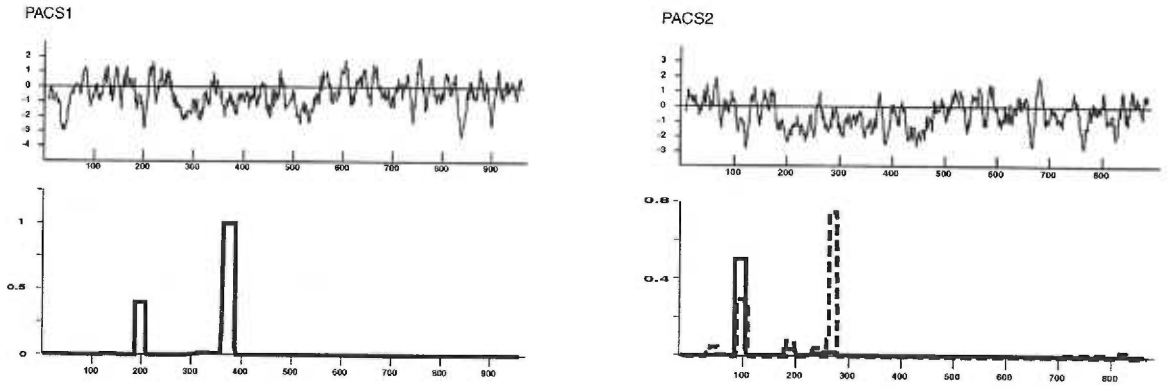
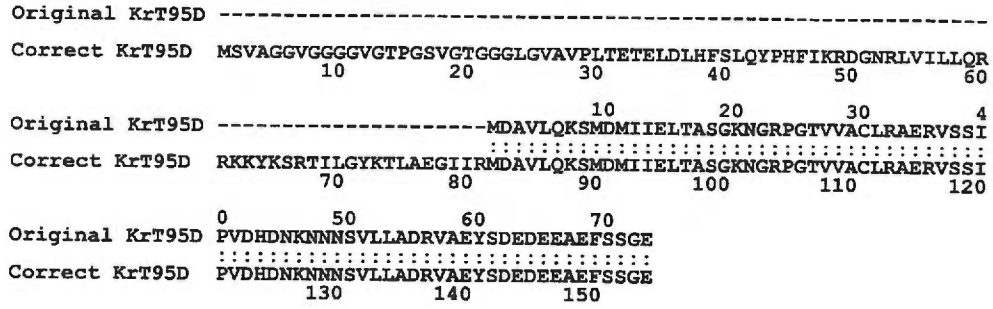

PACS1
PACS2

Fig.1c


Original KrT95D
Correct KrT95D
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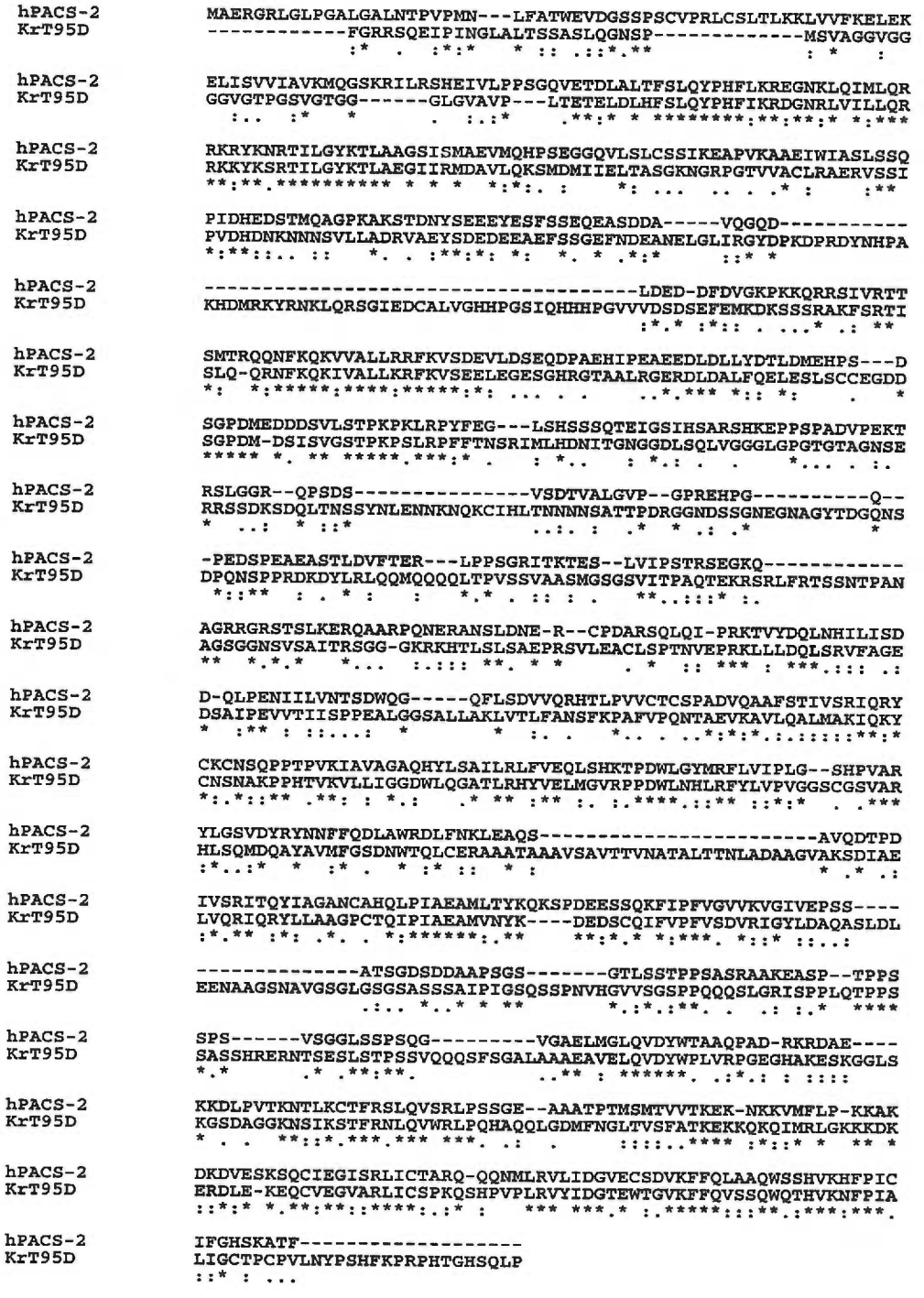
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KrT95D
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RKRYKNRTILGYKTLAAGSISMAEVMQHPSEGGQVLSLCSSIKEAPVKAAEIWIASLSSQ
PIDHEDSTMQAGPKAKSTDNYSEEEYESFSSEQEASDDA-----VQGQD----------
SMTRQQNFKQKVVALLRRFKVSDEVLDSEQDPAEHIPEAEEDLDLLYDTLDMEHPS---D
SGPDMEDDDSVLSTPKPKLRPYFEG---LSHSSSQTEIGSIHSARSHKEPPSPADVPEKT
RSLGGR--QPSDS---------------VSDTVALGVP--GPREHPG----------Q--
-PEDSPEAEASTLDVFTER---LPPSGRITKTES--LVIPSTRSEGKQ----------
AGRRGRSTSLKERQAARPQNERANSLDNE-R--CPDARSQLQI-PRKTVYDQLNHILISD
D-QLPENIILVNTSDWQG-----QFLSDVVQRHTLPVVCTCSPADVQAAFSTIVSRIQRY
CKCNSQPPTPVKIAVAGAQHYLSAILRLFVEQLSHKTPDWLGYMRFLVIPLG--SHPVAR
YLGSVDYRYNNFFQDLAWRDLFNKLEAQS----------AVQDTPD
IVSRITQYIAGANCAHQLPIAEAMLTYKQKSPDEESSQKFIPFVGVVKVGIVEPSS----
ATSGDSDDAAPSGS-------GTLSSTPPSASRAAKEASP--TPPS
SPS------VSGGLSSPSQG---------VGAELMGLQVDYWTAAQPAD-RKRDAE----
KKDLPVTKNTLKCTFRSLQVSRLPSSGE--AAATPTMSMTVVTKEK-NKKVMFLP-KKAK
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Figure 2

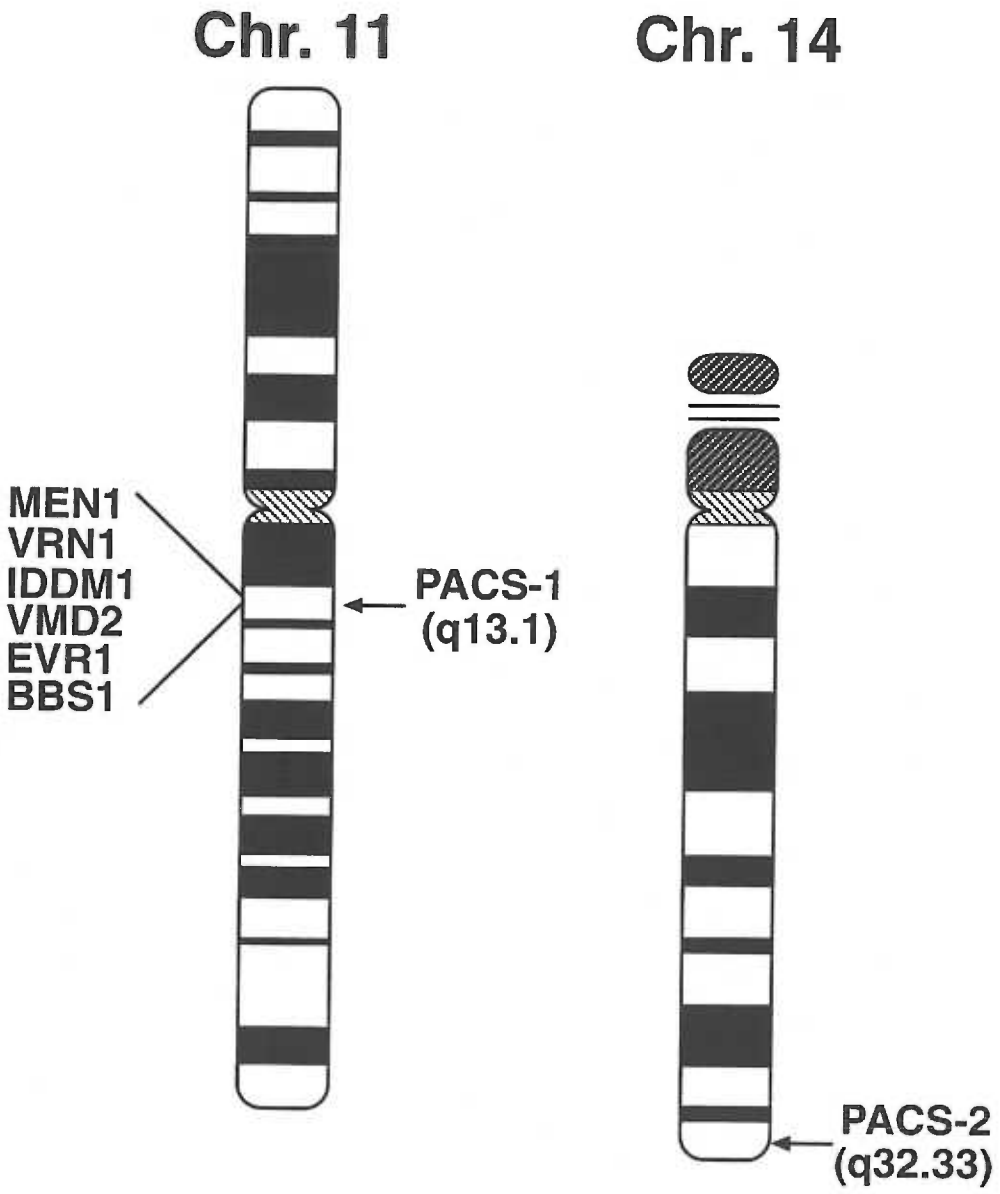
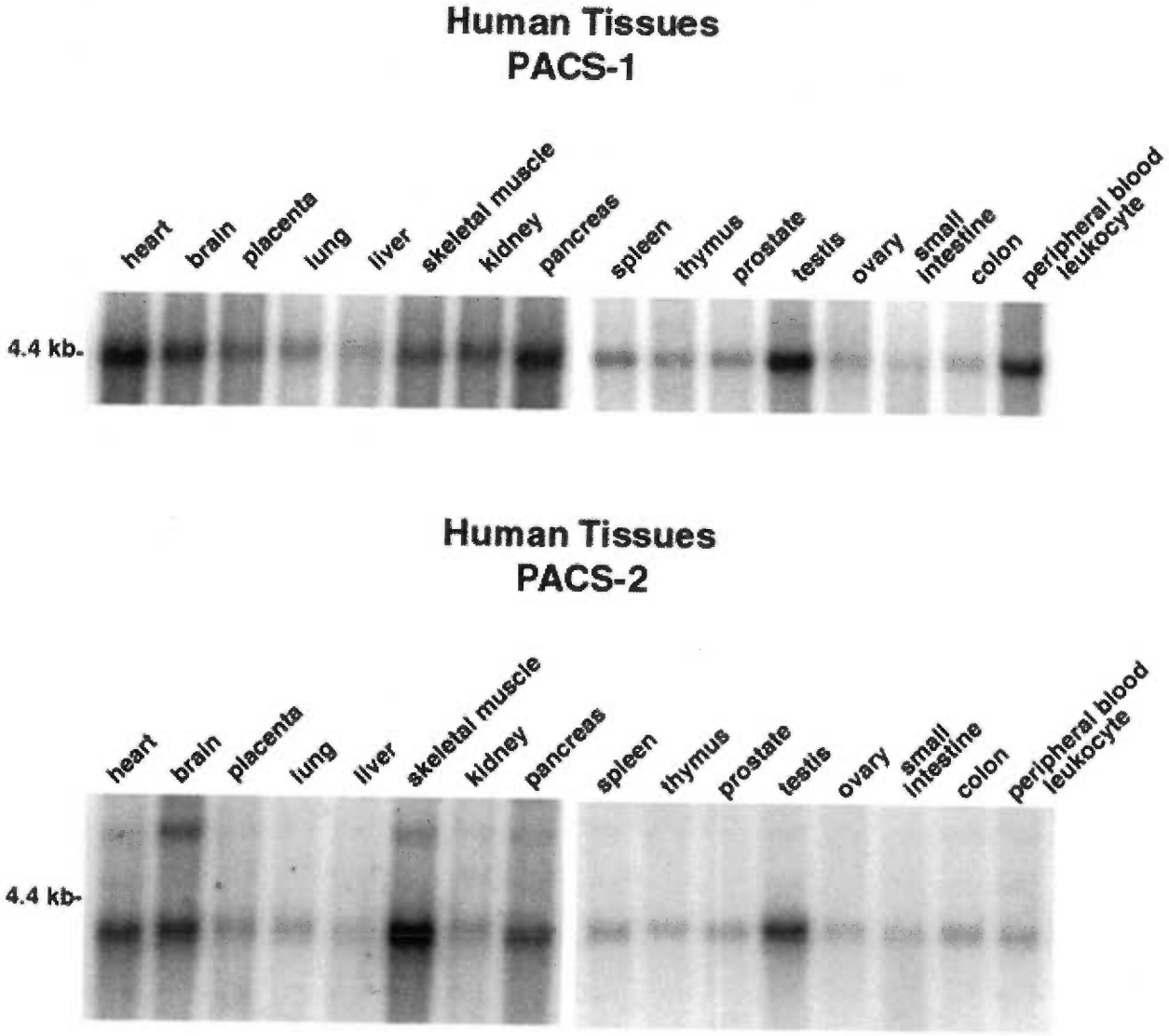

Chr. 11
Chr. 14
MEN1
VRN1
IDDM1
VMD2
EVR1
BBS1
PACS-1
(q13.1)
PACS-2
(q32.33)

Figure 3


Human Tissues
PACS-1
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
peripheral blood leukocyte
4.4 kb-
Human Tissues
PACS-2
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
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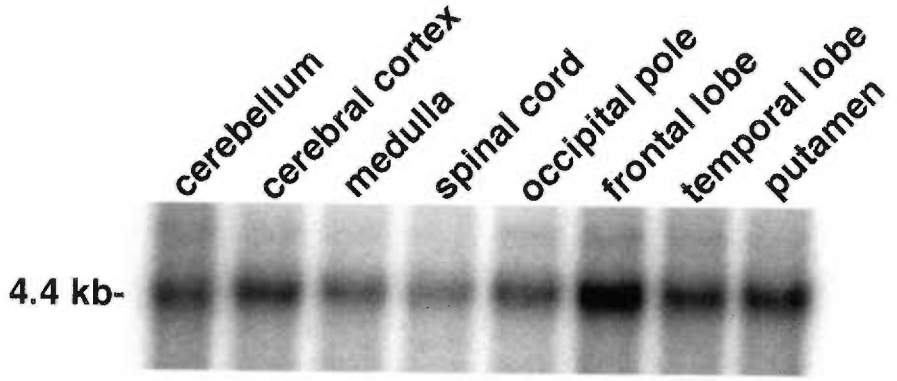
4.4 kb-

Human PACS-1 Brian Regions



Human PACS-2 Brain Regions

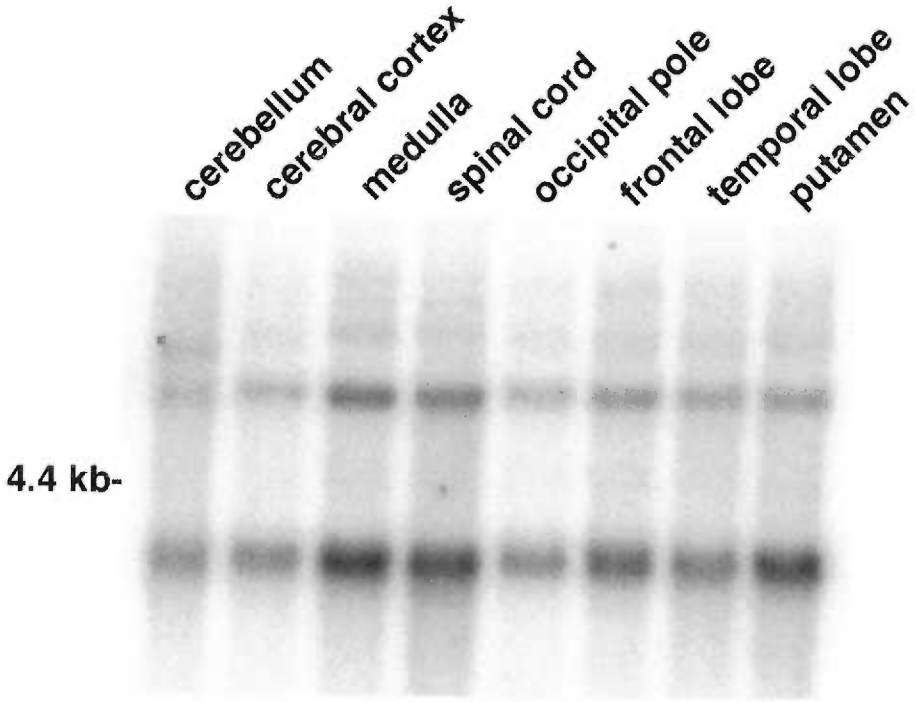


Figure 4

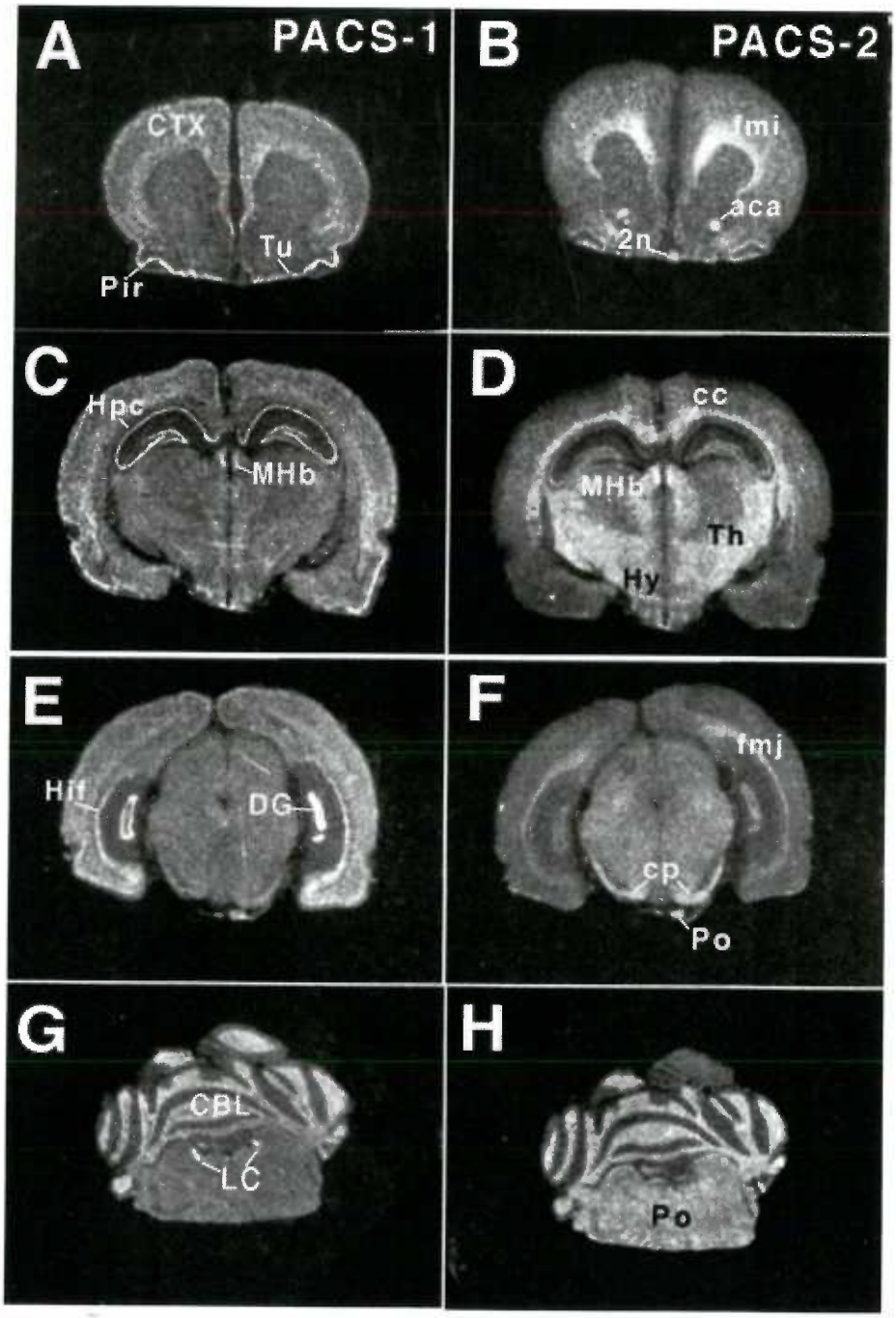

A
PACS-1
CTX
Tu
Pir
B
PACS-2
fmi
aca
2n
C
Hpc
MHb
D
cc
MHb
Th
Hy
E
Hif
DG
F
fmj
cp
Po
G
CBL
LC
H
Po

Figure 4.2

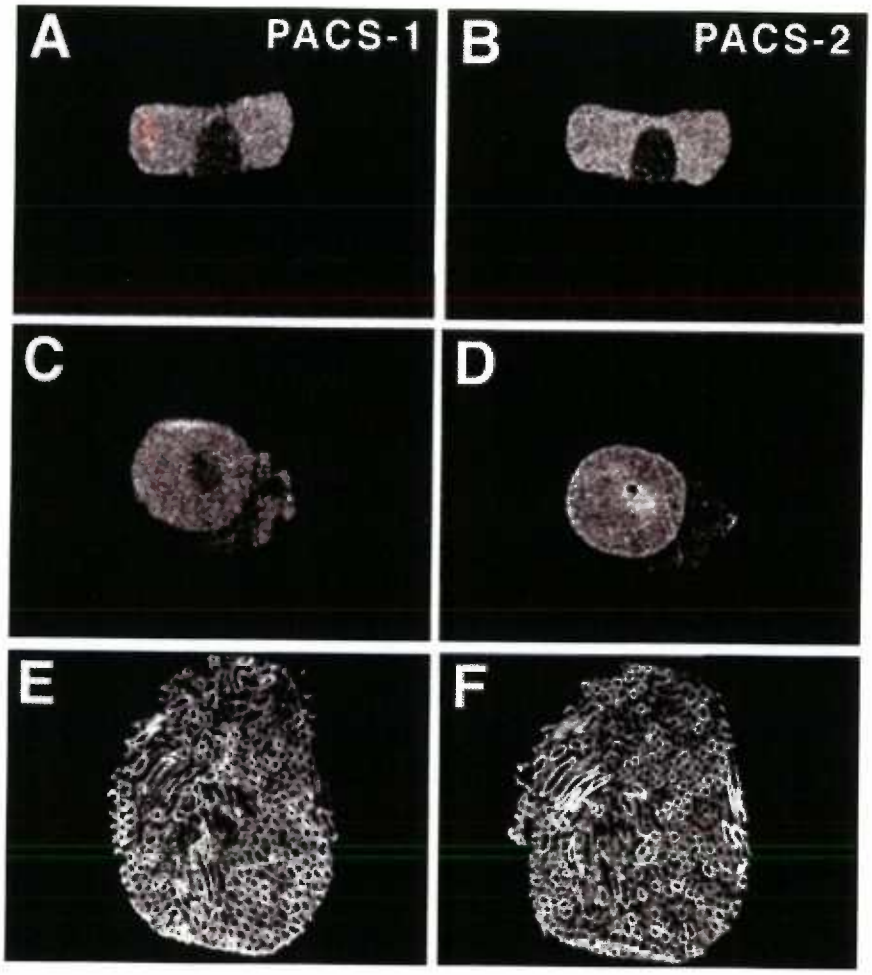


Figure 4.3

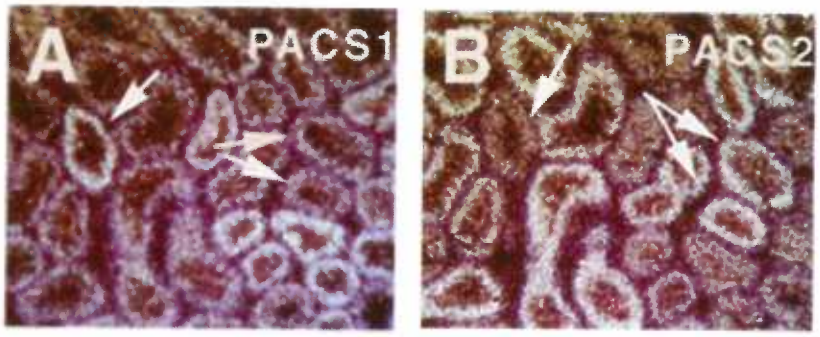
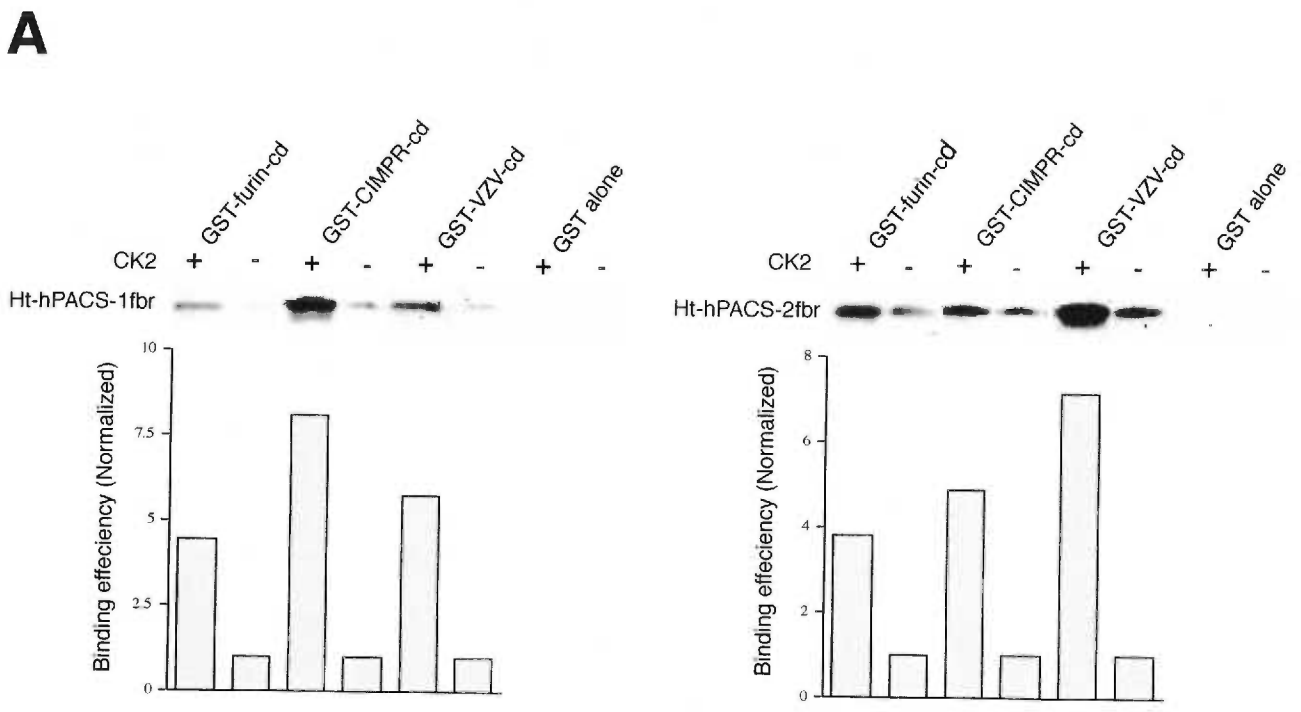
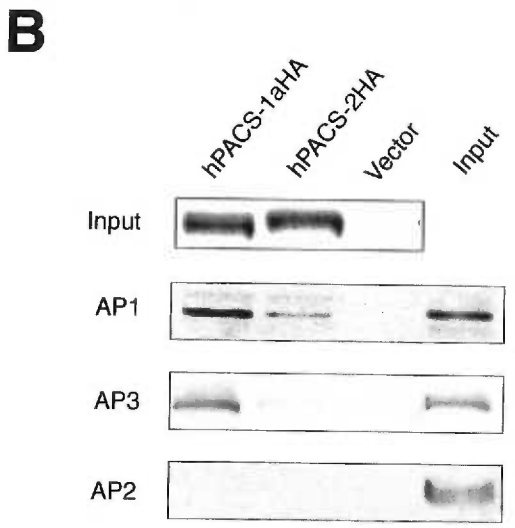


Figure 5


A
GST-furin-cd
GST-CIMPR-cd
GST-VZV-cd
GST alone
CK2
+ - + - + - + -
Ht-hPACS-1fbr
Binding effeciency (Normalized)
10
7.5
5
2.5
0
Ht-hPACS-2fbr
8
6
4
2
0


B
hPACS-1aHA
hPACS-2HA
Vector
Input
Input
AP1
AP3
AP2

Chapter 4

Interaction of PACS-1 with adaptor complexes: A role in protein sorting in neuroendocrine cells.

