The Role of Cleavage Site Sequence and Furin Specificity in Endoproteolysis of Pro-β-Nerve Growth Factor

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Abstract

A majority of neuroendocrine hormones derive from precursor proteins which require endoproteolysis at sequences containing paired (-K-R-, or -R-R-) or sometimes multiple basic amino acids. Processing of these precursors occurs in a tissue-specific manner and requires sorting to the regulated secretory pathway. Many growth factors and other constitutively secreted proteins also require this type of processing at similar sequences, though a broad distribution of cell types are capable of cleaving these precursors. The general motif of cleavage at sequences containing paired basic amino acids is found in all eukaryotic cells, including yeast and mammals, leading to the speculation of conservation of the enzymes responsible for this activity.

Recently, a family of subtilisin-like enzymes have been identified in mammals based on conserved sequence homology with the yeast KEX2 protease which cleaves pro-α-mating factor. The first of these mammalian enzymes to be identified at the genetic level was h*fur*. Our initial expression of hfur with a vaccinia virus expression vector demonstrated a functional gene product, furin, which was capable of cleaving paired-basic amino acids *in vitro*, and which facilitated processing of pro-β-NGF *in vivo*. This study also provided evidence for the expression of furin in many different cell types, both endocrine and nonendocrine, and for intracellular Golgi localization. The results are consistent with a role for furin in cleaving growth factors such as pro-β-NGF and at paired or multiple basic residues in the constitutive secretory pathway.

In order to further characterize the specificity of furin activity *in vitro*, we next isolated the enzyme from culture medium. This study established a strict calcium dependence for furin, activity within a broad pH range, and a distinct preference for cleavage at sequences

containing an arginine in position -4 to the cleavage site (toward the N-terminus) in addition to a -1 arginine. Basic residues at positions -2 and -3 appeared less significant for furin activity. These results agree with a number of reports of the requirement for a -4 arginine for cleavage of precursors in the constitutive secretory pathway. The *in vitro* characterization also distinguishes furin from other biochemically isolated activities related to neuroendocrine processing (proinsulin and POMC) and processing of proalbumin. Notably, furin is not inhibited by either the native α1-antitrypsin or mutant α1-antitrypsin Pittsburgh, which distinguishes this activity from thrombin and KEX2, as well as the convertase responsible for cleaving proalbumin.

Finally, we have mutated the cleavage site sequence of pro-β-NGF at the -4 arginine and determined the ability of the endogenous protease activity present in BSC-40 epithelial cells and of furin to recognize the mutant cleavage site *in vivo*. Both enzyme activities prefer the native sequence to a mutant containing a methionine at the -4 position. Mutation of the -4 arginine to lysine does not have a large affect on either activity. When expressed at high levels with a recombinant vaccinia virus, furin was able to convert both the -4 lysine and the -4 methionine mutant precursors to lower molecular weight mature form of NGF. Preliminary results are presented which suggest that the pro-β–NGF may be cleaved by furin *in vitro*, and thus suggests the feasibility of an assay for determining furin preference for the native or mutant forms of this precursor in the presence of limiting amounts of enzyme.

Introduction and Background

A common step required for the synthesis of many biologically active proteins and peptides is endoproteolysis of inactive precursor proteins, usually at pairs of basic amino acids (especially -Lys-Arg- and -Arg-Arg-). This mechanism was initially inferred from the sequences of several endocrine and neuroendocrine precursor proteins, including proinsulin (1) and the ACTH/β-endorphin precursor, proopiomelanocortin (POMC) (2). Subsequent studies have revealed a broad spectrum of precursor proteins, both endocrine and non-endocrine, that are endoproteolytically processed at single or multiple basic amino acid residues. The biochemical steps as well as the intracellular compartmentalization of neuroendocrine prohormone processing in the regulated secretory pathway have been extensively studied. In the last decade, however, studies of the biosynthesis of several non-endocrine proteins, including growth factors, cell adhesion molecules, receptors and viral envelope glycoproteins have demonstrated an essential role for intracellular proteolytic processing of precursors outside of the regulated secretory pathway. Furthermore, because expression of non-endocrine bioactive proteins is not restricted to the regulated secretory pathway, their biosynthesis can occur in a large variety of cell types and within several subcellular compartments.

A general consideration of the endoproteases active in the mammalian secretory pathway includes discussion of non-endocrine protein processing, both endogenous cell products and proproteins from bacterial and viral pathogens which also depend on host cell proteases for cleavage. Based on conservation of cleavage site sequences used in processing these non-endocrine proproteins, three classes of precursor emerge which reflect distinct enzyme activities: (i) those containing the sequence -Arg-X-Lys/Arg-Arg, (ii) -Lys/Arg-Arg, or (iii) single basic (Lys or Arg) residues. Together, the diversity of cleavage site structure as well as the compartmentalization of precursor protein processing suggest that multiple enzymes may be

responsible for endoproteolytic maturation of non-endocrine precursors such as pro-β-NGF in the constitutive secretory pathway. Thus a brief overview of some enzymes proposed to participate in precursor biosynthesis, including kallikreins, cathepsins and a group of mammalian subtilisin-like endoproteases structurally similar to the yeast Kex2p precursor processing endoprotease will be presented.

I. Compartmentalization and Processing of Mammalian Precursor Proteins

Proteins destined for the plasma membrane, the endoplasmic reticulum (ER), the Golgi, the lysosome or for secretion are cotranslationally translocated across the membrane of the ER and are then sorted and transported to the various compartments by intracellular vesicles. Import into the ER is directed by a signal sequence, generally located on the N-terminus of the translocated protein (3). Once in the lumen, the signal sequence may be removed by proteolytic cleavage and the protein is subject to many possible fates: localization through a transmembrane anchor in a membrane compartment (e.g. the ER, Golgi, lysosome, plasma membrane); cycling through these compartments by "trafficking" signals involving transport to and reclamation by individual compartments; or secretion from the cell by localization to a secretory vesicle, subsequent fusion of this vesicle with the plasma membrane and release of its contents to the extracellular environment (4, 5). The sorting process which takes place in the mammalian membrane compartment can determine not only the localization of a protein (intra- or extracellular), but the postranslational modifications which may occur by exposure to modifying enzymes located in the various compartments. Regulation of cellular function through intracellular protein trafficking and processing is one of the central issues in cell biology today. In this section I will briefly discuss some of the intracellular membrane compartments in the exocytic and endocytic pathways and some of the enzymatic processes which take place there. Specific enzymes involved in endoproteolytic cleavage, the main subject of this thesis, will be discussed in a later section (see section IV).

The endoplasmic reticulum is the initial site of membrane protein folding and sorting in the biosynthetic pathway. Different proteins exit the ER at different rates (6, 7), suggesting that the rate of exit may be affected by interaction with transport proteins, which either facilitate or inhibit transport from the ER to the Golgi. The rate-limiting step for "bulk flow" through the biosynthetic transport pathway is thought to be exit from the ER to the Golgi (4, 8). Thus transit through the Golgi occurs relatively quickly compared to a protein's residence in the ER. The lumen of the ER is the initial site of folding for a protein in this pathway. Improperly folded proteins may be retained until correct folding occurs or degraded by enzymes in the ER (4).

From the ER, proteins are transported through the Golgi membrane stack sequentially to the cis, medial and trans compartments (4, 8) (see figure 1). The individual compartments are marked by resident enzymes and reactions which take place there. Among the modifications which take place in these compartments are the addition and removal of sugar residues, phosphorylation and dephosphorylation, and endoproteolysis. It is the trans Golgi at which much of the sorting of proteins is thought to occur (4, 5). This membrane compartment is the site of formation for secretory and lysosome-bound vesicles (8) (see figure 1).

Two types of secretion occur in cells. Constitutive secretion occurs at a continuous rate, whereas regulated secretion occurs in response to an extracellular stimulus such as a hormone or depolarizing ion flux. Distinct secretory vesicles participate in constitutive and regulated secretion (9). Regulated vesicles bud off from the trans Golgi initially containing clathrin patches, which may aggregate to initiate formation of the vesicle . As these vesicles mature and become more electron-opaque the clathrin dissociates (9). A positive sorting signal is thought to be carried by proteins destined for regulated secretion, with sorting at the trans Golgi membrane (4). Constitutive secretory vesicles are not found to have clathrin associated with their outer coat, and are considered to carry "bulk flow" proteins from the Golgi to the plasma membrane (see figure 1). Recently adaptin-like molecules, called COPs for Coat-

associated Proteins, have been found associated with the non-clathrin coated, constitutive secretory vesicles (10, 11). These proteins may function like clathrin to initiate vesicle formation or to direct vesicle transport.

Vesicles bound for the lysosome compartment also bud off from the trans Golgi membrane, and carry mannose-6-phosphate receptors (M-6-PR's). These vesicles are thought to fuse with endosomes which then fuse with lysosomes (12). A number of degradative enzymes, including cathepsins (discussed later) are located in the lysosome. While components of the endosome and lysosome compartments are able to intermingle, the precise nature of this trafficking is not completely understood. The precursor to at least one growth factor precursor, that of TGF-β, may cycle through the lysosomal compartment by virtue of M-6-P residues on pro-TGF-β binding to M-6-PR (13, 14).