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Key words: PACS, adaptors, secretory granules, furin, casein kinase-2

Manuscript has been submitted to the *Journal of Biological Chemistry*. In the following section, I performed all the experiments except the Figure 1B by Colin Crump.

ABSTRACT

PACS-1 is a recently identified cytosolic protein involved in controlling the correct sub-cellular localization of certain integral membrane proteins, such as furin, that contain clusters of acidic residues. PACS-1 has also been shown to be in association with the AP-1 adaptor complex. To further understand the role of PACS-1 in membrane traffic pathways we have investigated the interaction of PACS-1 with heterotetrameric adaptor complexes. PACS-1 interacts with both AP-1 and AP-3 but not AP-2. A short motif within PACS-1 that is essential for these interactions has been identified. Mutation of this motif has yielded a dominant negative form of PACS-1 which can still bind to acidic cluster motifs but not to adaptor complexes. Over-expression of dominant negative PACS-1 causes a mislocalization of furin from the TGN to secretory granules in neuroendocrine cells but has no effect on the localization of other proteins which do not contain acidic cluster motifs. Thus we have generated a useful tool for the study of PACS-1 mediated membrane traffic and given an insight into a role for PACS-1 in neuroendocrine cells.

INTRODUCTION

The *trans*-Golgi network (TGN) and endosomal system is a series of highly dynamic and interconnected membrane bound organelles involved in the trafficking and modification of numerous proteins of the secretory and endocytic (Mellman, 1996; Traub and Kornfeld, 1997). The localization and sorting of membrane proteins in the TGN / endosomal system often requires the presence of specific sorting motifs within their cytosolic domains. The most well characterized sorting motifs found within the cytosolic domains of numerous integral membrane proteins are the tyrosine- and di-leucine-based signals (Marks *et al.*, 1997). These motifs are important for a variety of protein trafficking steps including endocytosis, targeting to the late endosome / lysosome compartments (and their derivatives such as melanosomes), targeting to the vacuole in yeast and targeting to the basolateral surface of polarized cells (Sandoval and Bakke, 1994; Trowbridge and Collawn, 1993). Certainly many, and potentially all, of the functions ascribed to tyrosine-based and di-leucine-based signals arise from the interaction of these motifs with heterotetrameric adaptor complexes AP-1, -2, -3 and -4 have so far been identified (Kirchhausen, 1999).

A more recently identified family of sorting motifs are based upon clusters of acidic residues often containing one or more consensus sites for phosphorylation by CK2. Acidic-cluster sorting motifs are found in many proteins with varied membrane trafficking itineraries. These sorting motifs are known to be important for the trafficking of the proprotein convertases furin and PC6B, the mannose 6-phopshate receptors (MPR) and some herpes virus envelope glycoproteins such as the varicella-zoster virus (VZV) gE protein (Alconada *et al.*, 1999; Chen *et al.*, 1997a; Dittie *et al.*, 1997; Jones *et al.*, 1995). In the case of furin and VZV-gE, the integrity of their phosphorylatable acidic

clusters are crucial for the correct subcellular localization of these proteins to the TGN (Alconada *et al.*, 1999; Jones *et al.*, 1995). Furthermore, the acidic clusters within the cytosolic domains of the cation independent (CI) and cation dependent (CD) MPR have been shown to be important for transport of lysosomal hydrolases by these receptors (Chen *et al.*, 1997a; Mauxion *et al.*, 1996).

Recently a novel protein termed PACS-1 was identified through its ability to specifically interact with the cytosolic domain of furin in a CK2 phosphorylation dependent manner (Wan *et al.*, 1998). PACS-1 interacts with the cytosolic domains of other integral membrane proteins containing acidic clusters (such as VZV-gE and CI-MPR) and antisense studies showed PACS-1 is important for the correct subcellular localization of both furin and CI-MPR (Wan *et al.*, 1998). PACS-1 was also shown to interact with the AP-1 adaptor complex, thus prompting the proposal that PACS-1 functions as a connector protein linking the cytosolic domains of proteins containing acidic clusters to clathrin adaptor complexes (Molloy *et al.*, 1999). In agreement with this proposal are previous data demonstrating that CK2 mediated phosphorylation of the acidic cluster within the cytosolic domain of furin enhances the association of furin with AP-1 (Dittie *et al.*, 1997). PACS-1 has also been recently shown to interact with the HIV-1 protein Nef and that this interaction has a functional role in the Nef mediated down regulation of class I major histocompatibility complexes (Piguet *et al.*, 2000).

Here we show that PACS-1 interacts with both AP-1 and AP-3 but not AP-2 from cytosol and that the binding of adaptor complexes to PACS-1 is through a direct protein-protein interaction. We have identified a determinant on PACS-1 necessary for binding AP-1, and mutated this binding region in PACS-1. The mutant PACS-1 functions in a dominant negative manner with respect to adaptor binding and provides a powerful means of

disrupting the PACS-1-mediated sorting of membrane proteins. Finally, we have shown this dominant negative PACS-1 interferes with furin trafficking in neuroendocrine AtT-20 cell, causing furin to be missorted from the TGN into secretory granules.

MATERIAL AND METHODS

DNA constructs

PACS-1ha was generated by inserting the coding sequence for HA at the 3' end of the human PACS-1 open reading frame in the pBssk(+) vector (Stratagene). The PACS-1ha construct was subcloned into pZVneo plasmid to make recombinant vaccinia viruses as previously described (Hayflick *et al.*, 1992; VanSlyke *et al.*, 1995).

Vectors encoding GST fusion proteins of four fragments covering the entire PACS-1 protein sequence were generated as follows. A fragment of first half of human PACS-1 was amplified with oligonucleotides XY-010 (5'-CGGTACCATGGCGGAACGCGGAGGGGCG) and YX-010 (5'-CAGGGCCCTCACACTTTGCTGGGGGAGCAGA), digested with *KpnI* and *ApaI*, blunt ended with T4 polymerase and ligated into the *SmaI* site of pGEX4T-1 (Pharmacia). This recombinant vector was digested with *EcoRI* and the fragment encoding residues 1-153 was cloned into the *EcoRI* site of pGEX4T-1. A DNA fragment encoding residues 117-266 was amplified with oligonucleotides XY-011 (5'-CGGGATCCCGGGTACCGTGCCTAGGCTATTCAGC) and YX-111 (5'-CGTGGATCCTCACTTGGATTTGATTCCTTCAT) and cloned into the *BamHI* of pGEX3X. A DNA fragment encoding residues 240-499 was amplified with oligonucleotides XY-051 (5'-AGGGTACCGTCTCTGTGCCTGTGGCA) and YX-013 (5'-CAGGGCCCTCACACTTTGCTGGGGGAGGCAGA), digested with *KpnI* and *EcoRI* and ligated into pGEX3X (Pharmacia) cut with same enzymes. A DNA fragment encoding residues 500-963 was amplified with the oligonucleotides XY-012 (5'-CAGGGTACCGAGGGGGTGCACACACCCCGG) and YX-012 (5'-GCAGGGCCCTCAGGTGGCCTTGCTGCCACTGAA), digested with *KpnI* and *ApaI*,

blunt ended with T4 polymerase and cloned into the *SmaI* site of pGEX4T-1. Truncation constructs of the FBR region of PACS-1 were subcloned into the *BamHI* site of pGEX3X following amplification with the following oligonucleotide pairs. Δ1: XY-011 + YX-050 (5'-AGGGATCCTCAATCTTCAACGTTGCTGTG); Δ2: XY-011 + YX-101 (5'-ACGGGATCCTCACAAGGTCTTATAGCCCAA); Δ3: XY-011 + YX-114 (5'-ACGGAATTCTCAATCTTTGTCCATTTCTTT); Δ4: XY-105 (5'-CTGGATCCCGGGTACCCTTCGCTCCAACGAGATC) + YX-011; Δ5: XY-106 (5'-CTGGATCCCGGGTACCTTAACCTTCTCCCTTCAG) + YX-011; Δ6: XY-119 (5'-CTGGATCCCGGGTACCATCATGCTGCAAAGGAGA) + YX-011; Δ7: XY-105 (5'-CTGGATCCCGGGTACCCTTCGCTCCAACGAGATC) + YX-100 (5'-ACGGGATCCTCATTGGAGCTCTGTTTCCAC); Δ8: XY-115 (5'-CTTGGATCCCCAGCTAGTGGACTGGTG) + YX-124 (5'-CATGAATTCTCAGTACTGAAGGGAGAAGGT).

An eight amino acid stretch of PACS-1 (E_{168}TELQLTF_{175}) was replaced with eight alanine residues by PCR mutagenesis using overlapping oligonucleotides encoding the mutated codons. The mutated fragment was then subcloned into the *EcoNI* and *AvrII* sites of pGEX-PACS-$1_{117-266}$ and pZVneo-PACS-1ha for expression of GST-PACS-$1_{117-266}$-8A and production of recombinant vaccinia virus expressing PACS-1ha-8A respectively.

The pET32-furin-cd vector was constructed by amplification of the furin cytosolic domain with oligonucleotides COL007 (5'-GGATCCCGCTCTGGCTTTAGT) and COL008 (5'-CTCGAGTCAGAGGGCGCTCTGGTC) followed by subcloning into the *BamHI* and *XhoI* sites of pET32a (Novagen).

In vivo co-immunoprecipitation

BSC-40 cells were grown to 100% confluence on 10 cm plates and infected with recombinant viruses as described previously (Molloy *et al.*, 1994). At 16 hr post-infection cells were washed once with PBS and harvested with 2 ml co-IP buffer (PBS, pH7.5, 1% NP-40, 1mM E64, 1mM pepstatin A, 1mM leupeptin, 1mM PMSF, 1mM aprotinin). The cell lysates were incubated for 20 min on ice and centrifuged at 8000 rpm for 5 min at 4°C. 500µl of each supernatant sample were incubated with 7.5µl anti-HA antibody (HA.11, Berkley Molecular) at room temperature for 2 hours. Samples were incubated with 25µl of protein G-sepharose at room temperature for 90 min. Beads were harvested by centrifugation (5000 rpm, 2 min, room temperature) and washed four times with 750µl co-IP buffer without protease inhibitors. Samples were then analyzed by western blotting followed by chemiluminescence detection using antibodies against γ-adaptin (100.3, Sigma; 1:500), α-adaptin (100.2, Sigma; 1:200), δ-adaptin (generous gift from R. Kelly, UCSF, USA; 1:5000), or the HA-epitope on the PACS proteins (HA.11; 1:3000).

Protein purification and cytosol preparation

pGEX vectors encoding the various GST-PACS-1 constructs described above were co-transformed into the bacterial strain BL21 (DE3) pLysS (Invitrogen) with the pT-Trx plasmid (Yasukawa *et al.*, 1995) and GST fusion proteins were expressed and purified using glutathione-sepharose following the manufacturers guidelines (Pharmacia). Thioredoxin tagged furin cytosolic domain (TRX-furin-cd) encoded by the pET32-furin-cd vector was expressed following transformation into the bacterial strain BL21 (DE3) pLysS and TRX-furin-cd was purified using Ni-NTA agarose following the manufacturers instructions (Qiagen). Purified AP-1 was obtained as described (Austin *et al.*, 2000).

BSC-40 cytosol was prepared as follows: BSC-40 cell were grown to 100% confluence on 15cm plates and harvested with 2ml GST binding buffer (150mM NaCl, 50mM Tris pH7.5, 2mM $MgCl_2$, 1% NP40) supplemented with protease inhibitors (1mM E64, 1mM pepstatin A, 1mM leupeptin, 1mM PMSF and 1mM aprotinin). The cell lysates were incubated for 20 min on ice and centrifuged at 8000 rpm for 5 min at 4°C to remove cell debris.

GST protein binding assays

50µl aliquots of BSC-40 cytosol were incubated with 5µg of each respective GST fusion protein in GST binding buffer for 45 min at 37°C. Each sample was then incubated with 20µl of glutathione-agarose for 30 min at room temperature. Glutathione beads were harvested by centrifugation (5000 rpm, 2 min, room temperature) and washed 5 times with 750µl GST binding buffer. Samples were then analyzed by western blotting followed by chemiluminescence detection using antibodies against γ-adaptin (100.3).

For the interaction assays with purified AP-1 and TRX-furin-cd, 1µg purified AP-1 or 2µg CK2 phosphorylated TRX-furin-cd (CK2 phosphorylation performed as previously described, (Jones *et al.*, 1995)) were incubated with 5µg of each respective GST fusion protein in the GST binding buffer for 45 min at 37°C. Each sample was incubated with 20µl of glutathione-agarose for 30 min at room temperature. Glutathione beads were harvested by centrifugation (5000 rpm, 2 min, room temperature.) and washed 5 times with 750µl buffer. Samples were then analyzed by western blotting followed by chemiluminescence detection using antibodies against γ-adaptin or the His-tag of TRX-furin-cd (anti-tetra His, Qiagen; 1:1000)

Two peptides containing the amino acid sequences LVETELQLTFSQLY (corresponding to PACS-1 residues 166-179) or EGIKSKLSDRSPDI (corresponding to PACS-1 residues 261-274) were synthesized by Macromolecule (Colorado State University, USA). Peptides were reconstituted in GST binding buffer + 5% DMSO. The peptides were added to the GST binding tubes at various concentrations as shown in the figure legends.

In vitro translation

cDNA fragments encoding μ1 and γ adaptins were excised from the plasmids pACT/μ1 and pACT/γ (kindly provided by J. Bonifacino; NIH, Bethseda, USA) with *EcoRI / XhoI* and *BamHI / EcoRI* respectively. The cDNA fragment encoding β1 adaptin was excised from the plasmid pGAD10/β1 (kindly provided by K. Nakayama, University of Tsukuba, Ibaraki, Japan) with *EcoRI*. All three cDNA fragments were subcloned into pBssk(+) cut with the same enzymes. The recombinant pBssk-adaptin vectors (1μg/50μl) were used as templates for *in vitro* translation from the T7 promoter according to the manufacture instructions (Promega, USA). Reaction tubes were centrifuged at 14000 rpm for 10 min at 4°C to remove aggregation. 10μl of each translation reaction was incubated with either GST-PACS-$1_{117-266}$ or GST alone in GST binding buffer for 45 min at 37°C. Each sample was then incubated with 20μl of glutathione-agarose for 30 min at room temperature. Glutathione beads were harvested by centrifugation (5000 rpm, 2 min, room temperature) and washed 5 times with 750μl GST binding buffer. Bound proteins were resolved by SDS-PAGE and signals detected by autoradiography.

Membrane fractionation

100% confluent BSC-40 cells were infected with the recombinant vaccinia viruses expressing PACS-1ha, PACS-1ha-8A or WT virus at moi 5 as previously described (Molloy *et al.*, 1994). Cell were harvested on ice with HEPES buffer (10mM HEPES, pH

7.2, 1mM EDTA, 1mM E64, 1mM pepstatin A, 1mM leupeptin, 1mM PMSF and 1mM aprotinin), lysed by 8 passes through a 25 gauge needle and centrifuged at 8000 rpm for 5 min at 4°C to remove cell debris. Supernatants were centrifuged at 45000 rpm for 60 min at 4°C and the resulting membrane pellets were resuspended in GST binding buffer. Samples were then analyzed by western blotting followed by chemiluminescence detection using antibodies against γ-adaptin (100.3), α-adaptin (100.2), δ-adaptin, or the HA-epitope on the PACS proteins (HA.11). The signals were quantified with the NIH image software. The data represent the mean of three independent experiments plus and minus one standard deviation, and are expressed as relative levels of membrane association normalized to control levels (WT virus).

Immunofluorescence

A7 cells were grown to 70-80% confluency and fixed and processed for immunofluorescence as previously described (Molloy *et al.*, 1994). Primary antibodies to PACS-1 (polyclonal 599, 1:50) together with monoclonal antibodies to AP-1 (100.3, 1:50) or AP-3 (generous gift from J. Bonifacino, NIH, Bethseda, USA; 1:50) were incubated for 4 hours at room temperature. AtT-20 cell were grown to 80% confluence and infected with recombinant vaccinia viruses expressing either PACS-1ha or PACS-1ha-8A alone (moi 10) or together with vaccinia viruses expressing either fur/f or fur/f$S_{773,775}$A (moi 5, described in (Jones *et al.*, 1995)) as previously described (Molloy *et al.*, 1994). Cells were fixed and processed for immunofluorescence as previously described (Molloy *et al.*, 1994). Primary antibodies to FLAG-tag (monoclonal M1, IBI; 1:300) together with polyclonal antibodies to ACTH (AS29; 1:200) or TGN38 (1479, generous gift from S. Milgram, University of North Carolina, USA; 1:1000) were incubated overnight at 4°C. Following incubation with fluorescently labeled secondary

antisera (Southern Biotech), images were captured using a 63x oil immersion objective on a Leica DM-RB microscope and processed with the Scion Image 1.62 program.

RESULTS

Previous data has shown that full-length rat PACS-1 can associate with the AP-1 adaptor complex in cytosol (Wan *et al.*, 1998). To determine whether human PACS-1 could also interact with adaptor complexes, recombinant HA tagged human PACS-1 (PACS-1ha) was expressed in cells and immunoprecipitated with anti-HA antibodies. The resulting samples were then analyzed by western blotting using antisera directed against specific subunits of the AP-1, AP-2 and AP-3 adaptor complexes (γ, α and δ adaptins respectively). In agreement with previous data, human PACS-1ha co-immunoprecipitated AP-1 (Figure 1A, lane 1). Furthermore human PACS-1ha co-immunoprecipitated AP-3 but not AP-2 (Figure 1A, lane 1). The selective association of PACS-1 with AP-1 and AP-3 but not AP-2 is consistent with previous results showing a requirement for PACS-1 for sorting in TGN /endosomal system but not for endocytosis (Molloy *et al.*, 1998; Wan *et al.*, 1998). A greater interaction level for PACS-1 with AP-1 than AP-3 was apparent from the same co-immunoprecipitation samples even though similar levels of both AP-1 and AP-3 signals were present in the cytosol (Figure 1A, lane 1 and 3). This may suggest a higher affinity interaction of PACS-1 for AP-1 than AP-3. Samples immunoprecipitated with anti-HA antibodies from cells infected with vector alone showed no presence of any adaptor complex (Figure 1A, lane 2).

Immunofluorescence analysis of human cells with specific anti-PACS-1 antisera demonstrated a punctate distribution of endogenous PACS-1 mainly localized to a perinuclear region (Figure 1B, panel i and iv). Double labeling of cells with antisera to PACS-1 and AP-1 or AP-3 demonstrated a partial co-localization of endogenous PACS-1 with both AP-1 and AP-3 in agreement with the ability of PACS-1 to interact with both adaptor complexes (Figure 1b, panels i-vi).

To identify the region of PACS-1 responsible for binding to adaptor complexes the entire 963 amino acid human protein was subdivided into four regions. The four regions of PACS-1 were chosen to cover different potential structural domains. These were the N-terminal atrophin related region (ARR, residues 1-116); the furin binding region containing a predicted coiled coil domain (FBR, residues 117-266); the middle region containing a second predicted coiled-coil region (MR, residues 267-499); and the C-terminal region of which most is absent from the minor isoform of the rat protein, PACS-1b (CTR, residues 500-963) (Figure 2A). GST fusion proteins covering each of the four regions of PACS-1 were expressed as GST fusion proteins, incubated with cytosol and recovered with glutathione agarose. Western blot analysis of recovered proteins demonstrated only GST-PACS-$1_{117\text{-}266}$ was able to associate with AP-1 suggesting all the information necessary for adaptor interaction is contained within this 149 amino acid sequence (Figure 2B). To determine if the association between PACS-1 and adaptor complexes is a direct protein-protein interaction not involving other cytosolic factors, GST-PACS-$1_{117\text{-}266}$ was tested for its ability to interact with purified AP-1. As shown in Figure 2C, purified AP-1 interacts with GST-PACS-$1_{117\text{-}266}$ but not with GST alone. This shows that PACS-1 can interact directly with the adaptor complex AP-1 (and presumably AP-3). 30-50% of the total purified AP-1 input was found to interact with GST-PACS-$1_{117\text{-}266}$ in these assays (data not shown).

Of the four subunits of the AP-1 heterotetrameric adaptor complex, three have so far been shown to interact with cytosolic factors: β1 with clathrin heavy chain and di-leucine-like motifs (Rapoport *et al.*, 1998; Shih *et al.*, 1995), γ with γ-synergin (Page *et al.*, 1999) and μ1 with tyrosine based motifs (Ohno *et al.*, 1995). To determine which of the subunits of AP-1 is responsible for interaction with PACS-1, the GST-PACS-$1_{117\text{-}266}$ protein was

tested for interaction with in vitro translated β1-, γ- and μ1 adaptins, a technique previously used to show interactions between tyrosine based motifs and μ2 (Ohno *et al.*, 1995). PACS-1 demonstrated a specific interaction with μ1 but not with β1 or γ-adaptins (Figure 2D). These data imply PACS-1 interacts with the μ chains of heterotetrameric adaptor complexes.

To further characterize the residues within PACS-1 required for interaction with AP-1, a series of truncation constructs were generated containing incremental deletions from both the N- and C-termini of GST-PACS-$1_{117\text{-}266}$ (Figure 3A). Each construct was incubated with cytosol and bound AP-1 was detected by western blot analyses. Analysis of the GST-PACS-$1_{117\text{-}266}$ C-terminal deletion mutants (Δ1 and Δ2) showed that the C-terminal 55 amino acids (residues 212-266) are not required for binding to AP-1. However, further truncation of additional 75 residues (Δ3) completely blocked the interaction with AP-1. Analysis of the GST-PACS-$1_{117\text{-}266}$ N-terminal deletion mutants (Δ4 and Δ5) showed binding to AP-1 does not require the N-terminal 55 amino acids (residues 117-172). However, further deletion of additional 19 amino acids (residues 173-192, construct Δ6) abolished binding. Two additional GST fusion proteins were generated to more rigorously identify the PACS-1 amino acids required for binding to AP-1. Construct Δ7, containing the 18 amino acid PACS-1 sequence between residues 154-172, failed to bind AP-1 consistent with the ability of construct Δ5, which lacks these residues, to bind AP-1. However, the overlapping construct Δ8 (residues 163-180) demonstrated AP-1 binding. These data suggest determinants sufficient to bind AP-1 are present within the 18 amino acid sequence of PACS-1 encoded by the Δ8 construct.

A further series of GST fusion proteins of PACS-$1_{117\text{-}266}$ were constructed which contained mutations of 3 or 4 sequential amino acid stretches to alanines covering the

whole potential AP-1 interaction domain (residues 163-180). None of these mutations were able to abolish the interaction with AP-1 suggesting the interaction site within PACS-1 for adaptors involve more than 3 or 4 consecutive residues (data not shown). Therefore, a mutant GST-PACS-$1_{117\text{-}266}$ was constructed containing eight consecutive residues (E_{168}TELQLTF_{175}) substituted alanines (GST-PACS-$1_{117\text{-}266}$-8A, Figure 3A, labeled as 8A). When incubated with cytosol, GST-PACS-$1_{117\text{-}266}$-8A failed to bind AP-1 suggesting this eight amino acid stretch contains essential determinants for adaptor binding. Given the interaction of GST-PACS-$1_{117\text{-}266}$ with μ1 (Figure 2D) it is of interest to note that a tyrosine residue is present just downstream of the eight amino acid substitution and is found within a potential consensus motif for interaction with μ chains (Y_{179}PHF_{182}). This potential tyrosine-based motif cannot be essential for interaction between PACS-1 and adaptors because mutation of the tyrosine residue to alanine during the 3-4 residue alanine substitutions over this region failed to significantly inhibit interaction (data not shown). However, the possibility that this tyrosine residue plays some role in the binding of PACS-1 to adaptors cannot be discounted.

To determine whether the eight-alanine mutation was causing a gross structural deformation of this domain of PACS-1, GST-PACS-$1_{117\text{-}266}$ and GST-PACS-$1_{117\text{-}266}$-8A were tested for their ability to interact with phosphorylated furin cytosolic domain. Both of these two constructs, but not GST alone, were able to interact with phosphorylated furin cytosolic domain with similar efficiency (Figure 3B). These data imply the eight-alanine substitution does not significantly alter the structure of this region of PACS-1 because GST-PACS-$1_{117\text{-}266}$-8A can still interact with cargo molecules. This eight-alanine mutation thus allows cargo and adaptor binding to be uncoupled.

The importance of PACS-1 residues 168-175 for the interaction of PACS-1 with AP-1 was further confirmed using peptide competition studies. Two peptides were synthesized, one covering the potential AP-1 interaction domain (corresponding to residues L_{166}VETELQLTFSQLY$_{179}$) and another covering the C-terminal end of the FBR domain (corresponding to residues E_{261}GIKSKLSDRSPDI$_{274}$). The ability of GST-PACS-$1_{117-266}$ to interact with AP-1 from cytosol in the presence or absence of increasing concentrations of these two peptides was analyzed. Presence of 50μM peptide corresponding to residue 166-179 peptide (AP-PEP$_{166-179}$) reduced the interaction level of GST-PACS-$1_{117-266}$ with AP-1 and 500μM AP-PEP$_{166-179}$ almost completely abolished interaction with AP-1. By contrast, the control peptide corresponding to residues 261-274 (CON-PEP$_{261-274}$) failed to interfere with the interaction between GST-PACS-$1_{117-266}$ and AP-1 at any concentration tested (Figure 3C).

The protein-protein interaction data detailed above has identified a short sequence of eight amino acids (168-175) that are involved in the binding of a fragment of PACS-1 to AP-1 but not to cargo molecules containing acidic cluster motifs. To determine the role of this adaptor complex binding domain *in vivo* an expression construct was generated containing a HA-tagged version of the full coding sequence of PACS-1 with residues 168-175 replaced by alanines (PACS-1ha-8A). Two sets of experiments were conducted to test if PACS-1ha-8A could interact with adaptor complexes. Firstly, cells expressing PACS-1ha, PACS-1ha-8A, or a vector control, were harvested, proteins immunoprecipitated with anti-HA antibodies, and the presence of co-immunoprecipitated AP-1 was determined by western blot analyses. In agreement with the GST protein binding assays, PACS-1ha but not PACS-1ha-8A could co-immunoprecipitate AP-1 (Figure 4A). Secondly, the ability of PACS-1ha and PACS-1ha-8A to recruit adaptor complexes to cellular membranes was examined. Low-density membrane fractions were

prepared from cells expressing either PACS-1ha, PACS-1ha-8A or vector control. Western blot analyses showed that PACS-1ha over-expression increased the membrane association of both AP-1 and AP-3 (approximately 4-fold and 2.5-fold respectively) but not AP-2 (Figure 4B). These data are consistent with the ability of PACS-1 to interact with both AP-1 and AP-3 (but not AP-2) and to interact with integral membrane proteins. In contrast, over-expression of PACS-1ha-8A failed to increase the association of any adaptor complexes with membranes above control levels (Figure 4B). Similar levels of both PACS-1ha and PACS-1ha-8A associated with membranes in cells expressing these proteins (Figure 4B, inset) consistent with the ability of PACS-1ha-8A to still associate with integral membrane proteins. Taken together these data demonstrates that PACS-1ha-8A functions as a *bona fide* dominant negative construct with respect to adaptor binding but not interaction with cargo, enabling these two activities to be uncoupled.

Previous data have demonstrated that PACS-1 is important for the correct subcellular localization of integral membrane proteins containing acidic cluster motifs within their cytosolic domains, namely furin and CI-MPR (Wan *et al.*, 1998). In the case of furin, the role of PACS-1 is thought to be in the retrieval of furin from post TGN compartments back to the TGN. However the localization of other integral membrane proteins which lack acidic cluster motifs (such as TGN38) to the TGN is thought to be independent of PACS-1. To test whether the interaction of PACS-1 with adaptor complexes is necessary for the localization of furin to the TGN, PACS-1ha or PACS-1ha-8A were co-expressed in AtT-20 cells with an epitope (FLAG)-tagged furin protein (fur/f) and the distributions of both furin and TGN38 were analyzed by immunofluorescence. In cells over-expressing PACS-1ha, furin demonstrated a perinuclear distribution that overlapped well with the staining pattern of TGN38, reminiscent of the normal subcellular localization of furin (Figure 5a-c). In cells over-expressing PACS-1ha-8A, furin demonstrated a dispersed,

punctate distribution throughout the cytosol including localization to the cell processes whereas TGN38 showed a normal, perinuclear type, staining pattern (Figure 5d-f). These data suggest that PACS-1 molecules, which are defective in adaptor complex binding, can no longer retrieve furin from post TGN compartments but cause no gross defect in the morphology of the TGN or the localization of proteins that use distinct sorting machinery (such as TGN38).

In the morphogenesis of secretory granules in neuroendocrine cells, furin is incorporated into immature secretory granules (ISGs) and then removed from the ISGs during their maturation into dense-core secretory granules (Dittie *et al.*, 1997). The retrieval of furin from ISGs is thought to require the recruitment of AP-1 and clathrin to the granules (Dittie *et al.*, 1997). Furthermore phosphorylation of furins acidic cluster by CK2 increases the recruitment of AP-1 to ISGs (Dittie *et al.*, 1997). To determine whether the redistribution of furin observed in the presence of PACS-1ha-8A expression was due to an increased proportion of furin in secretory granules, PACS-1ha-8A and fur/f were co-expressed in AtT-20 cells and the distribution of furin and the secretory granule marker ACTH were analyzed by immunofluorescence. The redistributed furin caused by over-expression of PACS-1ha-8A showed significant co-localization with ACTH within punctate structures throughout the cytosol (Figure 5g, h and insets). These data imply PACS-1 that is able to interact with adaptor complexes is important for the efficient retrieval of furin from ISGs. To further confirm the importance of PACS-1 in the retrieval of furin from ISGs, the effect of mutating the binding site for PACS-1 within the cytosolic domain of furin was examined. The interaction of PACS-1 with furin requires the CK2 phosphorylation of two serine residues within furins acidic cluster. A FLAG-tagged furin construct, in which these two serines has been mutated to alanines (fur/f-S773, 775A) was expressed in AtT-20 cells and the distributions of the furin mutant and

ACTH were analyzed by immunofluorescence. In agreement with previous data (Dittie *et al.*, 1997) the mutation of the acidic cluster serine residues in furin caused a mislocalization of mutant furin to punctate structures throughout the cytosol which co-localized well with ACTH (Figure 5i, j and insets). Taken together, these data imply recruitment of PACS-1 and associated adaptor complexes by furin is essential for efficient retrieval of furin from immature secretory granules in neuroendocrine cells.

DISCUSSION

Acidic-cluster motifs found within the cytosolic domains of many integral membrane proteins have been shown to be important for their correct subcellular localization and trafficking events (Molloy *et al.*, 1998). A protein termed PACS-1 which can specifically interact with acidic cluster motifs has recently been identified (Wan *et al.*, 1998). PACS-1 was shown to be essential for the normal subcellular localization of both furin and CI-MPR and furthermore data suggested PACS-1 could interact with the AP-1 adaptor complex. These results suggested a connector role for PACS-1 whereby this novel protein could link adaptor complexes to integral membrane proteins containing acidic cluster motifs. Further examples of proteins that may play an analogous role to the connector function proposed for PACS-1 can be found in the literature. β-arrestin is known to mediate the internalization of G-protein coupled receptors and interact with both clathrin and the AP-2 adaptor complex (Goodman *et al.*, 1996; Laporte *et al.*, 1999). The HIV protein Nef interacts with the cytosolic domain of CD4 and AP-2 causing the endocytosis and down regulation of CD4 (Piguet *et al.*, 1998). Therefore both β-arrestin and Nef can function by connecting integral membrane protein cargo to adaptor complexes, and in the case of β-arrestin, also to the clathrin lattice directly, serving to control the trafficking of these proteins. Nef has recently been shown to also interact with PACS-1 and this interaction functions in the down regulation of MHC class I complexes (Piguet *et al.*, 1998). In this report we have investigated the interaction of human PACS-1 with adaptor complexes and the determinants within PACS-1 that are important for these interaction.

The data presented show that PACS-1 interacts with both AP-1 and AP-3 in cytosol but not AP-2, consistent with a known functional role of PACS-1 for trafficking events in the

TGN/endosomal system but not for endocytosis (Molloy *et al.*, 1998; Wan *et al.*, 1998). Also consistent with these data, endogenous PACS-1 shows a partial co-localization with both AP-1 and AP-3 in the perinuclear region of cells. PACS-1 appears to associate with higher levels of AP-1 than AP-3 from cytosol compared to the level of these two adaptor complexes present in the cytosol samples. Whether this difference is due to a truly higher affinity of PACS-1 for AP-1 or just a greater accessibility of AP-1 than AP-3 for interaction with the overexpressed PACS-1 has yet to be determined. Whilst PACS-1 can associate with adaptors in cytosol it was unclear whether the interaction was a direct protein-protein binding event or through some other intermediates. The use of purified AP-1 and *in vitro* translated AP-1 adaptin subunits demonstrated PACS-1 does indeed interact directly with AP-1 and that this interaction appears to be via the μ1 subunit. By inference PACS-1 seems likely to interact directly with AP-3 through its μ3 subunit. The interaction of PACS-1 with the newly identified AP-4 adaptor complex has not been tested but given that the proposed function of AP-4 is also in the TGN/endosomal system (Dell'Angelica *et al.*, 1999a; Hirst *et al.*, 1999), an interaction between PACS-1 and AP-4 may be expected. The observation that acidic cluster motifs are found in a wide variety of membrane proteins which have very different trafficking itineraries may suggest that PACS-1 and/or functionally equivalent proteins could control multiple secretory and endocytic pathway trafficking events (Molloy *et al.*, 1998). For example PACS-1 has also been shown to be essential for maintaining a sub-population of furin in a cycling loop between the plasma membrane and early endosomes (Molloy *et al.*, 1998). Such a variety of roles for PACS-1 could be reconciled with the potential differential binding of PACS-1 to the various adaptor complexes or the presence of PACS-1 isoforms with different adaptor complex binding properties.

Mapping of the region of PACS-1 necessary for interaction with AP-1 defined an eighteen amino acid sequence that was capable of interacting with AP-1 *in vitro*. Whilst mutation of any 3 or 4 consecutive residues to alanines over this region of PACS-1 failed to abolish *in vitro* interaction with AP-1, a mutant in which 8 consecutive residues (168-175) were replaced by alanines failed to interact with AP-1. This eight alanine mutation had no observable effect on the interaction of PACS-1 with cargo (phosphorylated furin cytosolic domain) *in vitro* suggesting the inhibition of AP-1 binding caused by this mutation is not purely due to a gross distortion of the structure of this PACS-1 domain. A synthetic peptide covering this potential adaptor binding region of PACS-1 was also able to inhibit the interaction between PACS-1 and AP-1 whilst a peptide sequence from another area of PACS-1 was not inhibitory. These peptide inhibition data further support a role for this short motif (168-175) in the interaction of PACS-1 with adaptor complexes. Given the interaction between PACS-1 and *in vitro* translated μ1, it is of interest to note the presence of a tyrosine residue (position 179) within the eighteen amino acid sequence that is capable of interacting with AP-1. In the full sequence of PACS-1 this tyrosine resides within a YPHF motif, very similar to the consensus tyrosine-X-X-bulky hydrophobic residue motif required for interaction of integral membrane proteins with μ chains (Ohno *et al.*, 1998). The mutation of this tyrosine during the triple and quadruple alanine scanning of this region failed to abolish interaction of PACS-1 with AP-1 suggesting that the tyrosine is not essential for interaction of PACS-1 with adaptors. However it is conceivable that this tyrosine could be involved in the interaction of PACS-1 with the tyrosine-binding pocket of μ chains, but multiple residues within the sequence upstream of Y_{179} are also required for the binding of PACS-1 to the AP-1 complex. Consistent with this hypothesis, previous data have shown that modification of residues upstream and downstream of tyrosine based motifs within integral membrane protein cytosolic domains can alter the affinity and

specificity of their interactions with μ chains (Stearns *et al.*, 1990; Stephens and Banting, 1998; Stephens *et al.*, 1997).

The eight-residue mutation to alanine that abolished *in vitro* interaction of PACS-1 with AP-1 was introduced into full length PACS-1 for expression in mammalian cells to analyze the potential dominant negative effect of this mutation *in vivo*. This mutation abolished the ability of PACS-1 to co-immunoprecipitate AP-1 from cytosol. Furthermore whilst this mutant PACS-1 (PACS-1ha-8A) could still be recruited to internal membranes as efficiently as wild type PACS-1 it could no longer mediate the recruitment of AP-1 or AP-3 to these internal membranes. However, expression of PACS-1ha-8A was unable to significantly reduce the amount of membrane-associated adaptors beyond control cells, suggestive that the membrane proteins containing acidic cluster motifs (e.g. furin and CI-MPR) are not the principal receptors for adaptor complexes. These findings are in agreement with earlier studies showing that reduced levels of PACS-1 generated by antisense expression cause mislocalization of furin and CI-MPR but not AP-1 (Wan *et al.*, 1998).

When expressed in cells PACS-1ha-8A caused a mislocalization of furin from the TGN to secretory granules as demonstrated by co-localization with the secretory granule marker ACTH. The distribution of TGN38, which is localized to the TGN by a PACS-1 independent mechanism, was not affected by overexpression of the PACS-1 mutant suggesting the disruption of furin trafficking was not due to a gross change in TGN morphology or function. The PACS-1ha-8A mediated mislocalization of furin to secretory granules is consistent with previous studies of the phosphorylation state-dependent sorting of furin in neuroendocrine cells (Wan *et al.*, 1998). Cell fractionation studies showed furin buds from the TGN into ISGs together with cargo molecules

destined for storage in dense-core secretory granules (e.g. secretogranin II and ACTH) (Dittie *et al.*, 1997). However, furin molecules are retrieved from ISGs prior to granule maturation and this retrieval of furin from ISGs requires CK2-phosphorylation of the furin cytosolic domain. Taken together these data suggest the recruitment of both PACS-1 and associated AP-1 to the phosphorylated acidic cluster within the cytosolic domain of furin is essential for furins efficient retrieval from ISGs prior to maturation of the granules.

In summary these data show a clear role for the interaction of PACS-1 with adaptor complexes being essential for the transport of integral membrane proteins containing acidic cluster motifs. These data should enable many more studies to determine the exact roles of PACS proteins in multiple membrane trafficking events.

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FIGURE LEGENDS

Figure 1: Association of PACS-1 with adaptor complexes. A) PACS-1 can co-immunoprecipitate bot AP-1 and AP-3 but not AP-2. BSC-40 cells were infected with recombinant vaccinia viruses expressing PACS-1ha or wild type viruses and proteins were immunoprecipitated from cytosol with anti-HA antibodies (HA.11). Resulting samples were analyzed for the presence of co-immunoprecipitated adaptor complexes by western blotting followed by chemiluminescence detection using specific antisera to AP-1 (monoclonal anti-γ-adaptin, 100.3; row 1), AP-2 (monoclonal anti-α-adaptin, 100.2; row 2) and AP-3 (polyclonal anti-δ-adaptin; row 3). Expression and immunoprecipitation of PACS-1ha was confirmed by detection with monoclonal antisera to the HA tag (HA.11; row 4). B) PACS-1 partially colocalizes with both AP-1 and AP-3. A7 cells were fixed and co-stained with antibodies against PACS-1 (polyclonal 599; panels i and iv) and AP-1 (monoclonal anti-γ-adaptin, panel ii) or AP-3 (monoclonal anti-AP-3, panel v) followed by fluorescein conjugated anti-rabbit and Texas red conjugated anti-mouse antisera. The co-localization of PACS-1 (green) with AP-1 or AP-3 (red) are shown (panels iii and vi respectively).

Figure 2. Interaction of PACS-1 domains with AP-1. A). Schematic representation of PACS-1 and GST-PACS-1 domain fusion proteins (ARR: atrophin-related region; FBR: furin binding region; MR: middle region; CTR: C-terminal region). Predicted coiled-coil domains are shown (zigzags). B) The FBR domain of PACS-1 interacts with AP-1. Each GST-PACS-1 domain fusion protein and GST alone were incubated with BSC-40 cytosol, isolated with glutathione resin and analyzed for the presence of AP-1 by western blotting followed by chemiluminescence detection using monoclonal anti-γ-adaptin (100.3). C) The PACS-1 FBR domain interacts directly with purified AP-1. The purity of

isolated AP-1 was demonstrated by separation of purified AP-1 preparation by SDS-PAGE and Coomassie Blue staining (panel 1). GST-PACS-$1_{117-266}$ and GST alone were incubated with purified AP-1, isolated with glutathione resin and analyzed for the presence of AP-1 by western blotting followed by chemiluminescence detection using monoclonal anti-γ-adaptin (100.3; panel 2). D) The PACS-1 FBR domain interacts with the μ1 subunit of AP-1. GST-PACS-$1_{117-266}$ and GST alone were incubated with ^{35}S labeled *in vitro* translated μ1, β1 and γ-adaptins, isolated with glutathione resin, separated by SDS-PAGE and the presence of radiolabeled adaptins detected by autoradiography.

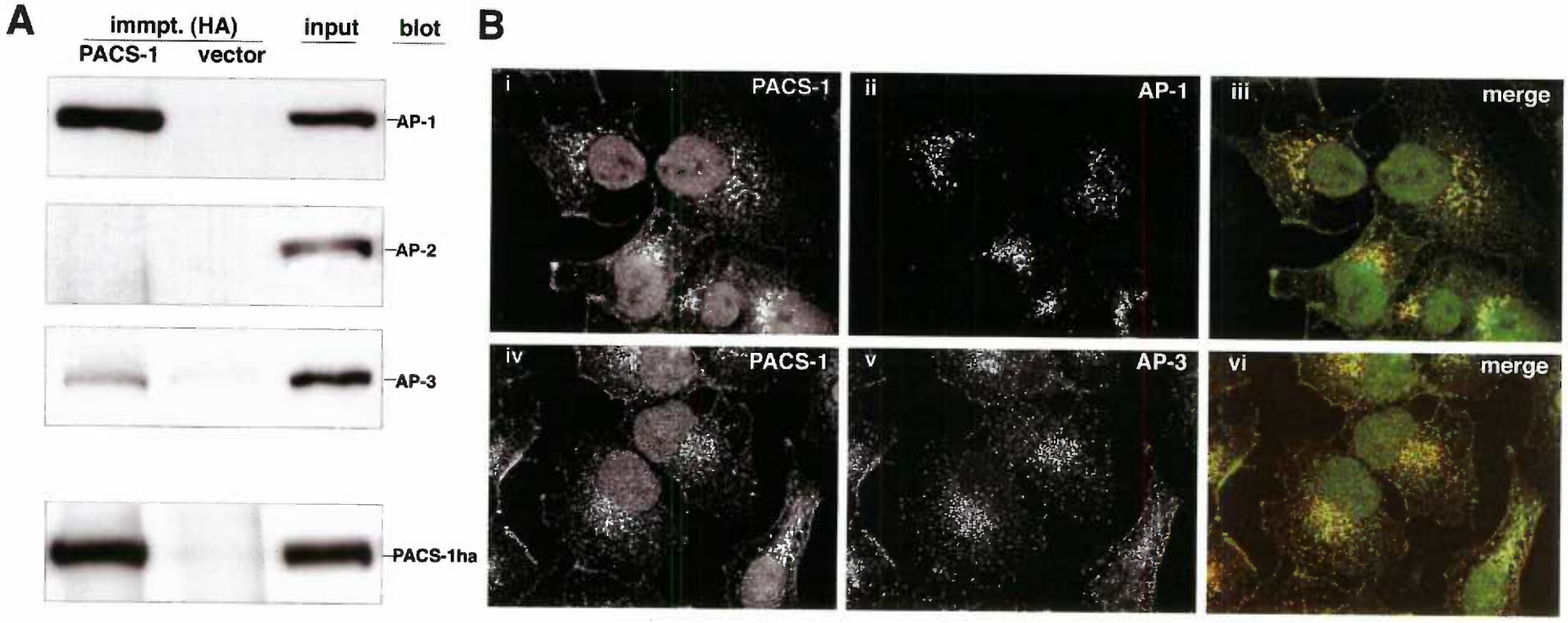
Figure 3. Sequence determinants required for PACS-1 interaction with adaptor complexes. A) Schematic representation of the FBR region of PACS-1 (residues 117-266), the various truncation mutants generated (Δ1-Δ8) and an eight amino acid section (residues 168-175) that was replaced with alanines (PACS-$1_{117-266}$-8A, labeled as 8A). GST fusion proteins of each of these constructs were incubated with BSC-40 cytosol, isolated with glutathione resin and analyzed for the presence of AP-1 by western blotting followed by chemiluminescence detection using monoclonal anti-γ-adaptin (100.3). Interaction between each GST fusion protein and AP-1 is signified by a + (positive interaction) or - (negative interaction). B) Mutation of PACS-1 residues 168-175 to alanines does not effect interaction of PACS-1 with phosphorylated furin cytosolic domain. GST fusion proteins of native PACS-1 FBR (GST-PACS-$1_{117-266}$), the eight alanine mutant PACS-1 FBR (GST-PACS-$1_{117-266}$-8A) and GST alone were incubated with CK2 phosphorylated TRX-furin-cd, isolated with glutathione resin and the presence of TRX-furin-cd was analyzed by western blotting followed by chemiluminescence detection using monoclonal anti-tetra-His antisera. C) A peptide corresponding to PACS-1 residues 166-179 inhibits the interaction of PACS-1 with AP-1. GST fusion proteins of native PACS-1 FBR (GST-PACS-$1_{117-266}$) were incubated with BSC-40 cytosol in the

presence of 50μM or 500μM control peptide (CON-$PEP_{261-274}$) or competing peptide (AP-$PEP_{166-179}$). GST-$PACS\text{-}1_{117-266}$ proteins were isolated with glutathione resin and samples were analyzed for the presence of AP-1 by western blotting followed by chemiluminescence detection using monoclonal anti-γ-adaptin (100.3).

Figure 4. Effect of mutation of PACS-1 residues 168-175 on *in vivo* interaction with adaptor complexes. A) Mutation of PACS-1 residues 168-175 to alanines abolishes the ability of PACS-1 to co-immunoprecipitate AP-1. BSC-40 cells were infected with recombinant vaccinia viruses expressing PACS-1ha, PACS-1ha-8A or wild type viruses and proteins were immunoprecipitated from cytosol with anti-HA antibodies (HA.11). Resulting samples were analyzed for the presence of co-immunoprecipitated AP-1 by western blotting followed by chemiluminescence detection using monoclonal anti-γ-adaptin antisera (100.3; panel 1). Expression and immunoprecipitation of PACS-1ha and PACS-1ha-8A was confirmed by detection with monoclonal antisera to the HA tag (HA.11; panel 2). B) Overexpression of PACS-1ha but not PACS-1ha-8A increases the membrane association of AP-1 and AP-3. BSC-40 cells were infected with wild type viruses (WT) or recombinant vaccinia viruses expressing PACS-1ha (P) or PACS-1ha-8A (8A) and intracellular membranes were harvested. Resulting samples were analyzed for the presence of adaptor complexes by western blotting followed by chemiluminescence detection using specific antisera to AP-1 (monoclonal anti-γ-adaptin, 100.3; columns 1-3), AP-2 (monoclonal anti-α-adaptin, 100.2; columns 4-6) and AP-3 (polyclonal anti-δ-adaptin; columns 7-9). Membrane association of PACS-1ha and PACS-1ha-8A was analyzed by western blotting followed by chemiluminescence detection with anti-HA antisera (HA.11; see inset). Data were quantified using the NIH gel image analysis software. The mean of three independent experiments plus and minus one standard deviation is shown.

Figure 5. Overexpression of dominant negative PACS-1 disrupts the subcellular localization of furin and causes miss localization of furin to secretory granules in AtT-20 cells. AtT-20 cells were co-infected with recombinant vaccinia viruses expressing FLAG tagged furin and PACS-1ha (a-c) or PACS-1ha-8A (d-h) or infected with recombinant vaccinia viruses expressing $S_{773,775}A$ mutant FLAG tagged furin (i-j). Cells were fixed, permeabilized and double labeled with monoclonal anti-FLAG tag (M1; a, d, g and i) and either polyclonal anti-TGN38 (b and e) or polyclonal anti-ACTH (AS29; h and j) followed by anti-mouse-FITC and anti-rabbit-TXRD. Merged images of green and red signals are shown (FLAG-tagged furin shown in green, TGN38 or ACTH shown in red; c, f, insets in h and j).

Figure 1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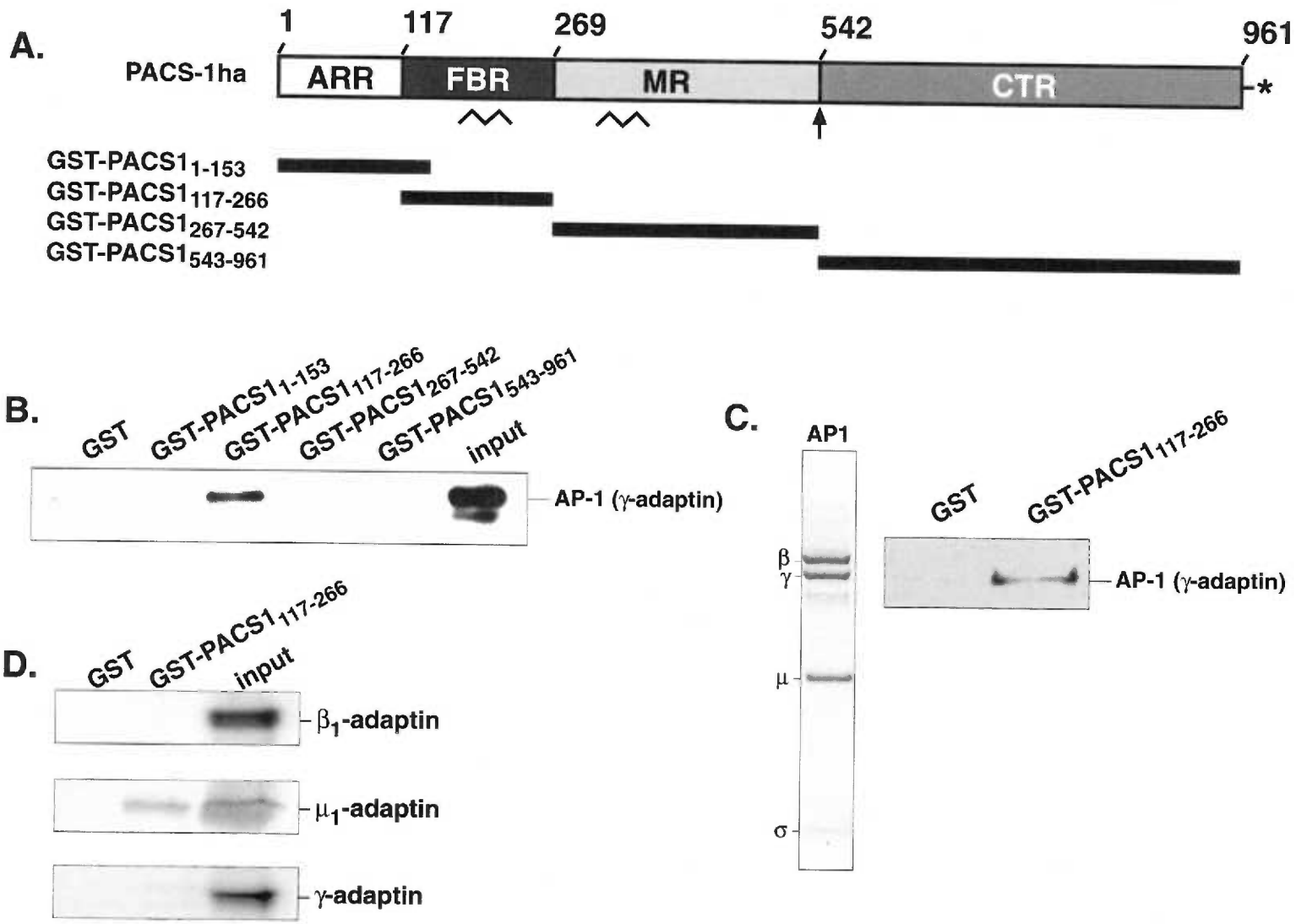
merge

Figure 2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input
AP-1 (γ-adaptin)
C.
AP1
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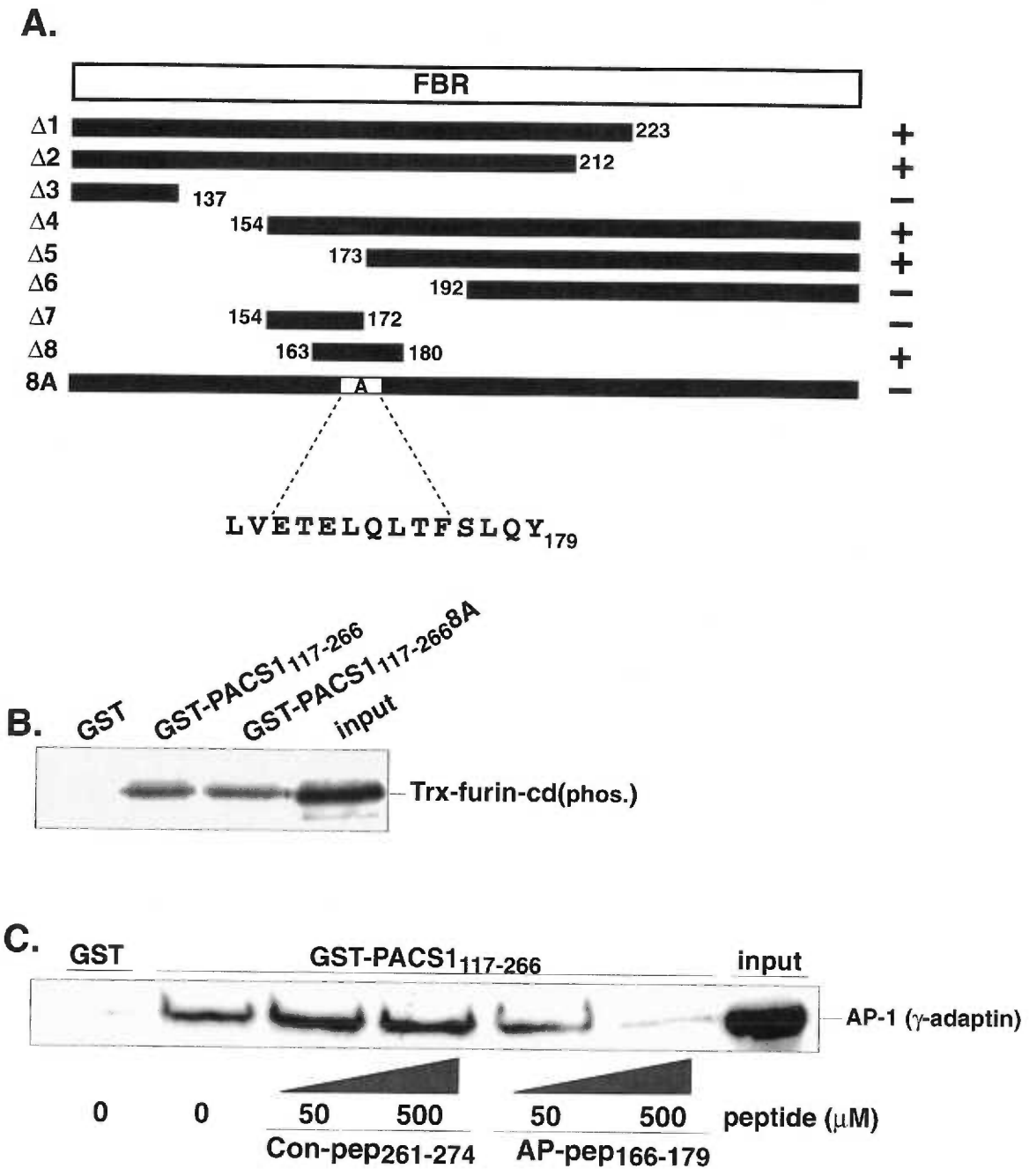
Figure 3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Δ2
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Δ3
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Δ5
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192
−
Δ7
154
172
−
Δ8
163
180
+
8A
A
−
L V E T E L Q L T F S L Q Y 179
B.
GST
GST-PACS1 117-266
GST-PACS1 117-266 8A
input
Trx-furin-cd(phos.)
C.
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Figure 4

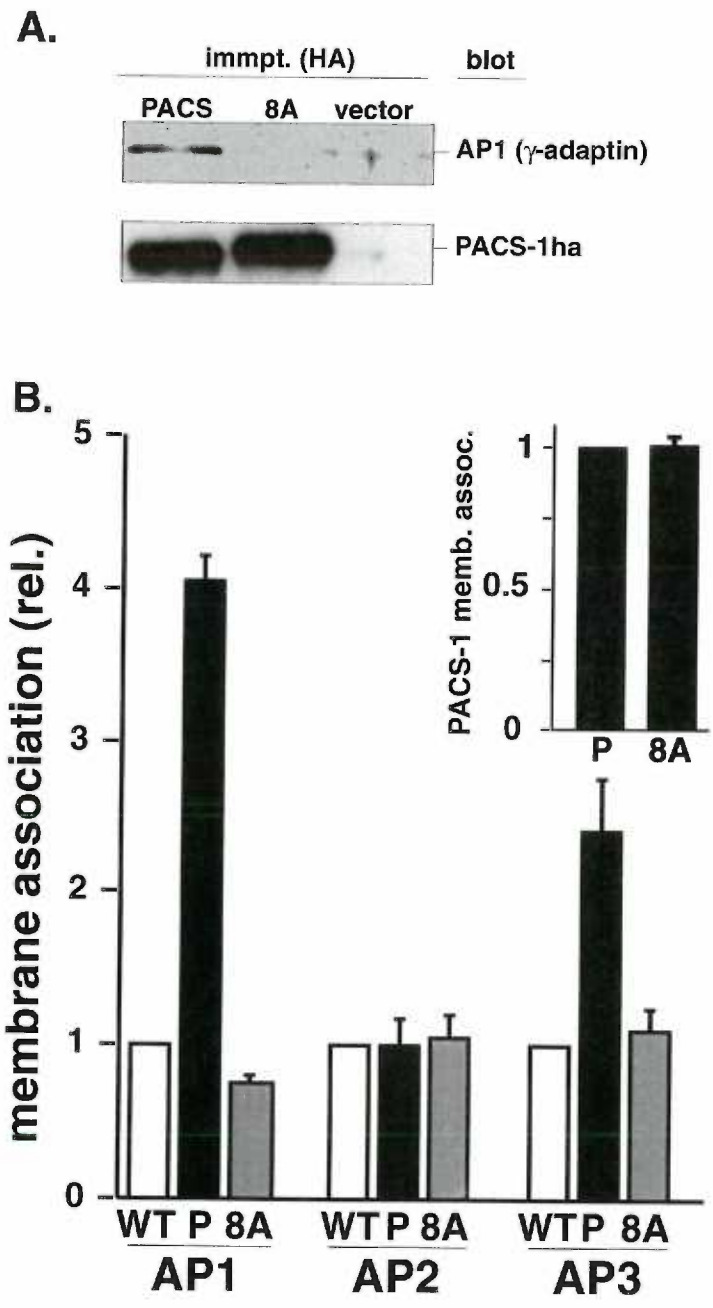
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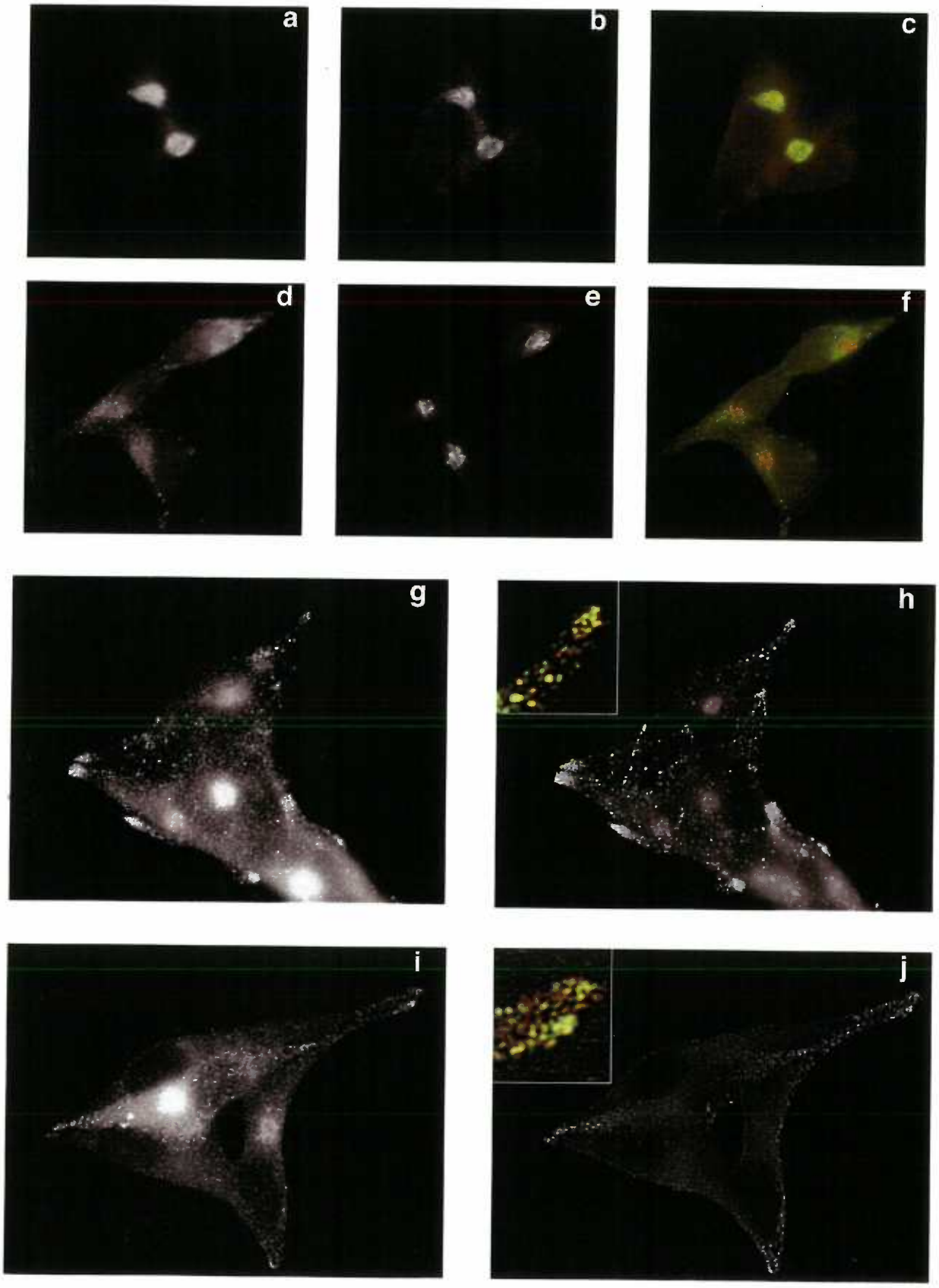
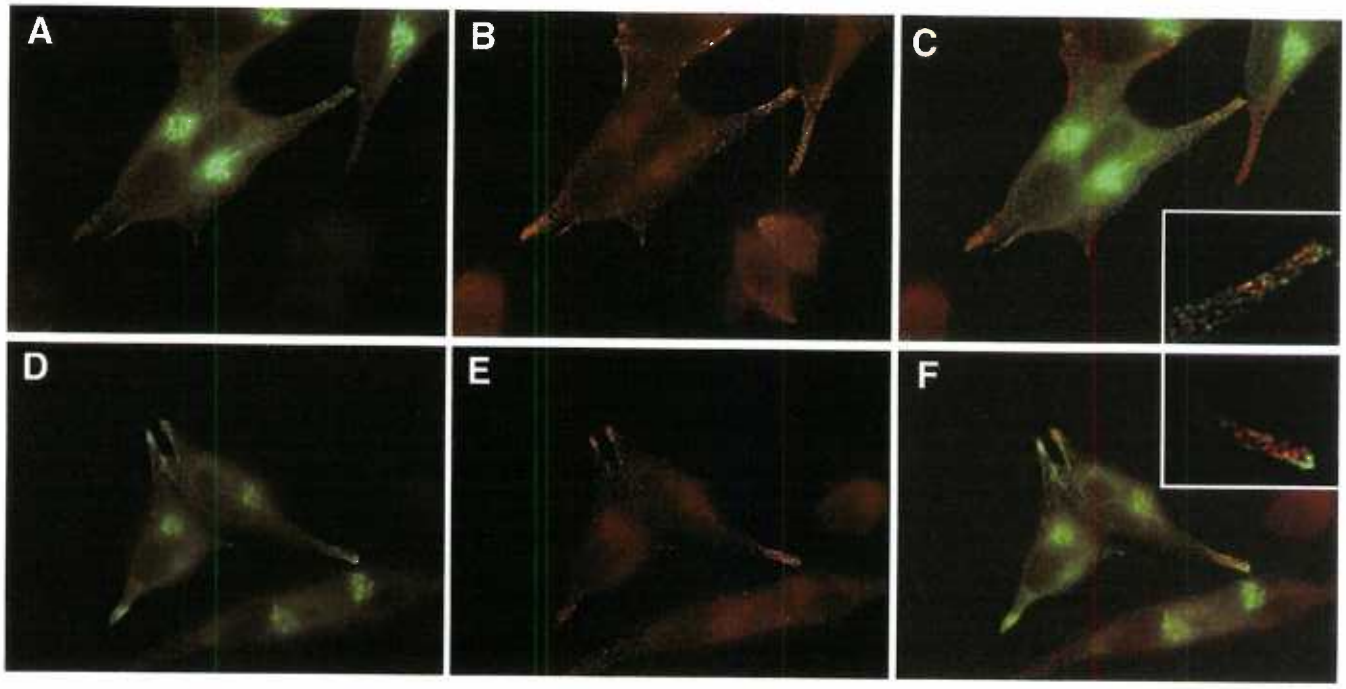
Figure 5



Additional data in chapter 4. Overexpression of dominant negative PACS-1 disrupts the subcellular localization of PC6B and causes mislocalization of PC6B to peripheral endosomes in AtT-20 cells. AtT-20 cells were co-infected with recombinant vaccinia viruses co-expressing Flag-tagged furin (fur/f as described in chapter 2) and PACS-1ha (a-c) or PACS-1ha-8A (d-h). Cells were fixed, permeabilized and double labeled with monoclonal anti-FLAG tag (M1; a, c, d and f) and polyclonal anti-ACTH (AS29; b, c, e and f) followed by anti-mouse-FITC and anti-rabbit-TXRD. Merged images of green and red signals are shown (fur/f shown in green, ACTH shown in red). Note in the insets in panel C and F, no significant overlap was observed between PC6B and ACTH staining.


A
B
C
D
E
F

Appendix

Through collaboration with other individuals in the lab, I have also worked on the enzymatic characterization of α1-PDX inhibition on different mammalian PCs. The results have been published with the title "alpha1-Antitrypsin Portland, a bioengineered serpin highly selective for furin: application as an antipathogenic agent" in the *Proceeding National Acdemic Sciences of USA*. 1998, 95(13): 7293-8 by Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A. J. and Thomas, G. It is summarized here:

The important role of furin in the proteolytic activation of many pathogenic molecules has made this endoprotease a target for the development of potent and selective antiproteolytic agents. Here, we demonstrate the utility of the protein-based inhibitor alpha1- antitrypsin Portland (α1-PDX) as an antipathogenic agent that can be used prophylactically to block furin-dependent cell killing by Pseudomonas exotoxin A. Biochemical analysis of the specificity of a bacterially expressed His- and FLAG-tagged α1-PDX (α1-PDX/hf) revealed the selectivity of the α1-PDX/hf reactive site loop for furin (Ki, 600 pM) but not for other proprotein convertase family members or other unrelated endoproteases. Kinetic studies show that α1-PDX/hf inhibits furin by a slow tight-binding mechanism characteristic of serpin molecules and functions as a suicide substrate inhibitor. Once bound to furin's active site, α1-PDX/hf partitions with equal probability to undergo proteolysis by furin at the C- terminal side of the reactive center -Arg355-Ile-Pro-Arg358- downward arrow or to form a kinetically trapped SDS-stable complex with the enzyme. This partitioning between the complex-forming and proteolytic pathways contributes to the ability of α1-PDX/hf to differentially inhibit members of the proprotein convertase family. Finally, we propose a structural model of

the α1-PDX-reactive site loop that explains the high degree of enzyme selectivity of this serpin and which can be used to generate small molecule furin inhibitors.

Through collaboration with other persons in the lab, I have characterized the function of the novel gene rat PACS-1. Specifically, I have shown the rat PACS-1 associates with adaptor complex AP-1 in vitro, which supports the connector role of PACS-1 in membrane protein sorting. The results has been summarized with other data and published with the title "PACS-1 defines a novel gene family of cytosolic sorting proteins required for *trans*-Golgi network localization" in *Cell*, 1998, 94(2): 205-16 by Wan, L., Molloy, S. S., Thomas, L., Liu, G., Xiang, Y., Rybak, S. L. and Thomas, G.. The paper is summarized here.

We report the role of one member of a novel gene family, PACS-1, in the localization of trans-Golgi network (TGN) membrane proteins. PACS-1 directs the TGN localization of furin by binding to the protease's phosphorylated cytosolic domain. Antisense studies show TGN localization of furin and mannose-6-phosphate receptor, but not TGN46, is strictly dependent on PACS-1. Analyses in vitro and in vivo show PACS-1 has properties of a coat protein and connects furin to components of the clathrin-sorting machinery. Cell-free assays indicate TGN localization of furin is directed by a PACS-1-mediated retrieval step. Together, these findings explain a mechanism by which membrane proteins in mammalian cells are localized to the TGN.

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α_1-Antitrypsin Portland, a bioengineered serpin highly selective for furin: Application as an antipathogenic agent

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ABSTRACT **The important role of furin in the proteolytic activation of many pathogenic molecules has made this endoprotease a target for the development of potent and selective antiproteolytic agents. Here, we demonstrate the utility of the protein-based inhibitor α_1-antitrypsin Portland (α_1-PDX) as an antipathogenic agent that can be used prophylactically to block furin-dependent cell killing by *Pseudomonas* exotoxin A. Biochemical analysis of the specificity of a bacterially expressed His- and FLAG-tagged α_1-PDX (α_1-PDX/hf) revealed the selectivity of the α_1-PDX/hf reactive site loop for furin (K_i, 600 pM) but not for other proprotein convertase family members or other unrelated endoproteases. Kinetic studies show that α_1-PDX/hf inhibits furin by a slow tight-binding mechanism characteristic of serpin molecules and functions as a suicide substrate inhibitor. Once bound to furin's active site, α_1-PDX/hf partitions with equal probability to undergo proteolysis by furin at the C-terminal side of the reactive center -Arg^{355}-Ile-Pro-Arg^{358}-$\downarrow$ or to form a kinetically trapped SDS-stable complex with the enzyme. This partitioning between the complex-forming and proteolytic pathways contributes to the ability of α_1-PDX/hf to differentially inhibit members of the proprotein convertase family. Finally, we propose a structural model of the α_1-PDX-reactive site loop that explains the high degree of enzyme selectivity of this serpin and which can be used to generate small molecule furin inhibitors.**

The recent discovery of a novel family of mammalian subtilisin-related proprotein convertases (PCs) that reside in the secretory pathway has led to a more detailed understanding of the molecular basis of proprotein maturation (1). Members of the PC family identified to date include furin, PACE-4, PC2, PC1/3 (hereafter termed PC3), PC4, PC5/6A and B (hereafter termed PC6A and PC6B), and LPC/PC7/PC8 (hereafter termed PC7) (2).

One PC, furin, trafficks through the *trans*-Golgi network/endosomal system where it cleaves a wide range of proproteins at the consensus sequence -Arg-Xaa-Lys/Arg-Arg-$\downarrow$ (3). Mutational analyses showed that the minimal consensus sequence required for furin processing is -Arg-Xaa-Xaa-Arg-$\downarrow$ (4). By contrast, PC2 and PC3 reside in the regulated secretory pathway of neuroendocrine cells where they process prohormones at -Lys/Arg-Arg- sites (1, 5). The roles of the remaining PCs have yet to be determined.

In addition to endogenous proproteins, many pathogens require furin to become active (3). For example, several viral coat proteins including HIV type 1 (HIV-1) gp160, Newcastle-disease virus F_o, measles virus F_o, and human cytomegalovirus glycoprotein B, require processing by furin in the biosynthetic pathway to generate infectious virions (3). Similarly, cleavage of many bacterial toxins by furin at the plasma membrane (e.g., anthrax toxin) and/or in endosomal compartments [e.g., *Pseudomonas* exotoxin A (PEA), and diphtheria and Shiga toxins] is required for their cytotoxicity (6).

The discovery that a diverse group of viral and bacterial pathogens requires furin for proteolytic activation has fostered the development of furin inhibitors. These include active-site-directed chloromethyl ketone inhibitors (7, 8), reversible peptide inhibitors (9, 10), and several protein-based inhibitors (11–14). The protein-based inhibitors include a recently described native serpin, PI8 (14), which contains two consensus furin sites, -Arg-Asn-Ser-Arg^{339}- and -Arg^{339}-Cys-Ser-Arg^{342}-, in its reactive site domain. Other protein-based inhibitors represent engineered variants of either the turkey ovomucoid third domain (-Ala-Cys-Thr-Leu^{18}- $\rightarrow$ -Arg-Cys-Lys-Arg^{18}-) (11), α_2-macroglobulin (-Gly-Phe-Tyr-Glu^{686}-Ser-Asp- $\rightarrow$ -Arg-Ser-Lys-Arg^{686}-Ser-Leu-) (13), or α_1-antitrypsin (α_1-AT). The latter variant, α_1-AT Portland (α_1-PDX), is distinct in that it contains a single minimal furin consensus motif in its reactive site loop (RSL; Ala^{355}-Ile-Pro-Met^{358}- $\rightarrow$ -Arg^{355}-Ile-Pro-Arg^{358}-) (12).

α_1-PDX is a potent inhibitor of furin (IC_{50} = 0.6 nM) and when expressed in cells (either by stable or transient transfection), blocks the processing of HIV-1 gp160 and measles virus-F_o and correspondingly inhibits virus spread (12, 15). However, relative to the chymotrypsin superfamily of serine proteases, little is known regarding the mechanism of inhibition of subtilase superfamily members, including furin, by α_1-AT or its engineered variants. In addition, although α_1-PDX does not inhibit either elastase or thrombin (12), the selectivity of this furin-directed inhibitor for the other PCs has not been established. Finally, whereas genome expression of α_1-PDX effectively blocks proprotein maturation (12, 15), it remains to be determined whether the recombinant protein can be used as a therapeutic agent.

Here, we report the mechanism of furin inhibition by α_1-PDX and the intrinsic selectivity of α_1-PDX for furin but not for other PCs. Furthermore, we show that α_1-PDX can be used prophylactically to block cell killing by PEA, a clinically important pathogen gene product. Our model of the α_1-PDX-RSL provides a basis for determining the interactions important for substrate binding and enzyme selectivity. How this model may facilitate development of small molecule therapeutics is discussed.

MATERIALS AND METHODS

Materials. pGlu-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide (pERTKR-MCA) was obtained from Peptides International, *N*-tert-butoxycarbonyl-Asp(benzyl)-Pro-Arg-MCA and decanoyl-Arg-Val-Lys-Arg-CH_2Cl (Dec-RVKR-CH_2Cl) from

Abbreviations: α_1-PDX, α_1-antitrypsin Portland; α_1-PDX/hf, His- and FLAG-tagged α_1-PDX; RSL, reactive site loop; PC, proprotein convertase; PEA, *Pseudomonas* exotoxin A; α_1-AT, α_1-antitrypsin; pERTKR-MCA, pGlu-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide; Dec-RVKR-CH_2Cl, decanoyl-Arg-Val-Lys-Arg-CH_2Cl; α_1-PIT, α_1-antitrypsin Pittsburgh; RP-HPLC, reverse-phase HPLC; SI, stoichiometry of inhibition.

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Bachem, thrombin (EC 3.4.21.5) from Sigma, and mAb M2 from Kodak.

His-/FLAG-Tagged α_1-AT Variants. The DNA sequence encoding the human α_1-AT signal peptide in pBS-AT (12) was replaced with sequences encoding the epitope(FLAG)-tag (underlined) by insertion of the annealed complimentary oligos 5′-CTAGAGGATCCCATGGACTACAAGGACGACGATGACAAGGAA-3′ and 5′-GATCTTCCTTGTCATCGTCGTCCTTGTAGTCCATGGGATCCT-3′. The resulting cDNA was subcloned into pDS56–6His to generate pDS56α_1-AT/hf. To generate pDS56α_1-PDX/hf, the DNA sequences encoding the RSL were replaced by the complimentary oligos (5′-GGGGCCATGTTTTTAGAGCGCATACCCA-3′ and 5′-GATCTGGGTATGCGCTCTAAAAACATGGCCCCTGCA-3′) coding for Arg (bold) in positions P4 and P1. To generate pDS56α_1-PIT/hf, complimentary oligos (5′-GGGGCCATGTTTTTAGAGGCCATACCCA-3′ and 5′-GATCTGGGTATGGCCTCTAAAAACATGGCCCCTGCA-3′) encoding a P1 Arg were used. The resulting ORFs directed cytosolic expression of the recombinant proteins initiating with a Met followed by the His and FLAG tags and the mature sequences of either α_1-PDX/hf or α_1-PIT/hf (see Fig. 1*A*).

Expression and Purification of α1-PDX/hf. α_1-AT variants were expressed in *Escherichia coli* strain BL21 transformed with either pDS56α_1-PDX/hf or pDS56α_1-PIT/hf. Protein expression was induced by addition of 1 mM isopropyl β-D-thiogalactoside, and cultures were grown overnight at 31°C. The cells were washed in metal-chelation chromatography binding buffer (5 mM imidazole/0.5 M NaCl/20 mM Tris·Cl, pH 7.9) and disrupted by cavitation (French press, 1,000 psi). The clarified and filtered supernatants containing soluble α_1-AT variants were applied to a Ni^{2+}-agarose column (Pharmacia), and bound proteins were eluted with 100 mM EDTA. The eluates were adjusted to 3.5 M NaCl and applied to a phenyl-Sepharose column (Pharmacia). The bound α_1-PDX/hf or α_1-antitrypsin Pittsburgh (α_1-PIT)/hf was eluted with 20 mM Bis-Tris, pH 7.0 and concentrated (4 mg/ml final) by diafiltration [Diaflo membrane, 10-kDa cut-off (Pierce)] in the same buffer. Protein purity and composition were demonstrated by Coomassie blue staining of SDS/PAGE gels, Western blot (using mAb M2), reverse-phase HPLC (RP-HPLC) (Fig. 1*B*), amino acid analysis, and MS (Fig. 1*C*). Serpin preparations were stored at 4°C.

Analytical Characterization of α_1-AT Variants. RP-HPLC was performed as described previously (5) by using a Vydac C4 column (25 cm × 0.46 cm). Electrospray MS analysis and amino acid composition of RP-HPLC-purified α_1-AT variants were performed by SynPep, Dublin, CA.

Cell Culture. BSC-40, AtT20, LoVo, and A7 cells were grown as described (15–17). PEA assays were performed as described (17).

Vaccinia Virus Recombinants. To monitor enzyme expression the FLAG tag was inserted by loop-in mutagenesis using mutagenic primers C terminal to the proregion cleavage site of each PC (except for PC2) as follows: FLAG-tagged (underline) human PACE-4 (hPACE-4/f), 5′-CGAAGGGTGAAGA-GAGACTACAAGGACGACGATGACAAGCAGGTGCGAAGTGAC--3′ (C terminal to Arg^{149}) (18); murine PC3 (mPC3/f), 5′-AGAGAAGTAAACGTGACTACAAGGACGACGATGACAAGTCAGTTCAAAAAGACT-3′ (C terminal to Arg^{110}) (19); murine PC6B (mPC6B/f), 5′-AAAAAGAACCAAGAGGGACTACAAGGACGACGATGACAAGGATTATGACCTCAGCC-3′ (C terminal to Arg^{116}) (20). A secreted soluble FLAG-tagged human PC7 (hPC7/f) was generated by inserting the epitope tag C terminal to Arg^{141} (21) by using the mutagenic primer 5′-CTGCTAAGGCGGGCTAAGCGCGACTACAAGGACGACGATGACAAGTCGGTCCACTTCAACGACC--3′ and a second primer 5′-CCCAACACCCTCAAGTAGGCCTAGCTGGTAGGCTGTTTC-3′ to insert a stop codon (bold) in place of Thr^{667}. Generation of vaccinia virus recombinants expressing each of the PCs was performed as described previously (22). The human fur713t/f (hereafter termed hfurin/f) and human PC2 (hPC2) constructs were described previously (5, 22).

Expression of Recombinant PCs. Each PC was produced by infecting cultured cells with the corresponding vaccinia virus recombinant (5 pfu/cell) and collecting the secreted/shed enzyme after 16 hr at 37°C in a serum- and phenol red-free defined medium (MCDB202) (4). hFurin/f, mPC3/f, mPC6B/f, and hPC7/f were expressed in BSC-40 cells. hPACE-4 was expressed in LoVo cells, and hPC2 was expressed in AtT-20 cells. Conditioned medium containing each enzyme preparation was clarified (5,000 × *g* 10 min), concentrated [Biomax filter, 30-kDa cut-off (Millipore)], and stored at −70°C. Each enzyme preparation was enzymatically pure based on the absence of PC activity in medium from replicate cultures infected with wild-type vaccinia virus (F.J. and G.T., data not shown).

Enzyme Assays. The enzyme assay data were obtained by using a FluoroMax-2 spectrofluorometer equipped with a 96-well plate reader (Instrument SA, Edison, NJ) using excitation/emission wavelengths of 370/460 nm to measure released AMC (7-amino-4-methylcoumarin). Thrombin assays were performed using *N*-tert-butoxycarbonyl-Asp(benzyl)-Pro-Arg-MCA as a fluorogenic substrate (12) whereas pERTKR-MCA was used for each of the PCs (8). Furin, PC6B, PC7, and PACE-4 assays were performed in 100 mM Hepes, pH 7.5, containing 0.5% Triton X-100 and 1 mM $CaCl_2$. PC3 and PC2 assays were performed as described (8,

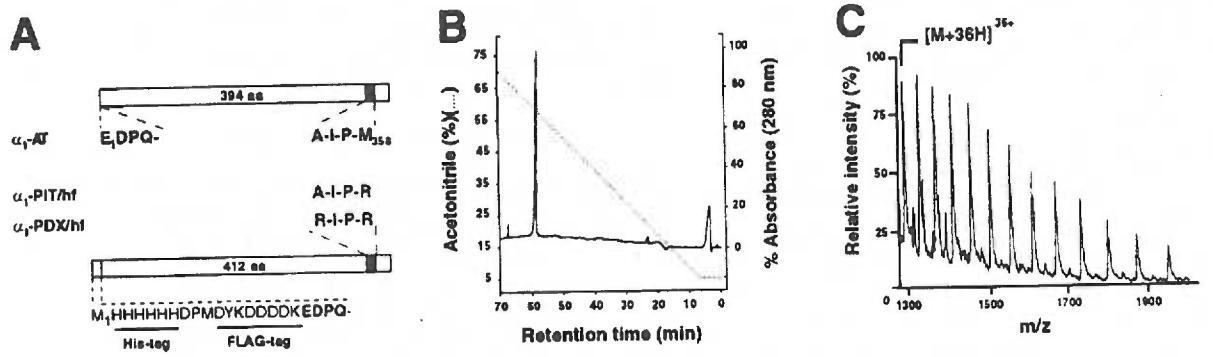

FIG. 1. Histidine-FLAG-tagged α_1-AT variants. (*A*) Diagram of α_1-AT variants including native α_1-AT, α_1-PIT/hf, and α_1-PDX/hf. The His and FLAG sequences that replace the signal sequence as well as the amino acid substitutions within the RSL are shown. (*B*) RP-HPLC analysis of purified α_1-PDX/hf. The chromatogram shows a single prominent A_{280} peak eluting at 58 min corresponding to purified α_1-PDX/hf. (*C*) Electrospray MS of purified α_1-PDX/hf. The measured molecular mass of α_1-PDX/hf and α_1-PIT/hf (46,719 ± 3 Da and 46,631 ± 3 Da, respectively) was in close agreement (relative error less than 0.02%) with the calculated values for α_1-PDX/hf (46,727 Da) and α_1-PIT/hf (46,642 Da).

23). Values for K_m and V_{max} were determined as described (8) whereas E_0 and K_i were obtained by fitting the data (v and I) to the equation: $v = SA(E_0 - 0.5\{(E_0 + I + K_i) - [(E_0 + I + K_i)^2 - 4E_0I]\}^{1/2}\})$ (SA, specific activity; E_0, enzyme concentration, and I, inhibitor concentration) by nonlinear regression (ENZFITTER, Elsevier-Biosoft, Cambridge, UK) (24).

Determination of the RSL Cleavage Site by MS. α_1-PDX/hf (100 μg) was incubated with furin for 1 hr at 25°C. Stoichiometries were chosen so that the RSL cleavage products were generated in sufficient concentration to be detected by MS. Reactions were stopped with EDTA (5 mM final), and the samples were separated by RP-HPLC as described above and subjected to matrix-assisted laser desorption ionization (MALDI)-MS. Positive ion MALDI-time of flight spectra were obtained by using a Voyager Elite Biospectrometry Research Station (PerSeptive Biosystems, Framingham, MA). Samples were dissolved in 0.1% aqueous trifluoroacetic acid and analyzed by using a matrix of sinapinic acid.

RESULTS

Expression of His-/FLAG-Tagged α_1-AT Variants. A rigorous determination of (*i*) the mechanism by which α_1-PDX inhibits furin, (*ii*) the enzyme specificity of α_1-PDX, and (*iii*) the ability of recombinant α_1-PDX to function prophylactically requires preparative amounts of the recombinant protein. To generate sufficient amounts of α_1-PDX for these analyses, we expressed this serpin in the cytosol of bacteria. Using PCR mutagenesis, the α_1-PDX signal sequence was replaced with an initiator methionine followed by histidine (His) and epitope-FLAG tags (Fig. 1*A*). The His tag in the resultant construct, α_1-PDX/hf, allowed rapid purification of the recombinant protein whereas the FLAG tag was used to assay for α_1-PDX/hf by immunodetection. To control for the effect of the His/FLAG tags on serpin activity, the same mutagenesis was performed on α_1-PIT (to generate α_1-PIT/hf), a naturally occurring α_1-AT variant, which inhibits thrombin and differs from α_1-PDX by a single residue at position P4 in the RSL (-Ala-Ile-Pro-Arg^{358}-). Plasmids expressing either α_1-PDX/hf or α_1-PIT/hf were transformed into *E. coli*, and protein expression was induced by the addition of isopropyl β-D-thiogalactoside at 31°C. After cell disruption, the α_1-AT variants were purified by Ni^{2+} binding- and hydrophobic interaction-chromatography. This procedure yielded recombinant serpins that were essentially pure and intact, as determined by Coomassie blue staining (data not shown) and Western blot analysis (Fig. 2 *C* and *D*). The purity and molecular composition of each preparation were confirmed by analytical RP-HPLC (Fig. 1*B*), amino acid analysis (data not shown), and electrospray MS (Fig. 1*C*).

Mechanism of Furin Inhibition by α_1-PDX/hf. The time- and concentration-dependent inactivation of furin by α_1-PDX/hf was examined. Characteristic of other serpin-enzyme interactions (25, 26), the inhibition of furin by α_1-PDX/hf obeyed slow-binding inhibition kinetics, as indicated by the biphasic plots, where maximal inhibition was achieved more rapidly with increasing concentrations of α_1-PDX/hf (Fig. 2*A*). The serpin-enzyme complexes were kinetically trapped (i.e., slow off-rate) because no furin activity was liberated when the assays were extended for up to 6 hr (data not shown). By contrast, α_1-PIT/hf was unable to inhibit furin at any concentration tested (Fig. 2*B*), although it potently inhibited thrombin (Table 1). Nonlinear regression analysis of the data in Fig. 2*A* showed that the initial velocity (v_0) was inversely proportional to the concentration of α_1-PDX/hf. This finding suggests that the inhibition of furin by α_1-PDX/hf follows a two-step mechanism whereby rapid formation of a loose α_1-PDX/hf·furin complex is followed by a slow isomerization to the tightly bound product (illustrated by the asymptotic component of each curve).

The stability of the α_1-PDX/hf·furin complex was examined by assessing its resistance to SDS denaturation. Increasing amounts

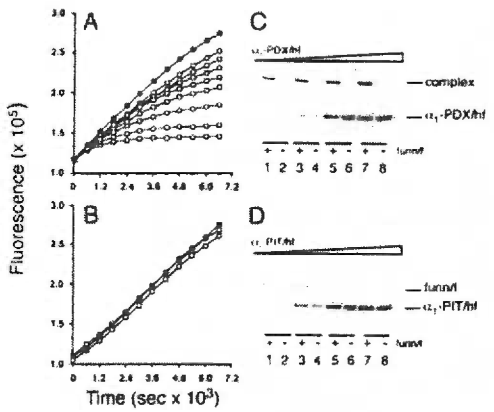

FIG. 2. Inhibition of furin by α_1-AT variants. Progress curves showing the effect of α_1-PDX/hf (*A*) or α_1-PIT/hf (*B*) on furin activity. Furin/f (5.4 nM) was incubated with pERTKR-MCA (100 μM) at room temperature in the absence (●) or presence (○) of 6.1 (top), 7.7, 15, 21, 31, 61, 123, or 184 (bottom) nM α_1-AT variant. Data are representative of at least three independent experiments. SDS/PAGE analysis of reactions of furin/f with α_1-PDX/hf (*C*) or α_1-PIT/hf (*D*). Increasing concentrations of α_1-AT variants (134, 268, 670, and 1,340 nM) were incubated with or without furin/f (5.4 nM) for 1 hr at room temperature. Enzyme activity was stopped with EDTA (5 mM final) followed by addition of SDS-SB (containing 2% SDS and 20 mM DTT). Samples were heated (95°C), resolved by SDS/PAGE (10% gel), and transferred to nitrocellulose and FLAG-tagged proteins detected by Western blot using mAb M2.

of α_1-PDX/hf or α_1-PIT/hf were incubated in the presence or absence of FLAG-tagged soluble furin (furin/f). After incubation to generate serpin-enzyme complexes, residual furin activity was inhibited by addition of EDTA, and the reaction mixtures were analyzed by Western blot (Fig. 2 *C* and *D*). In agreement with the predicted mass of α_1-PDX/hf, incubation of this serpin in the absence of furin showed a single 47-kDa protein (Fig. 2*C*, lanes 2, 4, 6, and 8). However, coincubation resulted in a shift of furin/f and α_1-PDX/hf to a single high M_r band (≈160 kDa) corresponding to the kinetically trapped, SDS-resistant α_1-PDX/hf·furin/f complex (Fig. 2*C*, lanes 1, 3, 5, and 7). By contrast, coincubation of α_1-PIT/hf with furin/f failed to generate an SDS-stable complex (Fig. 2*D*).

The temporal relationship between α_1-PDX/hf·furin complex formation and inhibition of furin activity also was examined (Fig. 3). The rate of formation (50% complete at ≈5 sec and ≈100% complete by 2 min) was coincident with the loss of furin activity. In addition, a less-abundant cleaved form of α_1-PDX/hf also accumulated in parallel with the SDS-stable complex. Matrix-assisted laser desorption ionization-MS analysis of the products revealed that the reaction between furin and α_1-PDX/hf resulted in P1-P1′ bond cleavage of the α_1-PDX/hf RSL (-Arg^{P4}-Ile-Pro-Arg^{P1}-↓Ser). The molecular mass observed for the liberated N-terminal fragment (42,652 ± 43 Da) was in agreement with the calculated molecular mass (42,614 Da, relative error 0.09%). Thus, the rate of complex formation, cleavage of the α_1-PDX/hf, and loss of enzymatic activity, are tightly coupled.

Stoichiometry of Inhibition (SI) and Determination of K_i. After formation of the α_1-PDX/hf·furin acyl intermediate (EI′), α_1-PDX/hf may either be hydrolyzed and released (I*) or it may trap the enzyme in a kinetically stable SDS-resistant complex (EI*) (Figs. 2*C* and 3). The relative flux of a serpin through these pathways reflects its efficiency as an inhibitor for a given endoprotease and is described as the SI (26);

$$\begin{array}{ll} I + E \Leftrightarrow EI \rightarrow EI' \rightarrow E + I^* & \text{(substrate pathway)} \\ \qquad\qquad\qquad\quad \downarrow & \\ \qquad\qquad\qquad\quad EI^* & \text{(inhibitor pathway).} \end{array}$$

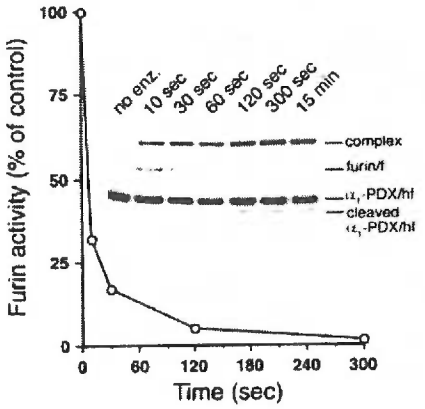

FIG. 3. Time course of inhibition and complex formation between α_1-PDX/hf and furin/f. Replicate tubes containing furin/f (10 nM) and α_1-PDX/hf (78 nM) were incubated at room temperature. At each time point pERTKR-MCA (100 μM) was added to one set of tubes to measure residual furin/f activity. Shown is one of three independent experiments (variability < 10%). (*Inset*) At the indicated times, the replicate reactions were stopped by addition of EDTA (final concentration 5 mM), and samples were processed for Western blot analysis.

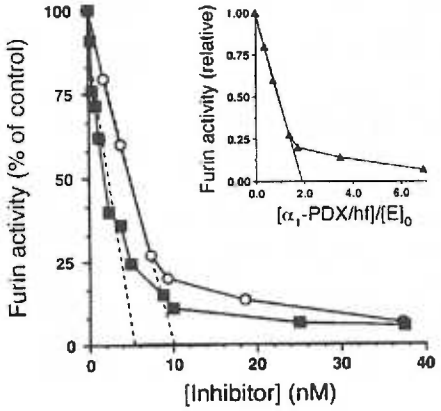

FIG. 4. Tight-binding titration of furin by α_1-PDX/hf and Dec-RVKR-CH_2Cl. Furin/f (5.4 nM) was incubated with increasing amounts of α_1-PDX/hf (○) and Dec-RVKR-CH_2Cl (■) for 30 min at room temperature. pERTKR-MCA (100 μM) was added to determine residual furin activity. Because valid titration curves can be obtained for tight-binding titrants when $[E]_0/K_i > 2$ (24), the K_i of α_1-PDX/hf for furin was obtained by fitting the data to an equation for equilibrium binding (24). This analysis revealed a value of $K_i = 0.60$ nM and a value of $[E]_0 = 10$ nM. Importantly, the calculated ratio of $[E]_0/K_i = 17$ confirmed the validity of the K_i value obtained by using α_1-PDX/hf as tight-binding titrants of furin. (*Inset*) The data shown were used to determine the stoichiometry of the reaction between α_1-PDX/hf and furin. The abscissa shows the inhibitor/enzyme ratio using a value for active furin ($[E]_0 = 5.4$ nM) determined by titration using the active-site-directed irreversible inhibitor Dec-RVKR-CH_2Cl. The data are averages of duplicate samples and are representative of three independent experiments.

Titration experiments were performed to determine the SI between α_1-PDX/hf and furin. First, the amount of active enzyme (E_0) was determined by titrating furin activity with the active-site-directed irreversible inhibitor Dec-RVKR-CH_2Cl. In a parallel analysis, furin activity was titrated with α_1-PDX/hf (Fig. 4). Regression analysis of residual furin activity as a function of $[I]_0$:$[E]_0$ showed that approximately 2 mol of α_1-PDX/hf are required to inactivate 1 mol of furin, indicating equal flux of this serpin through the inhibitory and hydrolytic pathways (Fig. 4, *Inset*).

The inhibition of furin by α_1-PDX/hf showed that α_1-PDX/hf is a tight-binding titrant of furin (Fig. 4). Indeed, α_1-PDX/hf fulfilled several criteria of an active site titrant including its stability (Fig. 2*A*), its formation of a kinetically trapped complex with furin (Fig. 2*C*), and its rapid combination with the furin active site (Fig. 3).

Intrinsic Specificity of α_1-PDX/hf. The potent inhibition of furin by α_1-PDX/hf coupled with its inability to inhibit either elastase or thrombin (Table 1 and ref. 12) suggests this serpin is selective for furin. Therefore, we evaluated the intrinsic specificity of α_1-PDX/hf by quantifying its inhibitory properties against the other PCs. To generate active preparations of the other PCs, vaccinia virus recombinants were constructed that expressed FLAG-tagged variants (inserted C terminal to the propeptide cleavage site) of PACE-4, PC3, PC6B, and PC7. Detection of the expressed enzymes was facilitated by the inserted FLAG tag. Because proPC2 activation is disrupted by insertion of the FLAG tag (unpublished results), a nonepitope-tagged PC2 construct was used. Vaccinia virus recombinants encoding each PC then were used to express the enzymes as described in *Materials and Methods*.

Table 1. Kinetic and stoichiometric parameters for the proprotein convertases

	Coumarinamide substrate	Peptide-based inhibitor	Protein-based inhibitor		
	pERTKR-MCA	Dec-RVKR-CH_2Cl	α_1-PIT/hf	α_1-PDX/hf	
Enzyme	K_m, μM	K_i, nM	K_i, nM	K_i, nM	SI
Furin	4.7	2.0	>500	1.4	2¶
Furin/f†	3.2	0.60	>500	0.60§	2¶
PC6B/f†	0.41	0.11	>500	2.3§	8¶
PC3/f†	25	2.0	>500	260§	40¶
PC2	54	0.36	>500	1,000	nd
PACE-4/f†	6.3	3.6	>500	>5,000	nd
PC7/f†	19	0.12	>500	>5,000	nd
Thrombin	12‡	nd	0.05	>5,000	nd

Assays were performed in duplicate. Reported values are the mean of three independent experiments (SEM <15%). All PCs were active-site titrated with Dec-RVKR-CH_2Cl. Calculations assume α_1-PDX/hf, α_1-PIT/hf, and Dec-RVKR-CH_2Cl are fully active. SI values were determined as described in legend to Fig. 4. nd, not determined.

†Molecular mass determined by Western blot using mAb M2: Furin/f (86 kDa); PC6B/f [144 kDa (major band) and 60 kDa (minor band)]; PC3/f [86 kDa (major band) and 67 kDa (minor band)]; PACE-4/f (102 kDa); PC7/f (79 kDa).

‡Boc-D(Bz)-PR-MCA.

§EI* complex determined by Western blot; α_1-PDX/hf·furin/f (160 kDa); α_1-PDX/hf·PC6B/f (two bands corresponding to the 144- and 60-kDa forms); α_1-PDX/hf·PC3/f (two bands corresponding to the 86- and 67-kDa forms).

¶Cleaved α_1-PDX/hf (I*) detected on incubation with the respective enzyme.

Initially, the K_m for hydrolysis of the synthetic peptide substrate pERTKR-MCA was determined for each PC (Table 1). Whereas the PCs display unique cleavage site specificity with proprotein substrates (1), this small fluorogenic substrate can be hydrolyzed by each member of the family. The similar K_ms for native- and epitope-tagged furin and PC3 constructs (Table 1 and ref. 8) demonstrate the lack of effect on enzyme expression and activity of this epitope-tag inserted C terminal to the propeptide cleavage site.

The selectivity and effectiveness of α_1-PDX/hf for each PC then was compared with α_1-PIT/hf and Dec-RVKR-CH_2Cl by determining the K_i values (Table 1). Using these criteria, the PCs exhibited similar reactivity toward the peptide inhibitor (K_i = 0.11–3.6 nM). Thus, like peptide substrates, this irreversible peptidyl inhibitor does not discriminate between the PCs. In contrast, α_1-PDX/hf displayed a striking selectivity between the PCs. α_1-PDX/hf potently inhibited both furin/f (K_i = 0.60 nM) and PC6B/f (K_i = 2.3 nM). However, the SI for PC6B/f was markedly higher (4-fold), indicating that it partitions through the hydrolytic pathway more frequently than furin. Also, α_1-PDX/hf inhibits PC3/f approximately 400-fold less effectively than it does furin (K_i = 260 nM) and partitioning greatly favors the hydrolytic pathway relative to the formation of the SDS-stable complex (SI = 40). Furthermore, α_1-PDX is a weak competitive inhibitor of PC2 (K_i = 1 μM), suggesting it is unable to form an acyl intermediate. Neither PACE-4/f nor PC7/f are inhibited by α_1-PDX at the concentrations tested.

Inhibition of PEA Cytotoxicity by Exogenous α_1-PDX/hf. The selective inhibition of furin by α_1-PDX/hf combined with the inherent stability of the α_1-PDX/hf·furin complex (EI*) suggested that this serpin could be used to inhibit the processing of furin substrates *in vivo*. Therefore, we investigated the capacity of α_1-PDX/hf to prevent the furin-dependent cytotoxicity of PEA. The 67-kDa protoxin requires cleavage within endosomes by furin at the consensus site -Arg^{P4}-Gln-Pro-Arg^{P1}-↓ to become cytotoxic (6). The excised 37-kDa C-terminal fragment translocates into the cytosol where it ADP ribosylates elongation factor 2 to inhibit protein synthesis (6).

Replicate plates of cells pretreated with increasing concentrations of α_1-PDX/hf or α_1-PIT/hf were incubated with 10 nM PEA. The furin-dependent activation of PEA was monitored by measuring the rate of protein synthesis. Whereas control cells were completely sensitive to PEA, cells pretreated with α_1-PDX/hf showed a concentration-dependent protection against toxin activation, with 10 μM α_1-PDX/hf completely blocking the effect of the toxin. By contrast, at the same concentrations, α_1-PIT/hf was unable to protect the cells from PEA (Fig. 5).

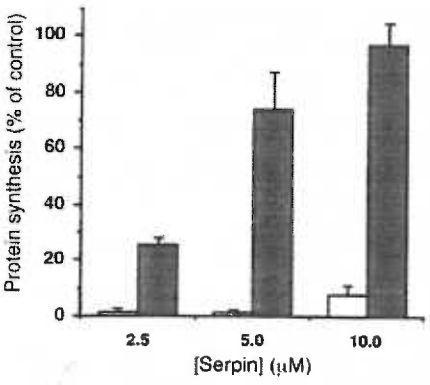

FIG. 5. Exogenous α_1-PDX/hf neutralizes PEA-induced cytotoxicity in cultured cells. Replicate cultures of A7 melanoma cells were incubated in the absence or presence of increasing concentrations of α_1-PIT/hf (open bars) or α_1-PDX/hf (filled bars) for 2 hr at 37°C. Subsequently, PEA (10 nM final) was added, and the cultures were incubated at 37°C for 6 hr. Cells then were incubated with [^{35}S]-Met/Cys for an additional 30 min at 37°C and harvested, and [^{35}S]-Met/Cys incorporation into cellular proteins was determined by scintillation counting of the trichloroacetic acid precipitates. Data shown are representative of three assays (error bars represent standard error).

DISCUSSION

The broad and important role of furin in the proteolytic maturation of proprotein substrates (3), including the activation of many pathogen molecules [e.g., viral envelope glycoproteins (7) and bacterial toxins (6)], has made this endoprotease a target for the development of potent and selective inhibitors. Here, we report the biochemical characterization of α_1-PDX/hf, an engineered α_1-AT variant containing the minimal consensus furin cleavage site (-Arg^{P4}-Xaa-Xaa-Arg^{P1}-) in its RSL. We show that preparative amounts of the active His- and FLAG-tagged α_1-AT variants can be expressed in bacteria and readily purified to homogeneity. Kinetic studies showed α_1-PDX/hf inhibits furin by a slow tight-binding mechanism characteristic of serpin molecules and operates as a suicide substrate inhibitor (26). Once bound to furin's active site, α_1-PDX/hf is partitioned with equal probability to undergo proteolysis by furin at the C-terminal side of the -Arg^{P4}-Ile-Pro-Arg^{P1}-↓ site (I*) or to form a kinetically trapped SDS-stable complex (EI*) with the enzyme. Analysis of the specificity of α_1-PDX/hf showed it is selective for furin and, to a lesser extent PC6B, but not for other PC family members. Finally, the utility of α_1-PDX/hf to serve as a potential therapeutic agent was demonstrated by its ability to block furin-dependent cell killing by PEA.

Our results show that α_1-PDX inhibits members of the PC family of mammalian subtilisins by the same mechanism used by α_1-AT to inhibit chymotrypsin family members. The ability of α_1-PDX to form SDS-stable complexes with furin, PC6B, and PC3, but not PC2, PACE-4, or PC7, partially illustrates the enzyme selectivity of α_1-PDX. Unlike chymotrypsin family en-

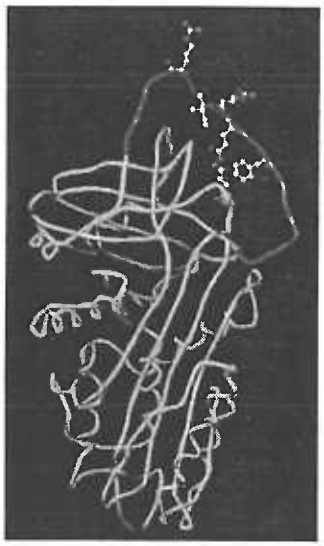

FIG. 6. Model structure of α_1-PDX. The global energy-minimum structure was compiled by using the SYBYL version 6.3 software (Tripos, St. Louis) based on the atomic coordinates reported for α_1-AT (28). The reactive loop P15-P'5 (Gly^{344}–Glu^{363}) is depicted in red. Introduction of the double Arg substitution (Arg^{358} and Arg^{355}) has negligible effect on the overall conformation because both side groups face the solvent (hence the furin active site). The RSL is stabilized by direct interactions with the core of the protein. These include hydrogen bonding between the P5 Glu (Glu^{354}) and Arg^{196} as well as hydrophobic interactions between the P3 Ile (Ile^{356}) and P7 Phe (Phe^{352}) and also between the prolines at positions P2, P3', and P4' (in addition to the rigidity contributed by these residues).

zymes, however, the SI between α_1-AT and subtilase superfamily members is highly variable (Table 1 and ref. 27). Thus, although α_1-PDX has a similar K_i for both furin and PC6B, the SI is strikingly different; four times as much α_1-PDX is required to inhibit PC6B at saturating concentrations compared with furin. The highly selective inhibition of furin by α_1-PDX compared with the other PCs illustrates that the structure of the reactive center of each PC is unique, implying a different substrate specificity for each family member.

Compared with previously reported furin inhibitors, the enzyme specificity of α_1-PDX is highly selective. For example, peptidyl chloromethyl ketones show little selectivity for any PC member (Table 1). Furthermore, their cytotoxicity and the unstable nature of the chloromethane group preclude their usefulness as therapeutic antiproteolytic agents. Similarly, both PI8 and the furin-directed α_2-macroglobulin variant show very little enzyme specificity. Both proteins inhibit many proteases in addition to furin greatly limiting their use as therapeutics. Thus, the unique specificity of the α_1-PDX/hf-RSL reported here demonstrates a major advantage of the α_1-AT scaffold for the design of furin-selective inhibitors.

The solved structure of α_1-AT shows that, unlike other serpins, its RSL is stabilized by direct interactions with the core of the protein (28). Based on this information, we have generated an energy-minimized model of α_1-PDX (Fig. 6). Ideal for furin, the P1 and P4 residues are predicted to be unnecessary for stabilization of the RSL, and instead they face the solvent where they can interact with the target enzyme's active site. Importantly, our model of the α_1-PDX/hf-RSL provides a basis for determining the interactions important for substrate binding and selectivity between the different PCs. This model also can be used to construct a three-dimensional template with which to develop combinatorial libraries for small molecule furin inhibitors or to synthesize conformationally constrained peptoids (29).

Our results conflict with those of Benjannet *et al.* (30), who reported, using overexpression methods, that (*i*) α_1-PDX is a competitive inhibitor of furin and (*ii*) α_1-PDX is not a selective but rather a general inhibitor of the PCs in the constitutive secretory pathway. However, because excessive amounts of enzyme interfere with detection of the serpin-enzyme complex (27), the methodology used by Benjannet *et al.* may have precluded determination of the inhibitory mechanism. Furthermore, it is likely that overexpression methods limit the ability to dissect a complex issue such as the inherent selectivity of serpin-based inhibition of proteinases (26). For example, overexpression of α_1-PIT (a poor inhibitor of furin, see Table 1 and ref. 12) in COS cells inhibits furin (31).

The inherent selectivity and stability of α_1-PDX suggest its broad applicability as a therapeutic agent. Indeed, the observation that α_1-PDX/hf blocks furin-dependent cell killing by PEA demonstrates its therapeutic potential. *Pseudomonas aeruginosa* is a clinically important pathogen constituting a major complication in burn patients and people afflicted with cystic fibrosis (32). Animal studies show the contribution of PEA to pathogen virulence (33). Indeed, the exotoxin A gene is amplified in *Pseudomonal* colonies isolated from the lungs of cystic fibrosis patients, suggesting that it may contribute to the complications of that disease (34). Studies examining the potential of α_1-PDX in the treatment of *Pseudomonal* infections and other pathogens that use furin to become virulent are in progress.

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PACS-1 Defines a Novel Gene Family of Cytosolic Sorting Proteins Required for *trans*-Golgi Network Localization

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Summary

We report the role of one member of a novel gene family, PACS-1, in the localization of *trans*-Golgi network (TGN) membrane proteins. PACS-1 directs the TGN localization of furin by binding to the protease's phosphorylated cytosolic domain. Antisense studies show TGN localization of furin and mannose-6-phosphate receptor, but not TGN46, is strictly dependent on PACS-1. Analyses in vitro and in vivo show PACS-1 has properties of a coat protein and connects furin to components of the clathrin-sorting machinery. Cell-free assays indicate TGN localization of furin is directed by a PACS-1-mediated retrieval step. Together, these findings explain a mechanism by which membrane proteins in mammalian cells are localized to the TGN.

Introduction

The regulation of membrane and protein traffic between secretory pathway compartments requires the orchestrated interaction of a large number of components, including various small molecules, lipid, cytosolic, and membrane proteins, and cytoskeletal elements (Cole and Lippincott-Schwartz, 1995; Schekman and Orci, 1996; Schmid, 1997). The secretory pathway compartments can be subdivided into two central membrane populations, the endoplasmic reticulum (ER)/Golgi system and the *trans*-Golgi network (TGN)/endosomal system. The ER/Golgi system performs the folding, oligomerization, and initial co- and posttranslational modifications of proteins transiting the secretory pathway. The TGN/endosomal system is central to the sorting, export, and recovery of numerous soluble and membrane-associated secretory pathway proteins. In addition to housing several biochemical reactions, the TGN orchestrates the routing of proteins to lysosomes, to regulated and constitutive exocytic pathways, and, in polarized cells, to apical and basolateral membranes.

For the ER/Golgi and TGN/endosomal systems to fulfill their manifold roles, resident proteins must be distinguished from the population of migrant molecules that transit through them. For example, the ER maintains populations of both soluble and membrane-anchored resident proteins that are localized by retrieval-based mechanisms. Soluble resident proteins (e.g., GRP78/Bip) contain a C-terminal -KDEL motif (Munro and Pelham, 1987) that binds to the KDEL receptor to direct retrieval from the Golgi, whereas resident membrane proteins contain a basic amino acid motif (e.g., KKXX) within their cytosolic domains that directs retrieval by binding to COPI coatomer (Letourneur et al., 1994). By contrast, resident Golgi cisternae glycosyltransferases are localized primarily by a retention mechanism, based in part on the length of their transmembrane domains (Munro, 1995). However, despite the central role of the TGN to the organization of the secretory pathway, little is known about the cellular machinery that directs the localization and distribution of membrane proteins within this dynamic compartment.

The endoprotease furin represents an excellent model with which to identify the factors that direct protein sorting within the TGN. Localization of furin to clathrin-coated regions of the TGN requires a motif within its cytosolic domain (cd) consisting of an acidic cluster (AC) of amino acids (..EECPS$_{773}$DS$_{775}$EEDE..) and containing a pair of casein kinase II (CKII) phosphorylatable serines (Molloy et al., 1994; Schäfer et al., 1995; Takahashi et al., 1995). Phosphorylation of the furin-cd AC is required for TGN localization of the endoprotease and enhances its association with the clathrin adaptor AP-1 (Dittié et al., 1997). These findings suggest that TGN localization of furin is mediated by an AP-1/clathrin-sorting step. Similarly, TGN localization of varicella-zoster virus gE (VZV-gE) also requires phosphorylation of an AC within its cd by CKII (Alconada et al., 1996; see also Figure 7A). By contrast, TGN localization of TGN38 requires a tyrosine-based motif (Wong and Hong, 1993), suggesting distinct sorting machinery is used to localize different membrane proteins to the TGN.

Like the plasma membrane-associated adaptor AP-2, AP-1 binds directly to many tyrosine-based and/or dileucine-like internalization/endosomal targeting signals (Ohno et al., 1996). However, direct binding of AP-1 to the structurally disparate furin TGN localization signal has not been demonstrated. Indeed, some membrane proteins contain sorting motifs that do not bind adaptors directly. Rather, additional cytosolic proteins are required to connect them to the clathrin-sorting machinery. For example, the HIV-1 *nef* gene product directs the internalization of CD4 receptor by connecting the cell surface protein to AP-2 (Foti et al., 1997). Similarly, β-arrestin directs the internalization of β-adrenergic receptor (β-AR) by connecting it directly to clathrin (Lin et al., 1997). These findings raise the possibility that localization of TGN proteins may similarly require the participation of connector proteins.

Here, we report the identification of one member of a novel gene family of cytosolic connector proteins, PACS (*p*hosphofurin *a*cidic *c*luster *s*orting proteins), that directs the localization of furin to the TGN. We show that PACS-1 binds directly to the TGN localization signal on the furin-cd and connects the endoprotease to the clathrin-sorting machinery. We use cell-free assays to show that the furin-cd AC is not necessary for TGN budding or retention but apparently functions in a PACS-1-mediated retrieval step. We also show that PACS-1 is required for the correct localization of the cation-independent mannose-6-phosphate receptor (CI-MPR),

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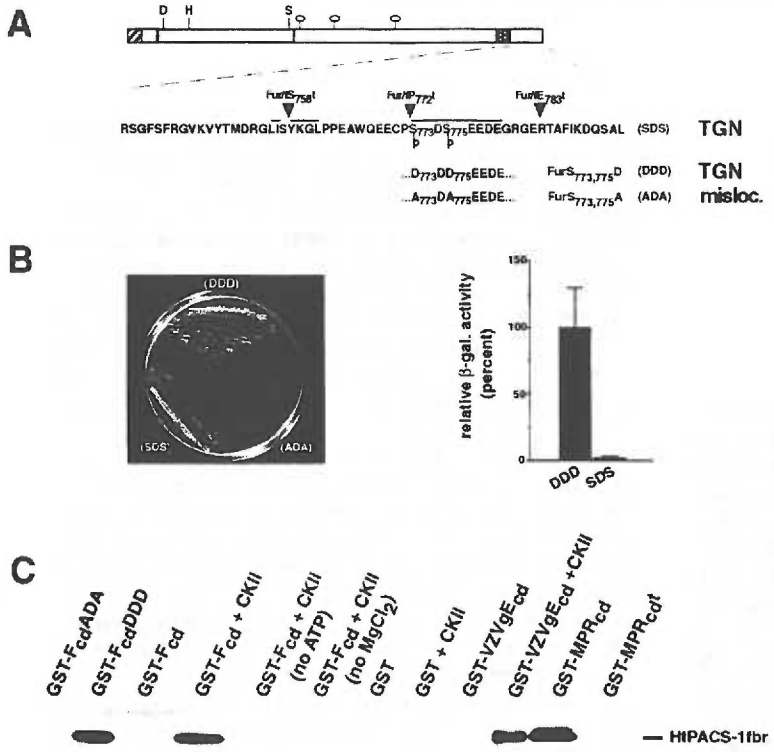

Figure 1. PACS-1 Binds to Acidic Cluster Sorting Motifs

(A) FLAG-tagged furin constructs and localization. The N-terminal cross-hatched box shows the FLAG epitope; the shaded area depicts the catalytic domain with active site residues; tailed circles depict N-linked carbohydrate; and the stippled box depicts the membrane-spanning domain. The 56-amino acid cd is shown. The Tyr-based and dileucine-like internalization motifs and the AC TGN localization motif are overlined.

(B) (Left) L40 yeast cells were cotransformed with pPACS (Leu^- selection) and bait plasmid (Trp^- selection) containing the furin-cd (SDS) or phosphorylation-state mutants (DDD, ADA) and then selected for growth on Leu^-/Trp^- YC minimal plates. Colonies from the Leu^-/Trp^- plates were streaked onto His^- plates and scored for growth. (Right) Measurement of β-gal activity provided a quantitative comparison of the binding affinity of PACS-1 for the native (SDS) and mutant (DDD) furin cd's (assay performed in triplicate, error bars = SD).

(C) (Left) HtPACS-1fbr was combined with either GST, GST-F_{cd}DDD, GST-F_{cd}ADA, GST-F_{cd}, or GST-F_{cd}, phosphorylated by CKII (Jones et al., 1995) in binding buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$, and 1% NP40) and incubated for 1 hr at RT. Protein complexes were recovered with glutathione-agarose beads (30 min, RT). Bound proteins were washed and analyzed by Western blot with antiserum 678 (PACS-1). Controls showed binding was strictly dependent on phosphorylation of GST-F_{cd}, since no binding occurred when either ATP or Mg^{2+} were omitted. (Right) Binding of PACS-1 to additional TGN/endosomal proteins. Binding of HtPACS-1 to GST-VZVgE_{cd} was examined as described above, using both nonphosphorylated and CKII-phosphorylated samples. Binding of PACS-1 to the CI-MPR cd was assessed using either a GST-MPR fusion protein containing both AC motifs (GST-MPR_{cd}) or a truncated construct lacking the C-terminal AC (GST-MPR_{cd}t; see Figure 7A). Results are representative of several independent experiments.

an itinerant TGN/endosomal membrane protein that, like furin, utilizes AC sorting motifs within its cd (Meresse and Hoflack, 1993; Mauxion et al., 1996; Chen et al., 1997). The presence of candidate PACS-binding motifs within many membrane protein cd's suggests a broad and important role for PACS-1 in protein sorting.

Results

Cloning of *PACS-1* by Two-Hybrid Screen

A yeast genetic screen was conducted to identify cytosolic proteins that bind directly to the phosphorylated furin-cd. Because furin-cd phosphorylation cannot be controlled in yeast, this approach required the generation of site-directed furin mutants that constitutively exhibit the phosphorylation-dependent localization of the endoprotease to the TGN (Figure 1A). Furin constructs with either the native sequence (fur/f) or a phosphorylation-mimic mutation, fur/f$S_{773,775}$D (DDD), are localized to the TGN, whereas fur/f$S_{773,775}$A (ADA), a construct that cannot be phosphorylated, is mislocalized (Takahashi et al., 1995; Dittié et al., 1997). Based on these data, a yeast two-hybrid analysis of a mouse embryo cDNA library was conducted that selected for clones that interact with the phosphorylation-mimic mutant $S_{773,775}$D and not the $S_{773,775}$A construct containing disrupted phosphorylation sites. The analysis for one such clone, *PACS-1*, is shown in Figure 1B. Both the native furin-cd and $S_{773,775}$D support growth in His^- media. However, only $S_{773,775}$D supports induction of β-galactosidase (β-gal). The higher affinity of PACS-1 for $S_{773,775}$D suggests the native furin-cd is phosphorylated inefficiently in yeast.

Protein–protein binding in vitro showed that the sequence encoded by the cloned *PACS-1* segment interacts directly with the phosphorylated furin-cd (Figure 1C). A His-tagged construct, HtPACS-1fbr, containing the 140–amino acid furin-binding region (fbr) was incubated with either GST, phosphorylated or nonphosphorylated GST-furin-cd (GST-F_{cd}), or constructs containing phosphorylation state mutations. In agreement with the two-hybrid analysis, the interaction between HtPACS-1fbr and GST-F_{cd}DDD was readily detected, whereas no interaction was observed with GST-F_{cd}, GST-F_{cd}ADA, or GST alone. However, phosphorylation of GST-F_{cd} by CKII promoted binding to HtPACS-1fbr. Similarly, HtPACS-1fbr bound the cd of VZV-gE in a phosphorylation-dependent manner, further suggesting a role for PACS-1 in directing the TGN localization of membrane proteins.

Although TGN localization of both furin and VZV-gE is strictly dependent upon the integrity of their cd ACs, additional proteins (e.g., CI-MPR) have similar AC motifs with important but less well-defined sorting functions (see Figure 7A). Indeed, HtPACS-1fbr also binds to the CI-MPR cd. Furthermore, binding requires the C-terminal AC, a motif important for receptor trafficking and AP-1 recruitment (Mauxion et al., 1996). Interestingly, whereas efficient binding of PACS-1 to the furin and

VZV-gE cd's requires phosphorylation, binding to the CI-MPR does not. Together, the binding assays indicate a broad role for PACS-1 in directing protein sorting in the TGN/endosomal system.

Screening of a rat brain cDNA library with the *PACS-1* segment yielded two cDNAs, representing splice variants of a single gene (Figure 2A). Both cDNAs encode complete open reading frames (ORFs) that contain the 140-amino acid fbr present in PACS-1(Val-115-Pro-255). The larger clone, *PACS-1a*, contains a 2886 nt ORF encoding a 961-residue protein. The smaller clone, *PACS-1b*, contains a 1680 nt ORF encoding a 559-residue protein. Northern blot hybridization with transcript-specific probes showed that, like furin, both *PACS-1* transcripts are expressed ubiquitously and the 4.4 kb *PACS-1a* transcript is present in great excess (>20-fold) over that of the 3.6 kb *PACS-1b* transcript (data not shown).

PACS-1 Has Properties of a Cytosolic Coat Protein

Analysis of homogenates from tissues and cultured cell lines revealed a 120 kDa anti-PACS immunoreactive protein that comigrated with recombinant PACS-1a (see Figure 3A). Consistent with the absence of a predicted transmembrane domain (Figure 2B, left), sedimentation at 100,000 × g resulted in 78% of the PACS-1a partitioning to the supernatant, while 22% remained membrane-associated but could be extracted with high salt (0.5 M) and carbonate (0.1 M, [pH 11]) (Figure 2B, right).

Immunocytochemical studies supported the membrane fractionation analysis and demonstrated that PACS-1 has properties characteristic of a coatomer protein. Analysis of PACS-1 localization by immunofluorescence microscopy revealed a punctate staining pattern present in the paranuclear region that coincided with that of γ-adaptin and extended into the cell periphery (Figure 2C). Treatment of cells with AlF_4^- prior to fixation, which enhances coatomer association with membranes (Ikonen et al., 1997), increased the intensity of the paranuclear PACS-1 signal. Consistent with previous studies, AlF_4^- produced an enhanced TGN staining pattern for γ-adaptin, which was more condensed than in control cells and in close juxtaposition to the PACS-1 staining. By contrast, treatment of cells with brefeldin A (BFA), which blocks the ARF-directed coatomer association with cellular membranes, resulted in dispersed PACS-1 and γ-adaptin staining patterns. These results indicate that PACS-1 has a dynamic, ARF-dependent, TGN-proximal localization that is distinct, yet overlapping with γ-adaptin.

TGN Localization of Furin Requires PACS-1

The importance of PACS-1 in the sorting of furin in vivo was investigated using an antisense strategy. A7 melanoma cells, which contain detectable levels of endogenous furin, were stably transfected with either pCEP-4 plasmid containing *PACS-1* in reverse orientation or with an empty vector. Following drug selection, the level of PACS-1 in the clonal cell lines was determined by Western blot analysis (Figure 3A). Multiple antisense clones were obtained with levels of PACS-1 protein reduced 3- to 9-fold relative to a control protein (α-tubulin).

The effect of the antisense knockdown of PACS-1 levels on endogenous furin localization was determined by immunofluorescence microscopy (Figure 3B). Analysis of control cells showed a paranuclear staining pattern for endogenous furin characteristic of its TGN localization (Molloy et al., 1994; Schäfer et al., 1995). By contrast, endogenous furin staining was markedly dispersed in the PACS-1 antisense cells. Western blot analysis showed similar levels of furin in control and antisense cells, demonstrating that the weaker staining in the antisense cells was due to a mislocalization of the endoprotease and not to differences in expression and/or stability (inset).

The mislocalization of furin did not result from a gross corruption of the TGN, since localization of both TGN46, the human ortholog of TGN38, and γ-adaptin were unaffected by PACS-1 depletion (Figure 3B). These results also demonstrate that furin and TGN38/46 are localized to the TGN by separate mechanisms. Other secretory pathway markers, including those for the ER (SSR), Golgi cisternae (mannosidase II), and plasma membrane/endosomes (transferrin receptor), were also unaffected by PACS-1 depletion. These results demonstrate a role for PACS-1 in the sorting of select proteins within the TGN/endosomal system.

A direct link between mislocalization of furin and suppression of PACS-1 expression was provided by rescue experiments (Figure 3C). Due to the low level of endogenous furin, fur/f was expressed alone or in combination with PACS-1a in antisense (AS19) and control (C1) cells in order to increase the sensitivity of this analysis. Consistent with the distribution of endogenous furin, the staining pattern of fur/f was dramatically different in the control and antisense cells. However, when fur/f and PACS-1a (or PACS-1b, data not shown) were coexpressed in the antisense cells, the TGN localization of fur/f was restored.

Localization of CI-MPR Requires PACS-1

The binding of HtPACS-1fbr to the C-terminal CI-MPR AC (Figure 1C) suggested that PACS-1 may also direct sorting of the CI-MPR. To examine this possibility, the localization of CI-MPR was compared in C1 and AS19 cells (Figure 4). In control cells, both CI-MPR and TGN46 colocalized with γ-adaptin (Figure 4A). Furthermore, in agreement with Figure 3, TGN46 in AS19 cells remained colocalized with γ-adaptin in the paranuclear region. By contrast, the CI-MPR staining pattern in AS19 cells was more distended relative to that of γ-adaptin and was redistributed to an endosomal population where it overlapped with internalized dextran. The extent of CI-MPR redistribution was quantified by morphometric analysis (Figure 4B). These data demonstrate that the routing of CI-MPR is directed by PACS-1. Furthermore, the uncoupling of CI-MPR and γ-adaptin staining patterns argues that recruitment of AP-1 to the TGN is independent of cargo protein. Finally, these data support a broad and important role for the PACS family in protein sorting within the secretory pathway.

TGN Retrieval, but Not Retention, Is Mediated by Furin-cd Acidic Cluster

The requirement of furin-cd phosphorylation for the TGN localization of furin suggested that the furin-cd AC directs either the retention or the retrieval of the endoprotease to the TGN. Furthermore, phosphorylation of the

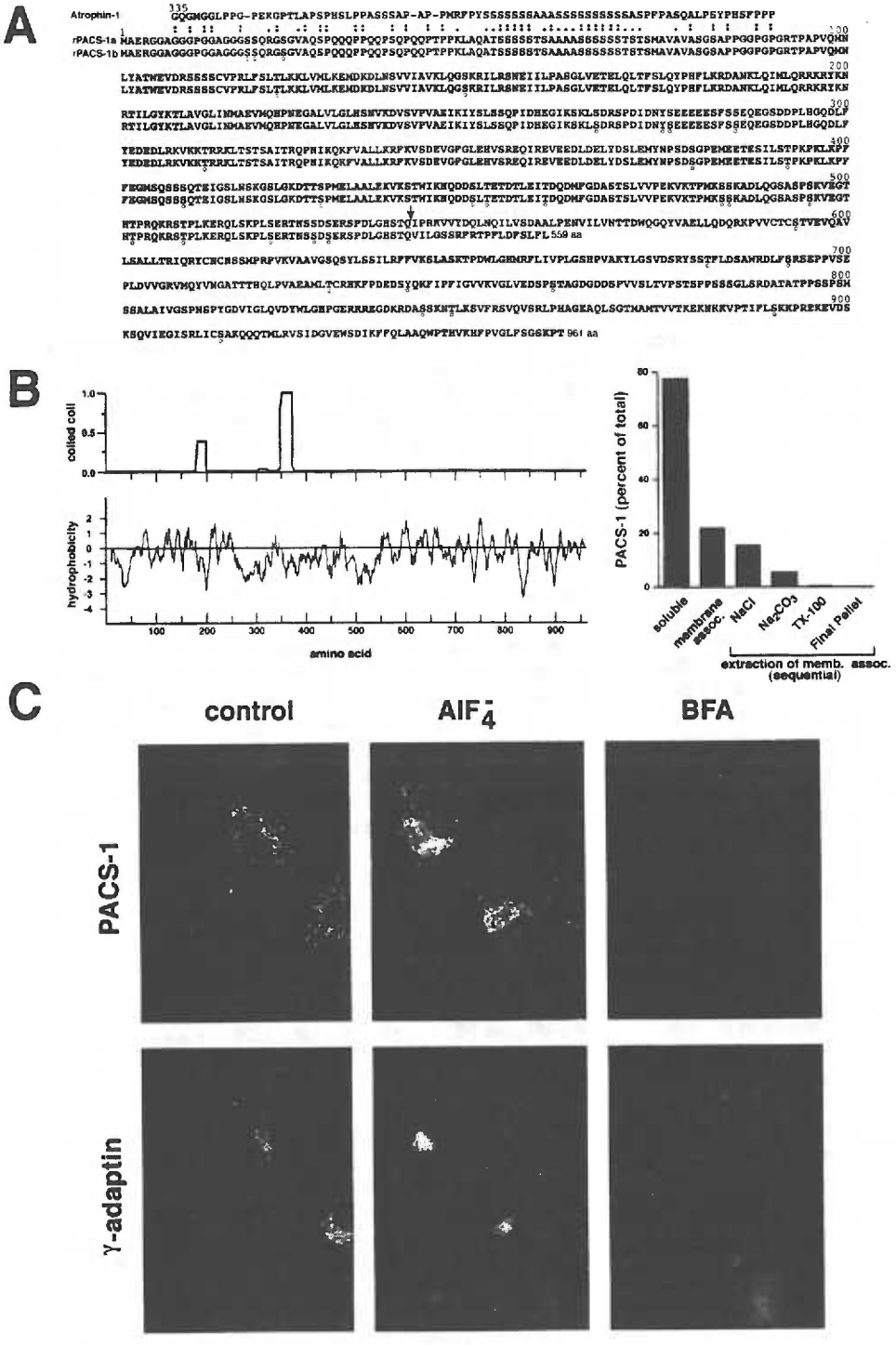

Figure 2. Predicted Amino Acid Sequence and Subcellular Localization of PACS-1

(A) Analysis of *PACS-1a* and *PACS-1b* cDNAs. The predicted translation start site in both open reading frames complies with Kozak's rules (Kozak, 1991) and is 93 nts downstream from an in-frame stop codon. The predicted splice site (↓) follows Gln-540. Hydropathy analysis (Kyte-Doolittle) predicts both proteins are hydrophilic and lack membrane-spanning domains. The fbr is shaded. A single amino acid difference is present in mouse PACS-1fbr (Ala-144→Gly). The N-terminal region shares limited homology with atrophin-1 (shown). Sequences between Leu-164 and the PACS-1a C terminus share 31% identity with the *Drosophila* gene product KrT95D. EST clones containing PACS-1 sequences were found for human (gbAA381307, gbH85491, embZ44004, gbAA296369, embZ40056, gbR50269, gbT11563, gbT11564, and gbAA159946), *C. elegans* (D36982, D37521), and rice (D48009). Open circles, potential phosphorylation sites; double closed circles, identical amino acids; closed circles, conserved amino acids.

(B) (Left) Lupas coiled-coil determination (window = 21 residues) (Lupas et al., 1991) and Kyte-Doolittle hydrophobicity plot (window = 11 res-

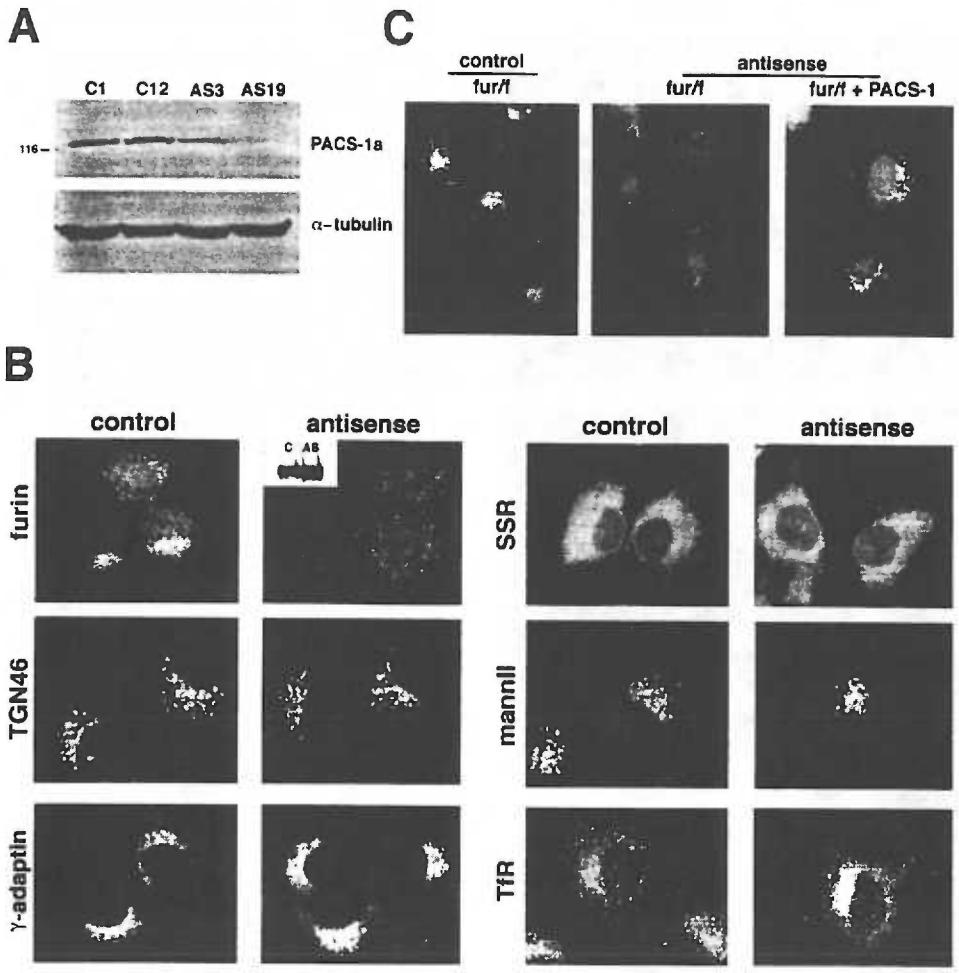

Figure 3. PACS-1 Is Required for TGN Localization of Furin

(A) An equivalent aliquot of lysate from control (C1,C12) and antisense (AS3,AS19) cell lines was resolved by SDS-PAGE and analyzed by Western blot using antiserum 7704 (PACS-1) and MAb N356 (α-tubulin), followed by chemiluminescent detection. Signals were quantified using NIH Image software. M, standard (kDa) is shown on left. Similar results were obtained for suppression of PACS-1b (data not shown).

(B) Parallel plates of C1 and AS19 cells were fixed, permeabilized, and stained with MAb MON152 (furin), anti-mannosidase II, anti-SSR, anti-TGN46, and MAb 100/3 (γ-adaptin). TfR was visualized by incubation of cells with rhodamine transferrin (r-Tf, 40 ng/ml) for 1 hr. The selective mislocalization of endogenous furin was observed in several independent PACS-1a lines. (Inset) Membrane samples from equal amounts of C1 and AS19 cells were separated by SDS-PAGE and analyzed by Western blot using a furin-cd antiserum (ABR).

(C) Parallel plates of C1 and AS19 cells were infected with VV recombinants expressing fur/f (m.o.i. = 3) or fur/f (m.o.i. = 3) and PACS-1a (m.o.i. = 3). At 4 hr postinfection, cells were fixed, permeabilized, and fur/f visualized with MAb M2. Coexpression of fur/f with wild-type VV had no effect on fur/f trafficking (data not shown).

CI-MPR-cd by CKII has been reported to recruit AP-1 to the TGN (Mauxion et al., 1996), suggesting that the furin-cd AC may also modulate TGN budding. To distinguish between these possibilities, a cell-free budding assay was performed on FLAG-tagged furin constructs expressed in AtT-20 cells (Figure 5). AtT-20 cells were used in this assay because the different N-glycosylated forms are readily resolved by SDS-PAGE. The efficacy of the budding reaction was demonstrated by showing that budding of TGN (i.e., sialylated) furin constructs—both the membrane-anchored construct and a soluble form generated by cleavage within the lumenal domain—required addition of cytosol, ATP, and hydrolyzable GTP (Figure 5A). Furthermore, budding was specific for TGN-accumulated furin, since a Golgi-stack form of furin (i.e., nonsialylated) was not released during the incubation. The relative efficiency of budding of each furin construct was determined quantitatively by normalization to the amount of soluble sialylated furin released in each sample (Figure 5B). Budding of furin from

idues) of PACS-1a ORF. (Right) Membrane fractionation of endogenous PACS-1a analyzed by Western blotting using antiserum 7704 (PACS-1).

(C) A7 cells were either fixed immediately or treated with AlF_4^- (50 μM $AlCl_3$, 3 μM NaF) or 10 μM BFA for 30 min prior to fixation. Cells were then permeabilized and double labeled with affinity-purified antiserum 678 (PACS-1) and MAb 100/3 (γ-adaptin). Identical results were obtained with antiserum 7704 (PACS-1).

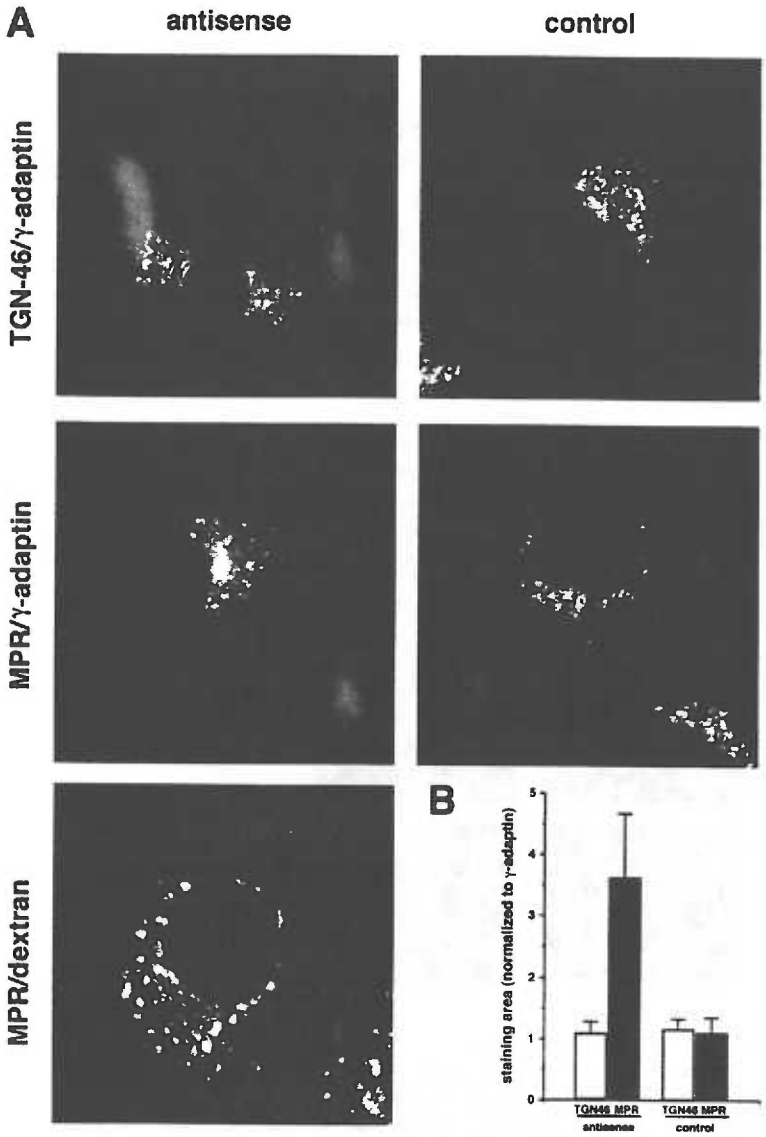

Figure 4. Localization of CI-MPR is PACS-1-Dependent

(A) Parallel plates of C1 and AS19 cells were fixed, permeabilized, and double-labeled with anti-TGN46 or anti-CI-MPR antisera and MAb 100/3 (γ-adaptin). Antibody staining was visualized with anti-rabbit-FITC (TGN46 and MPR, green) and anti-mouse IgG-TXR (γ-adaptin, red). Additional cells were incubated with TXR-dextran for 1 hr to label endocytic compartments prior to fixation and staining with anti-CI-MPR antiserum.

(B) CI-MPR mislocalization was assessed quantitatively by comparing the staining area of TGN46 and CI-MPR relative to γ-adaptin, using morphometric analyses (Scion Image 1.62). The summation includes only positive staining pixels (n = 50, error bars = SD).

the TGN was independent of cd phosphorylation state (fur/fDDD, fur/fADA) as well as the presence (fur/f, fur/fE_{783}t) or absence (fur/fP_{772}t) of the AC. By contrast, deletion of the tyrosine-based internalization signal (fur/fS_{758}t) blocked budding of furin. These results show that efficient budding of furin from the TGN is independent of the AC but requires furin-cd sequences containing the tyrosine-based motif previously shown to bind AP-1 directly (Ohno et al., 1996). Furthermore, the furin-cd AC has no effect on retention of the endoprotease to the TGN. Rather, these data argue that phosphorylated AC-dependent localization of furin to the TGN is mediated by a PACS-1-dependent retrieval mechanism. These findings are consistent with those in Figure 2, demonstrating a predominantly non-TGN localization of PACS-1, and Figure 4, showing that localization of the CI-MPR can be uncoupled from γ-adaptin.

PACS-1 Connects Furin-cd to Clathrin Adaptors

The selective binding of the PACS-1fbr to phosphorylated furin (Figure 1C), together with the observation that phosphorylation of the furin-cd increases its binding to γ-adaptin (Dittié et al., 1997), suggests that PACS-1 directs the TGN retrieval of furin by linking the endoprotease to components of the clathrin-sorting machinery. We tested this hypothesis by performing a series of protein binding assays (Figure 6). These analyses showed that GST-F_{cd}DDD, but not GST-F_{cd}ADA, bound endogenous PACS-1a in bovine brain cytosol (bbc) (Figure 6A). This selective binding was coupled with an enhanced association of γ-adaptin. Furthermore, γ-adaptin was also selectively recovered from bbc using HtPACS-1fbr but not the control protein HtABP-280 (containing sequences of the furin-sorting protein APB-280 that binds the furin-cd, independent of its phosphorylation state [Liu et al., 1997]) (Figure 6B). These results suggest that the association of AP-1 with the phosphorylated furin-cd is mediated by PACS-1.

Studies with cultured cells further supported our hypothesis that PACS-1 links furin to the clathrin-sorting machinery. Endogenous γ-adaptin could be recovered from cell extracts using Ni-agarose beads only in the

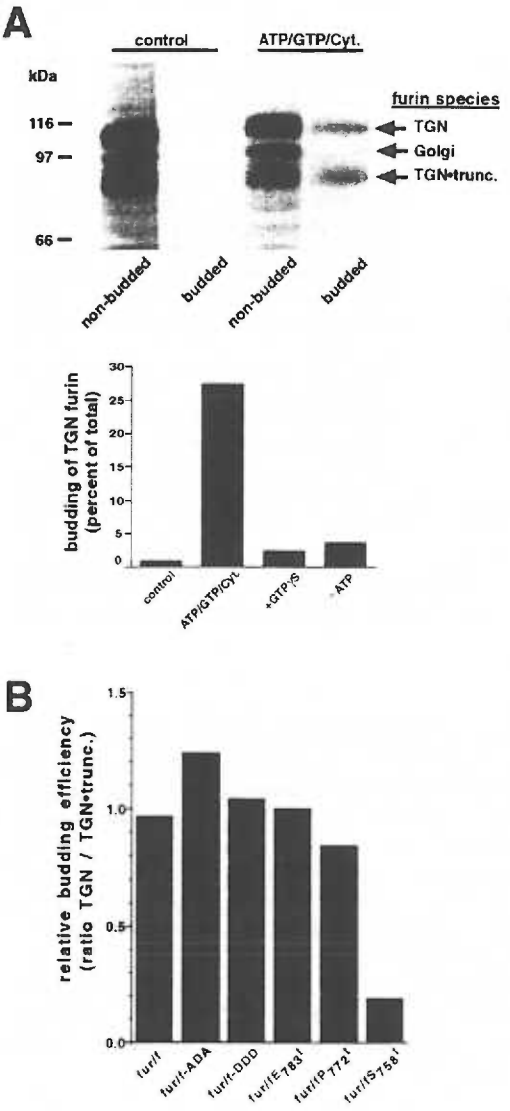

Figure 5. The Furin-cd Acidic Cluster Is Not Required for Efficient TGN Budding

(A) AtT-20 cells expressing fur/f were pulse-labeled with [^{35}S]Met/Cys, placed on ice, and scraped. Washed cells were incubated (37°C, 60 min) in the absence (control) or presence of budding mix and bbc (ATP/GTP/Cyt.) then placed on ice and released TGN-budded vesicles separated from broken cells by centrifugation. FLAG-tagged forms of furin were immunoprecipitated with MAb M1, separated by SDS-PAGE, and detected by fluorography. Only sialylated (TGN localized) furin was able to bud. The positions of sialylated, membrane-anchored furin (TGN) and sialylated, soluble furin proteolytically clipped in vivo, (TGN·trunc.), as well as endoglycosidase H-resistant, neuraminidase-insensitive furin (Golgi) are indicated. Control experiments showed that budding was ATP- and time-dependent and sensitive to GTPγS. Protease sensitivity demonstrated that budded fur/f was in the correct orientation. Molecular weight markers (kDa) are shown.

(B) The budding efficiency of a series of C-terminal furin truncations (Figure 1A) and CKII phosphorylation site mutants (DDD, ADA) was compared to native furin as described above. Budding efficiency is plotted as percentage of membrane bound/percentage soluble furin in the vesicle fraction. Results are representative of several independent experiments.

presence of coexpressed His-tagged PACS-1b (Figure 6C). Furthermore, GST-F_{cd}DDD bound substantially more γ-adaptin in extracts of control C1 cells than in extracts from PACS-1-depleted AS19 cells, despite equal levels of adaptor expression (Figure 6D). Together, our results show that PACS-1 directs the retrieval of phosphorylated furin to the TGN by connecting the endoprotease to the clathrin-sorting machinery in vivo.

Discussion

In this report, we describe PACS-1 as a member of a novel gene family of cytosolic connector proteins required for localization of membrane proteins to the TGN. PACS-1 is a partially membrane-associated cytosolic protein (Figure 2) that binds selectively to phosphorylated furin and VZV-gE cd's (Figure 1C), a modification required for their TGN localization (Takahashi et al., 1995; Alconada et al., 1996; Dittié et al., 1997). Antisense expression shows PACS-1 is required for TGN localization of furin but not TGN46 (Figure 3), demonstrating multiple sorting systems are used to maintain itinerant proteins in the TGN. In addition, PACS-1 binds to the CI-MPR-cd and is required for its correct localization (Figure 4), suggesting that PACS-1 directs the routing of a potentially large number of membrane proteins containing AC sorting motifs within their cd's (Figure 7A). Binding assays in vitro and coprecipitation studies in vivo show PACS-1 localizes furin to the TGN by connecting the endoprotease to the clathrin-sorting machinery (Figure 6). These studies, coupled with a demonstration of the lack of effect of the furin-cd AC on TGN retention or budding (Figure 5), argue that PACS-1 localizes the endoprotease to the TGN by a phosphorylation-dependent retrieval mechanism.

A Model for PACS-1-Mediated TGN Localization of Membrane Proteins

Based on our results, we propose a model for the localization of furin and other membrane proteins (e.g., CI- and CD-MPRs and VZV-gE) to the TGN in mammalian cells (Figure 7B). This model explains TGN localization by a selective retrieval mechanism, as opposed to the retention process used by resident membrane proteins of the Golgi cisternae (Munro, 1995). The initial step consists of the efficient budding of membrane-anchored furin from the TGN by virtue of its tyrosine motif interacting with AP-1. Evidence for this step is supported by several findings, including (1) the requirement for the tyrosine motif, but not the AC, for TGN budding of furin (Figure 5), (2) the ability of the furin-cd tyrosine motif to bind AP-1 (and AP-2) by two-hybrid analysis (Ohno et al., 1996), (3) the ability of VZV-gE-cd constructs containing only the tyrosine motif and not the AC to recruit AP-1 to TGN membranes (Alconada et al., 1996), and (4) the demonstration that export of lamp-1 from the TGN to clathrin-coated vesicles is directed by the binding of AP-1 to a tyrosine-based motif within the lamp-1 cd (Honing et al., 1996). The direct binding of AP-1 to tyrosine-based motifs does not, however, preclude the participation of additional AP-1-associated proteins (Mallet

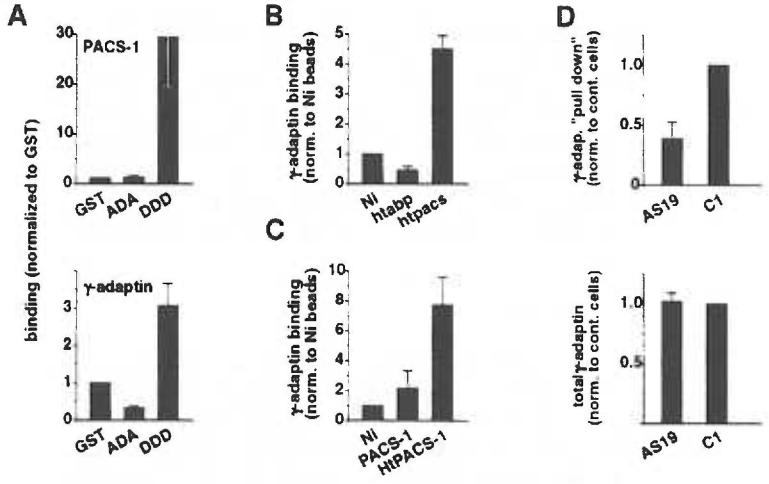

Figure 6. PACS-1 Mediates Association of Furin-cd with AP-1

(A) Aliquots of bbc were incubated with either GST, GST-F_{cd}ADA, or GST-F_{cd}DDD (37°C, 30 min). Bound proteins were recovered with glutathione-agarose beads and analyzed by Western blot.

(B) Aliquots of bbc were incubated (37°C, 30 min) in the absence or presence of either HtABP-280 or HtPACS-1fbr prebound to Ni-agarose in binding buffer containing 8 mM imidazole. Proteins were washed with binding buffer containing 50 mM imidazole, eluted in SDS-SB, analyzed by Western blot, and quantified.

(C) BSC-40 cells were infected with either wild-type VV or VV recombinants expressing either PACS-1b or HtPACS-1b (m.o.i. = 5). Clarified lysates (prepared in 50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM MgCl2, 1% NP-40, 0.2 mM PMSF, and 5 μg/ml leupeptin) were incubated with Ni-agarose beads in the presence of 3 mM imidazole (37°C, 30 min) and washed as described in (B), and bound proteins were analyzed by Western blot and quantified.

(D) Equivalent amounts of high-speed supernatant solution (290 μg total protein) containing equal amounts of γ-adaptin (bottom) were prepared from parallel plates of C1 and AS19 cells and incubated with GST-F_{cd}DDD (4°C, 16 hr; top). Protein complexes were recovered with glutathione agarose beads, analyzed by Western blot, and quantified. All data are averaged from at least three independent experiments and were quantified with NIH image software (error bars = SD).

and Brodsky, 1996; Seaman et al., 1996). Once incorporated into a post-TGN endosome, furin may either be exported to the cell surface or retrieved to the TGN. Export to the cell surface would require the activity of a furin phosphatase (S. S. M. and G. T., submitted), whereas retrieval to the TGN requires phosphorylation of the furin-cd AC by CKII (Jones et al., 1995; Takahashi et al., 1995; Dittié et al., 1997). Like coatomer proteins, PACS-1 is recruited to the endosome by ARF-1 (Figure 2), where it binds to phosphorylated furin and connects the endoprotease to the clathrin/adaptor complex (Figure 6). Phosphorylated furin is then retrieved back to the TGN. While we have demonstrated an interaction between PACS-1 and AP-1, the participation of other adaptor complexes (e.g., AP-3 [Simpson et al., 1997]) is not excluded. Thus, our model distinguishes the role of the tyrosine-based sorting motif (TGN budding) from the AC motif (retrieval). This phosphorylated AC-dependent retrieval step is consistent with the AP-1-associated removal of phosphorylated furin from clathrin-coated immature secretory granules (ISGs) (Dittié et al., 1997).

PACS-1 Homologs Are Broadly Expressed in Metazoans

Analysis of EST databases reveals at least three human PACS family members (Figure 2, and unpublished results). In addition, PACS homologs are also expressed in a wide variety of metazoans, including other mammals, nematodes, insects, and plants. PACS-1a shares significant sequence similarity with KrT95D, a Krüppel-regulated *Drosophila* gene product of unknown function (Hartmann and Jäckle, 1997). Sequence alignment of PACS-1a and KrT95D shows greater than 31% sequence identity between Leu-164 and Thr-961. Interestingly, the PACS-1 N-terminal region, composed of a glycine (G)-rich domain followed by domains rich in proline/glutamine (P/Q) and serine/alanine (S/A), is absent from the KrT95D sequence but shares limited homology with atrophin-1 (see Figure 2A), the gene product mutated in the neurodegenerative disease Dentatorubral-pallidoluysian atrophy (DRPLA, Nagafuchi et al., 1994). In addition to PACS-1 and atrophin-1, P/Q- and S/A-rich regions are present in many transcription factors and appear to direct protein–protein interactions (Gerber et al., 1994), suggesting a potential regulatory role for this domain of PACS-1. Despite the presence of PACS-1 homologs in evolutionarily distant organisms, none were identified in a search of the yeast genome database, suggesting metazoan cells require sorting steps not performed in yeast.

Functional Parallels between PACS-1 and Cell Surface Connectors

PACS-1 is functionally related to a small yet diverse set of cytosolic proteins that connect membrane proteins to the clathrin-sorting machinery. Previously identified members of this group, including HIV-1 Nef (Foti et al., 1997), Eps15 (Benmerah et al., 1998), and β-arrestin (Lin et al., 1997), mediate internalization of cell surface receptors (CD4, EGF, and G protein–coupled receptors, respectively) by connecting them to the clathrin/AP-2-sorting machinery. By contrast, PACS-1 connects membrane proteins to the clathrin/AP-1-sorting machinery. Interestingly, the regulation of protein sorting by PACS-1 and β-arrestin share many features. First, both connectors bind to membrane proteins phosphorylated on serine residues (Sohlemann et al., 1995, and Figure 1). The β-AR is phosphorylated by the β-adrenergic receptor kinase (βARK) to initiate receptor down-regulation (desensitization), whereas the furin-cd is phosphorylated by CKII to localize the endoprotease to the TGN. Second, PACS-1 and β-arrestin connect the membrane proteins to the clathrin-sorting machinery. β-arrestin connects the β-AR directly to clathrin-heavy chains (Goodman et

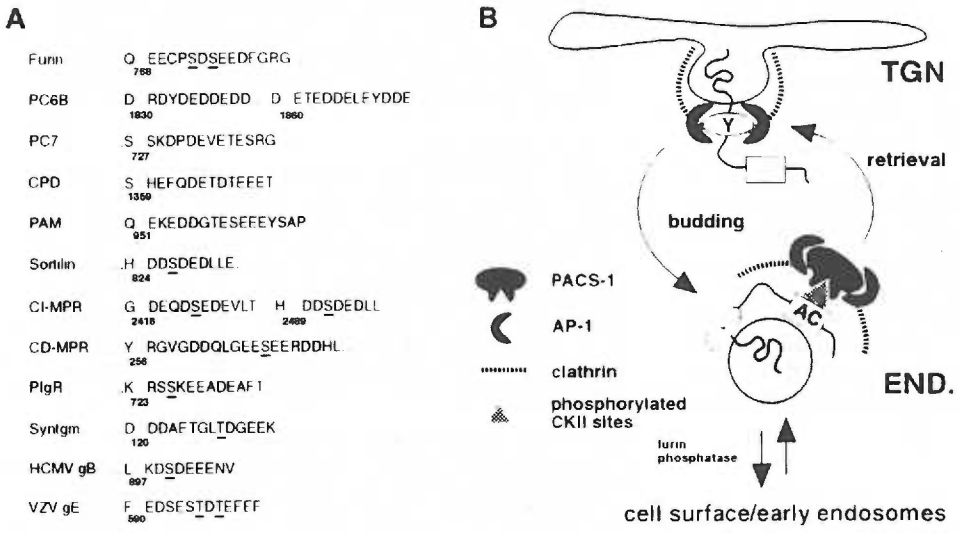

Figure 7. A Model for Acidic Cluster–Dependent Protein Trafficking

(A) Shown are (phosphorylatable) ACs of furin (Jones et al., 1995), PC6B (Nakagawa et al., 1993), PC7/8 (Bruzzaniti et al., 1996), carboxypeptidase D (Xin et al., 1997), PAM (Stoffers et al., 1991), sortilin (Petersen et al., 1997), CI-MPR (Meresse and Hoflack, 1993), CD-MPR (Korner et al., 1994), polymeric immunoglobulin receptor (pIgR) (Okamoto et al., 1994), synaptotagmin (Bennett et al., 1993), cytomegalovirus (HCMV) gB (Norais et al., 1996), and VZV-gE (Yao et al., 1993). Phosphorylated residues are underlined.

(B) A model for PACS-1-dependent TGN localization of furin. Budding of furin from the TGN is mediated by direct interaction of the furin-cd tyrosine motif (Y) with AP-1. The phosphorylated furin-cd acidic cluster (AC) is "silent" at this step (lightly shaded). The AC silencing is likely not due to lack of phosphate, since furin is phosphorylated as early as the endoplasmic reticulum (Jones et al., 1995). Rather, it may reflect exclusion of PACS-1 from TGN membranes. Once in a post-TGN endosome, furin is either shuttled to the cell surface/early endosomes or is retrieved to the TGN. Transit to the cell periphery would require dephosphorylation of the furin-cd (Jones et al., 1995; Dittié et al., 1997). By contrast, TGN retrieval requires binding of PACS-1 to the CKII-phosphorylated furin-cd AC and recruitment of AP-1. AP-1 is unable to bind the furin-cd AC directly and requires PACS-1 to serve as a connector protein. In this step, the Y motif is "silenced." The silencing mechanism may result from steric interference by PACS-1, compartment-specific modification of AP-1 (Wilde and Brodsky, 1996), or perhaps the exclusion from this compartment of accessory proteins that mobilize the Y-bound AP-1 molecules into clathrin coats (Mallet and Brodsky, 1996; Seaman et al., 1996).

al., 1997), whereas PACS-1 connects furin to AP-1 (Figure 6). Our results do not exclude the possibility that PACS-1 connects furin (or other itinerant membrane proteins containing PACS-1-binding motifs [e.g., CI-MPR, Figure 4]) directly to clathrin or to other adaptor complexes (e.g., AP-3). Third, both connectors are phosphorylated in vivo (Lin et al., 1997, and data not shown). While phosphorylation directs recruitment of β-arrestin onto cellular membranes, the role of PACS-1 phosphorylation has not been determined.

Regulation of Furin Trafficking by Phosphorylation

The sorting of furin through the TGN/endosomal system is dynamic and regulated by several factors, including identified components of the cytoskeleton (ABP-280; Liu et al., 1997) and the phosphorylation state of the furin-cd. In addition to localizing furin to the TGN, phosphorylation by CKII modulates localization of the endoprotease to an early endosome/cell surface cycling loop (S. S. M. and G. T., submitted) and, in neuroendocrine cells, the AP-1-coupled retrieval of furin molecules from ISGs (Dittié et al., 1997). By contrast, movement of furin between endosomes and the TGN is regulated by dephosphorylation of the furin-cd by specific PP2A isoforms (Jones et al., 1995, and S. S. M. and G. T., submitted). The phosphorylation-dependent trafficking of furin suggests that formation of furin-containing proprotein-processing compartments within the TGN/endosomal system is dynamic and acutely regulated by signal transduction pathways. The role of furin-cd phosphorylation in regulating the localization of the endoprotease to several cellular compartments suggests multiple sorting steps mediated by PACS-1. Indeed, in addition to localizing furin to the TGN, PACS-1 is also required for localization of the endoprotease to early endosomes (S. S. M. and G. T., submitted). Whether retrieval of furin from ISGs requires PACS-1 is currently being investigated. The importance of PACS-1 for localizing furin to multiple compartments is consistent with its distribution to vesicular compartments throughout the cytoplasm (Figure 2C).

A Broad Role for PACS Family Members in Protein Sorting

The presence of (phosphorylatable) ACs within the cd's of a large number of secretory pathway membrane proteins (Figure 7A) suggests PACS family members have a broad and important role in the organization of and trafficking between secretory pathway compartments. This is supported by the binding of PACS-1 to the VZV-gE and CI-MPR cd's as well as the requirement of PACS-1 for CI-MPR localization (Figure 4). Indeed, the TGN localization of VZV-gE, as well as the sorting of both the CI- and CD-MPRs, is directed by AC-sorting domains (Meresse and Hoflack, 1993; Alconada et al.,

1996; Mauxion et al., 1996; Chen et al., 1997). Very recently, a 47 kDa protein was identified that binds to a hydrophobic sorting signal in the CI- and CD-MPR cd's and participates in endosome-to-TGN sorting (Diaz and Pfeffer, 1998). Whether PACS-1 and TIP47 function in the same or in different sorting steps remains to be determined.

Our identification of a novel gene family of sorting proteins required for the phosphorylation state-mediated TGN localization of furin delineates core components of a novel signaling system used to sort proteins within the mammalian cell secretory pathway. How these signaling systems are used to regulate the dynamic localization of furin, and other itinerant membrane proteins, to specific compartments is currently being studied.

Experimental Procedures

Yeast Two-Hybrid Screen and Cloning

All methods used for yeast two-hybrid analysis are described elsewhere (Liu et al., 1997). The mouse embryo cDNA library was subcloned into pVP16 (HSV activation domain). DNA sequences encoding the native furin-cd or the DDD and ADA mutants were amplified by PCR and subcloned into pBTM116 (LexA DNA-binding domain). Quantitative β-gal assays were performed using dual transfected cells. Washed cells were resuspended in phosphate buffer and frozen/thawed, and clarified supernatants were incubated with ONPG (0.75 mg/ml) at 30°C and measured for β-gal activity (A-420). Cloning procedures were performed as described (Sambrook et al., 1989). The λgt10 rat brain cDNA library was provided by J. Adelman.

Bacterial Fusion Protein Production

pGEX-3X plasmids expressing either the native furin-cd or the DDD and ADA mutants are described elsewhere (Jones et al., 1995). pGEX-3X expressing the GST-CI-MPR cd's were provided by S. Tooze. The VZV-gE cd was obtained by PCR amplification (5′ primer-CGCGGATCCGGGTTAAAGCCTATAGGGTAG and 3′ primer-CGGAATTCCGGGTCTTATCTATATACACCGTG) of pTM1-VZVgE(wt) and subcloned into pGEX-3X following digestion with BamHI and EcoRI. Methods for isolation of GST-fusion proteins were performed as described (Pharmacia). HtPACS-1fbr was generated by PCR using pVP16:PACS as the template (5′ primer-GGAATTCCATATGCCCAGGCTCTTCAGCTTAAC; 3′ primer-CGGGATCCATGGGCTGGCTGGATAAAG). The PCR product was subcloned into pET16b (Novagen), digested with NdeI and BamHI, expressed in *E. coli* BL-21 lysS, and purified by Ni-chromatography. HtABP-280 containing amino acids P-1490→A-1607 of human ABP-280 is described elsewhere (Liu et al., 1997).

Cell Culture and Vaccinia Virus

BSC-40, AtT-20, and A7 cells were grown as described (Liu et al., 1997). To generate antisense cell lines, pZVneo:PACS-1b (below) was digested with BamHI and XhoI, and the DNA insert was subcloned in reverse orientation in pCEP-4. A7 cells were transfected with either pCEP-4:PACS-1as (antisense clones) or empty vector (control clones). Stable clones were selected with 300 μg/ml hygromycin B (Sigma).

Vaccinia virus (VV) recombinants expressing fur/f, fur/f$S_{773,775}$D, fur/f$S_{773,775}$A, fur/fE_{783}t, fur/fP_{772}t, and fur/fS_{758}t have been described (Molloy et al., 1994; Jones et al., 1995). To generate VV recombinants expressing PACS-1a or PACS-1b, the cDNA inserts were excised from pGEM7Zf(+)PACS-1a and pGEM7Zf(+)PACS-1b by digestion with SphI and EcoRI, blunt-ended with Klenow and T4 DNA polymerase, and subcloned into pZVneo. Vaccinia recombinants expressing PACS-1a and PACS-1b were generated by standard methods (Liu et al., 1997). To generate the VV recombinant expressing HtPACS-1b, a 10× His C tail was inserted into the *PACS-1b* cDNA by PCR [5′ primer-TACGACAGTCTAGAAATGTAC, 3′ primer- GGAATTCATCAA$(TGA)_{10}$CCCAAGATTACCTGTGTGC]. The mutant DNA fragment was cut with XbaI and EcoRI and subcloned into pGEM7Zf(+)PACS-1b. The *HtPACS-1b* cDNA was then subcloned into pZVneo as described above, and a VV recombinant was generated. HtPACS-1b contains all but the C-terminal 15 residues of PACS-1b ORF, which are replaced by the His-tag.

In Vitro Binding Assays

bbc was prepared as described (Dittié et al., 1997). Binding assays contained 200 μl bbc (4 μg/ml) combined with GST- or His-tagged proteins and were performed in 25 mM HEPES, 25 mM KCl, 2.5 mM KOAc (pH 7.2) (supplemented with 0.5 mM PMSF and 5 μg/ml leupeptin), except where indicated in the figure legends. Proteins were recovered with glutathione- or Ni-agarose, eluted with 2× SDS-sample buffer (SDS-SB), resolved by SDS-PAGE, and analyzed by Western blot with either MAb 100/3 (γ-adaptin) or antiserum 7704 (PACS-1) and HRP-conjugated secondary antibodies. Chemiluminescent signals were quantified using NIH Image software.

Immunofluorescence Microscopy and Antibodies

Immunofluorescence analyses were performed as previously described (Molloy et al., 1994). FLAG-tagged furin was detected with MAb M2 (IBI). Endogenous furin was detected with MAb MON152 (W. van de Ven) or a polyclonal antiserum against the furin-cd (ABR, Liu et al., 1997); γ-adaptin was detected using MAb 100/3 (Sigma). The anti-mannosidase II antiserum was provided by K. Moremen, the anti-TGN46 antiserum was provided by V. Ponnambalam, and the anti-CI-MPR antiserum was provided by B. Hoflack. Alpha-tubulin was detected with MAb N356 (Amersham). Staining of primary antibodies was visualized with species- or subtype-specific secondary antibodies (Fisher) conjugated to FITC or TXR. r-Tf and TXR-dextran internalization assays were performed as described (Liu et al., 1997).

To generate antiserum 678, the 422 nt DNA fragment encoding the fbr was amplified from pPACS-1a by PCR (5′ primer-AGCTCGGATCCGGCCGCTCAG; 3′ primer-GTTTTCCCAGTCACGAC), digested with BamHI and EcoRI and subcloned into pGEX-3X. The GST fusion protein was expressed in *E. coli* strain BL21, purified by glutathione agarose followed by SDS-PAGE, and the emulsified acrylamide gel was used to immunize rabbits. To generate antiserum 7704, the 859 nt fragment encoding residues Met-403 to the PACS-1b C-terminus (including 121 amino acids common to both PACS-1a and PACS-1b) was amplified by PCR (5′ primer- GGAATTCCATATGCCCAGGCTCTTCAGCTTAAC; 3′ primer- CGGGATCCATGGGCTGGCTGGATAAAG), using pGEM7Zf(+)PACS-1b as template. The fragment was digested with NdeI and BamHI and subcloned into pET16B. The His-tagged fusion protein was used to immunize rabbits as described above.

TGN Budding Assays

Methods were adapted from established protocols (Xu and Shields, 1993). AtT-20 cells (7×10^5) were infected with VV recombinants expressing either fur/f, fur/fDDD, fur/fADA, or one of the fur/f truncation mutants. At 5 hr postinfection, the cells were labeled with [^{35}S]Met/Cys for 30 min, chased in complete medium (90 min, 20°C), placed on ice, and rinsed two times with cold PBS and one time with hypotonic swelling buffer (15 mM KCl, 10 mM HEPES, [pH 7.2]). Cells were permeabilized by scraping in "breaking" buffer (90 mM KCl, 10 mM HEPES, [pH 7.2]) and were then pelleted at low speed and washed two times with assay buffer. The cell pellet was resuspended in 300 μl total volume including buffer, budding mix (ATP regenerating system and GTP), and bbc (~3 mg/ml final). The budding reaction was incubated (37°C, 60 min) and then placed on ice and the broken cells separated from post-TGN vesicles by centrifugation (15,000 × g, 2 min). The supernatant and pellet were diluted into mRIPA and the FLAG-tagged furin constructs were immunoprecipitated with MAb M1 and then resolved by SDS-PAGE.

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Brookhaven Protein Data Bank ID Codes

The PACS-1a and PACS-1b sequences have been deposited in the Brookhaven Protein Data Bank under ID codes AF076183 and AF075184, respectively.

Chapter 5

Conclusions and Discussion

Summary

Mammalian proprotein convertases (PCs) are a group of endoproteases that process molecules ranging from growth factors, hormones and serum proteins to bacterial toxins and virus envelope glycoproteins (Seidah *et al.*, 1998; Steiner, 1998). The processing of these substrates is critical for their bioactivities, which is important for the physiological functions of cells or organisms. Moreover, studies on processing of bacterial toxins and viral envelope glycoproteins have provided useful information for novel anti-pathgen strategies.

Biochemical and cellular studies have provided valuable information in understanding the function of these proteases. Both tissue and cellular distributions of the different proteases have indicated that these enzymes have distinct functions *in vivo*. For instance, PC1/3 and PC2 are specifically expressed in neuorendocrine cells and process prohormones during their maturation in mature secretory granules (Seidah *et al.*, 1998). Although PC6B and furin are ubiquitously expressed and they display nearly identical enzymatic characteristics, my studies presented in this thesis show that these two proteases are concentrated into distinct subcellular compartments. PC6B is localized into a late Golgi or post-TGN/endosome compartment distinct from furin (Chapter 2). Therefore, PC6B and furin may have different functions in processing substrates *in vivo*.

My findings are consistent with previous genetic studies showing that deficiency of furin cannot be complemented by endogenous PC6B (Roebroek *et al.*, 1998).

The PC6B cd contains information sufficient for the intracellular localization of the protease, and includes two novel sorting motifs that coordinate with each other to maintain the intracellular distribution of PC6B (Chapter 2). Both motifs contain multiple acidic residues and belong to a group of sorting signals named acidic cluster (AC) motifs, which are present on many membrane proteins such as furin, M6PR and VZV gE (Molloy *et al.*, 1999; Voorhees *et al.*, 1995; Zhu *et al.*, 1996). In addition, the PC6B-cd AC1 and the furin AC selectively bind to a novel cytosolic factor (PACS-1). PACS-1 associates with the AP-1/clathrin sorting machinery and is necessary for the correct localization of furin and PC6B (chapter 2).

PACS-1 belong a gene family including two broadly expressed human PACS genes (human PACS-1 and human PACS-2, Chapter 3). Both human PACS proteins share capacities in binding to cargo molecules and clathrin adaptor complexes (Chapter 3), suggesting they share similar roles in membrane protein trafficking. Biochemical studies identified a sequence in PACS-1 that binds to AP-1 complex. Mutation of this determinant blocked the interaction between PACS-1 and AP-1 complex, and behaved as a dominant negative when overexpressed in cells. The overexpressed PACS-1 mutant mislocalized furin and PC6B from the correct subcellular compartments (Chapter 4).

Together, these data illustrate a general mechanism of protein sorting in the TGN/endosomal system. Studies on this mechanism are not only useful for understanding

the function of the endoproteases, but also provide tools to study the other potential AC motif/PACS-1-mediated sorting events.

Localization of PC6B in the secretory pathway

As the center of protein sorting in the secretory pathway, the TGN is a dynamic system that hosts many sorting activities. In neuroendocrine cells, the sorting is more complex because of the presence of the regulated secretory pathway and the communications between the ISGs and the TGN, which is necessary to maintain correct targeting of secretory proteins (Tooze, 1998). I have shown that the PC6B compartment, also located in the perinuclear region, may represent another compartment with a possible connection to the TGN (as implicated by AC1 functioning as a TGN localization signal). All of these perinuclear compartments could integrate together to perform complicated sorting tasks in the post-Golgi region.

Several lines of evidence help our understanding of the nature of the PC6B compartment. First, pulse-chase experiments show that PC6B is sialylated in the TGN, suggesting that enzyme passes through the Golgi stack and concentrates in a compartment downstream from the Golgi complex. Second, the recycling of PC6B from the cell surface into the PC6B compartment suggests that the compartment is connected with the endocytic pathway. Third, the BFA-dispersable PC6B compartment is different from the condensation of TGN markers and tubular structure of endosomal markers by BFA (Lippincott-Schwartz *et al.*, 1991), but is similar to diffusion of Golgi stack markers by BFA (Lippincott-Schwartz *et al.*, 1990a). However, the possibility that PC6B is in the Golgi stack is less likely because of the sialylation of the PC6B protein in the TGN and the recycling of the protein from the cell surface. Together, the PC6B compartment could be a specialized perinuclear compartment connecting with endosomes in the

TGN/endosomal system. Recent structural observations on the Golgi suggests that different tubular structures can extend from the late Golgi cisternae and these structure function as sorting compartments in the late Golgi (Ladinsky *et al.*, 1999). Therefore, the PC6B compartment could also be speculated to be a tubular structure in the late Golgi compartment in parallel with the tubular structure hosting furin and TGN38/41. Finally, in neuroendocrine AtT20 cells, the PC6B compartment responds to secretagogue stimulation, suggesting a communication with the regulated secretory pathway. But the PC6B compartment is distinct from the ISGs or MSGs on the regulated secretory pathway since neither of them is accessible to the endocytic pathway. Further studies are necessary for understanding this PC6B compartment, and the relationship of this compartment to the TGN and other adjoining vesicular structures.

In AtT20 cells, the trafficking of PC6B is also different from that of furin which can be detected in ISG, but which does not respond to secretagogue. In the ISGs, furin is retrieved from the regulated secretory pathway by a PACS-1 and AP-1/clathrin coat mediated sorting event (Dittie et al., 1997 and Chapter 4). Overexpression of the dominant negative PACS-1 construct caused mislocalization furin into MSG, but did not mislocalize PC6B into MSG (Chapter 4). This could be explained by either of two possibilities. First, PC6B and furin are sorted into different vesicles from the TGN. While furin can be delivered into the ISGs, PC6B is never sorted into the ISGs. Second, both PC6B and furin are transported into the ISGs but they are retrieved by different sorting machinery. This observation is in agreement with the data showing that both MPRs and furin can be detected in distinct vesicle populations associated with the ISGs, suggesting that they are retrieved by different vesicles (Ditti *et al.*, 1999). These information support that PC6B and furin have distinct intracellular pathways with different sorting machinery,

which, together with their differentiated embryonic expression patterns (Beaubien *et al.*, 1995; Constam *et al.*, 1996; Zheng and Pintar, 1997), support the different functions of both enzymes *in vivo*. Moreover, as discussed in Chapter 2, PC6B and PAM-1 share similar properties in intracellular localization in neuroendocrine cells (Milgram *et al.*, 1997). These enzymes could reside in another processing compartments in addition to the TGN in the biosynthetic pathway.

Acidic Clusters

The intracellular trafficking of both PC6B and furin is mediated by acidic clusters on their cds. The AC motif was first identified on furin and VZV gE and is present on many other membrane proteins (Jones *et al.*, 1995; Molloy *et al.*, 1999; Voorhees *et al.*, 1995; Zhu *et al.*, 1996). An AC motif is defined as a sequence consisting of multiple acidic residues. Most AC motifs contain one or two CK2 phosphorylation site(s) whose phosphorylation state is critical for the functioning of the motifs (Jones *et al.*, 1995; Molloy *et al.*, 1999; Voorhees *et al.*, 1995). Several AC motifs have been shown to function as TGN localization signals in their phosphorylated states (Jones *et al.*, 1995; Takahashi *et al.*, 1995; Zhu *et al.*, 1996). Although the PC6B cd contains two functionally distinct AC motifs, neither can be phosphorylated. AC1 is a TGN localization signal that is functionally equivalent to the phosphorylated AC motifs on furin and VZV gE cds (Jones *et al.*, 1995; Zhu *et al.*, 1996). AC2 targets the protein to the PC6B specific compartment, and is functionally dominant over AC1 when both of them are present. The identification of the two functional AC motifs on the PC6B cd reveals the complexity of this group of sorting signals in at least three aspects: a nonphosphorylatable AC motif on one protein is functionally equivalent to a phosphorylated AC motif on another protein; multiple AC motifs can be present in the

same protein and together coordinate the intracellular sorting; and sequence-similar AC motifs can have distinct functions.

Like the phosphorylated furin AC motif, the PC6B AC1 selectively binds to the connector protein PACS-1, and this interaction is required for normal PC6B localization (Chapter 2). While the interaction between the furin cd and PACS-1 is regulated by CK2 phosphorylation (Wan *et al.*, 1998), the regulator of the interaction between AC1 and PACS-1 is unclear. However, PACS-1 is phosphorylated *in vivo*, and can be phosphorylated by CK2 *in vitro*. Therefore, the interaction between AC1 and PACS-1 could also be regulated by CK2 phosphorylation. Meanwhile, preliminary data shows that the PC6B AC2 selectively interacts with a 38kD protein from bovine brain cytosol, suggesting that, in addition to PACS genes, other genes (or gene families) mighy have role(s) in the AC motif-mediated protein sorting.

Acidic residues are involved in many sorting steps in the TGN/endosomal system (Heilker *et al.*, 1999; Matter *et al.*, 1992; Matter *et al.*, 1994; Simmen *et al.*, 1999) and Introduction 1.4.2). These residues contribute to the functioning of the motifs in two different ways. Some of the acidic residues are directly involved in protein-protein interactions whereas others are important for the phosphorylation of nearby serine/threonine residues, which in turn regulate protein-protein interactions (Heilker *et al.*, 1999; Le Borgne and Hoflack, 1998a; Mauxion *et al.*, 1996). Since the AC1 and AC2 of PC6B do not contain phosphorylation sites, their negatively charged residues are more likely to be involved in direct protein-protein interactions. The different roles played by these acidic residues reflect the sophistication of intracellular sorting.

PACS family

The PACS gene family is a novel gene family involved in protein trafficking in the TGN/endosomal system. PACS-1 was originally identified as a cytosolic protein that selectively recognized the CK2 phosphorylated furin AC motif and was necessary for the TGN localization of the protease. The PACS family is conserved in metazoans, but not in yeast, and is represented by at least three genes in human tissues. Two human PACS genes have been isolated and each has multiple isoforms produced by alternative splicing (Chapter 3). Both PACS genes are broadly expressed with distinct patterns in different tissues. *In situ* hybridization analyses show that they have distinct but complementary expression in rat brain and testis sections (Chapter 3).

Two human PACS proteins are highly conserved (54.4% identity) except that the N-terminus of PACS-1 is not present on PACS-2, which could be significant for the functional difference between these two proteins. Interestingly the human PACS-1 N-terminus contains a six glutamine residue stretch compared to the three-glutamine stretch in rat PACS-1, suggesting an expansion of the gluitamine stretch on human PACS-1. The expanded glutamine stretch has been shown to involve in the neurodegenerative diseases such as SCA (Sasaki and Tashiro, 1999). Moreover, this region shares high homology with a fragment of atrophin-1, a gene involved in neuronal disorder disease (Khan *et al.*, 1996; Loev *et al.*, 1995; Wan *et al.*, 1998). From these data, one speculation is that PACS-1 plays a role in neurodegenerative disease. Although a PCR-screening of a small number of donor samples has not revealed any further expansion on the human PACS-1 glutamine stretch, it remains to be seen whether PACS-1 mutants have a role in neurodegenerative diseases.

The complexity of the PACS family suggests diversified roles for these genes. PACS-2 is expressed at high levels in human muscle tissues that contain specialized cells with many mitochondria for energy generation. Preliminary observation shows that the amount of PACS-2 protein increases about four-fold during myoblast differentiation while the PACS-1 protein level does not change during this period, suggesting that PACS-2 may have a role in muscle differentiation. Coincidentally, muscle specific clathrin heavy chain has been isolated and its expression is up-regulated during myoblast differentiation (Sirotkin *et al.*, 1996). Both δ and σ3A subunits of AP-3 complex are heavily expressed in muscle tissues (Dell'Angelica *et al.*, 1997a; Ooi *et al.*, 1997). Together, this information suggests that muscle cells may have their own specific membrane trafficking system for their physiological function and PACS-2 may play a role in it.

Function of PACS in protein sorting

Both PACS proteins recognize many cargo molecules such as furin, M6PR and VZV gE, but the binding affinities remain to be characterized. The residues in AC motifs and PACS proteins necessary for interactions remain to be addressed. Structural analyses should be the key to understand how PACS proteins recognize these similar AC motifs. The interactions could be regulated in a similar way as those between tyrosine-based motifs (YXXΦ) and μ chains of AP complexes, in which each individual X residue in the tyrosine-based motifs (YXXΦ) is important for selectively binding to different μ chains (Ohno *et al.*, 1996; Owen and Evans, 1998). Such selective interactions have been proposed to be physiologically significant for the lysosomal delivery of CD63 and Lamp-2a (Bonifacino and Dell'Angelica, 1999). In a similar way, the selective interactions between cargo molecules and PACS proteins may play a critical role in their function *in vivo*.

Both PACS proteins associate with clathrin coats by binding clathrin adaptor complexes, including AP-1 and AP-3, but not AP-2. PACS-1 binds more strongly to AP-1 and AP-3 than PACS-2 does (~2-4 fold respectively, Chapter 4), showing different affinities in association with clathrin adaptor complexes. Thus, while both PACS proteins may function as connectors between cargo proteins and clathrin coats, the affinities of interactions among cargo, PACS and AP complexes are important in determining the function of each PACS protein in distinct sorting steps in the TGN/endosomal system. Future studies should be carried to measure these binding affinities.

The CK2 phosphorylation on the AC motifs has been shown to play a critical role in regulating their interaction with PACS proteins. PACS-1 is also phosphorylated *in vivo*, and several conserved putative CK2 phosphorylation sites exist on both PACS proteins although the function of the phosphorylation is not clear. However, studies have shown that clathrin assembly is highly regulated by phosphorylation (Slepnev *et al.*, 1998; Wilde and Brodsky, 1996). In addition, the formation of the complexes of AP-2/clathrin accessory proteins is regulated by phosphorylation (Chen *et al.*, 1999; Slepnev *et al.*, 1998). Therefore, CK2 phosphorylation may play a role of in PACS-1-mediated protein sorting. Since CK2 are ubiquitously expressed with constitutive activity and since the mechanism of how CK2 selectively target on proteins is unknown, the dephosphorylation by specific phosphatases could have a more significant role in the regulation of protein sorting.

The PACS family is functionally similar to other gene families including nef and β-arrestin. All these proteins selectively interact with cargo molecules and couple them

either to adaptor complexes or clathrin coats directly (Aiken *et al.*, 1994; Foti *et al.*, 1997; Goodman *et al.*, 1997; Goodman *et al.*, 1996; Piguet *et al.*, 1998). Nef can couple CD4 to AP-2/clathrin and COPI coat for sequential delivery from the cell surface to the late endosomes (Foti *et al.*, 1997; Piguet *et al.*, 1998). Although PACS can associate with AP-1 and AP-3, the physiological relevance of these interactions is not clear. However, both nef and PACS proteins can couple cargo molecules to multiple membrane protein sorting mechanisms. Furthermore, nef associates with PACS-1 to form a co-connector complex to mediate the TGN localization of MHC-I molecules (Piguet *et al.*, 2000). Together, these connector proteins have diversified roles in recruiting cargo molecules into different sorting vesicles.

Perspectives

The studies reported in this thesis have made significant progress in understanding the mechanism of intracellular localization and sorting of proprotein convertases and other TGN/endosomal proteins. The TGN sorting remains elusive due to lack of suitable molecular tools and techniques. Functional identification of AC sorting motifs and of the novel mechanism of PACS proteins provides a powerful means to understand protein sorting in the TGN. However, several issues remain to be clarified. First, PC6B has been clearly distinguished from furin in the subcellular localization, but the nature of the PC6B compartment is still not clear. Although PCs are present in the different compartments on the secretory and endocytic pathway, how a substrate is selectively processed by PCs in certain compartments is still unknown. Second, the regulation of association between cargo molecules (AC motifs) and PACS proteins and between PACS proteins and adaptor complexes needs to be further characterized. CK2 phosphorylation could be the key to understanding this regulation. Further studies will help us to understand how PACS

proteins recruit cargo molecules to clathrin sorting machinery. Finally, the function of PACS-2 in comparison to PACS-1 needs to be addressed in future studies.

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