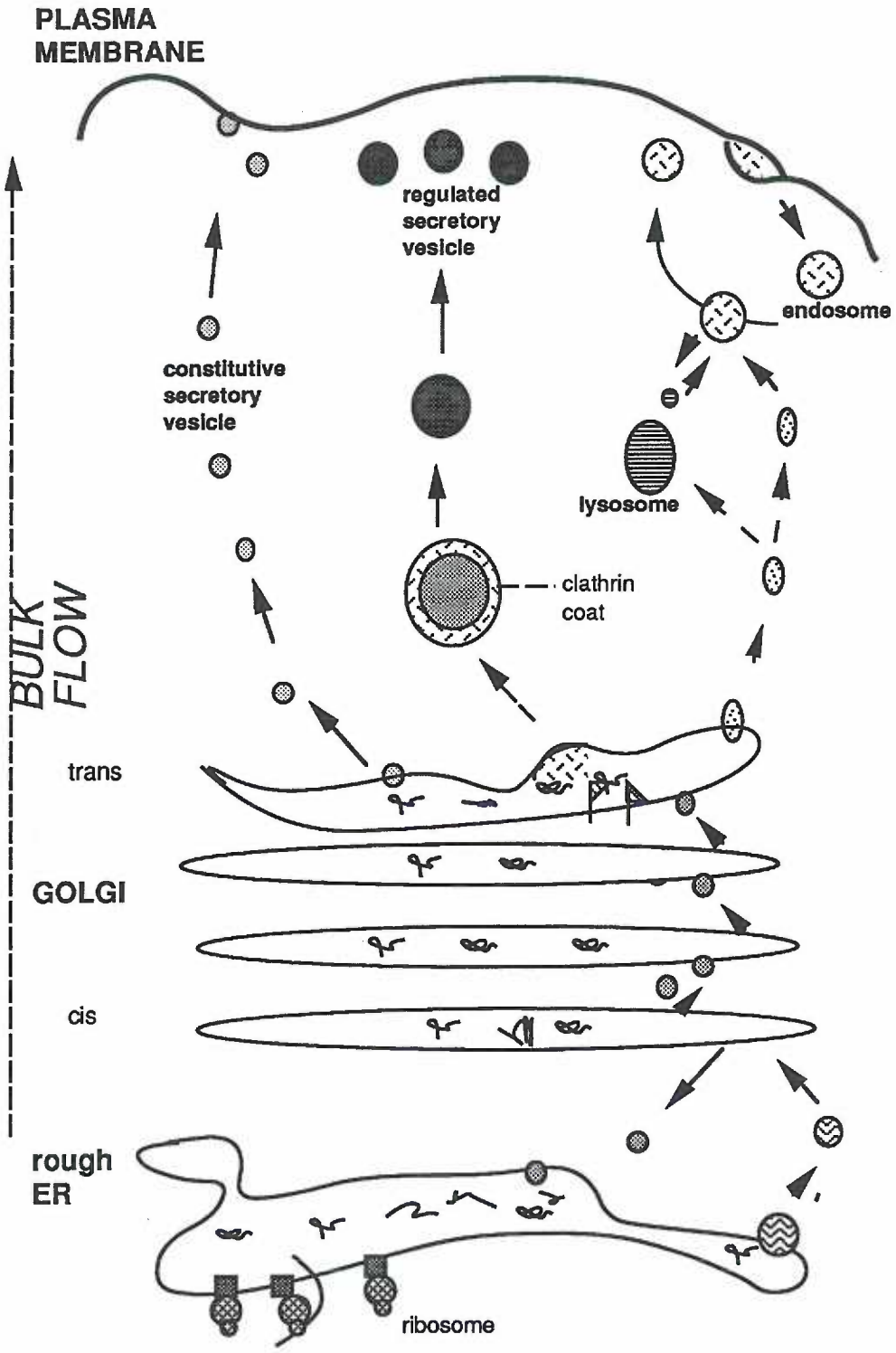


Figure 1. Schematic diagram of general intracellular traffic in protein biosyntethic transport

II. Biosynthesis and Secretion of Nerve Growth Factor

Nerve growth factor was first identified as an agent released from murine sarcoma cells which stimulated neurite outgrowth in embryonic neurons (15, 16). Subsequent investigations have established NGF as a neurotrophic factor, necessary for the proper development and maintenance of sensory and sympathetic neurons. Since its original description, the synthesis, structure and sites of action of NGF have been extensively studied (17-22). However, extremely low tissue levels of NGF protein have limited studies of its distribution to the transcriptional level, and relatively little is known about its post-transcriptional regulation. In peripheral cells, targets for sensory or sympathetic innervation, NGF is secreted through a calcium-dependent, constitutive pathway (23). Recent evidence suggests that in hippocampal neurons, a primary source of NGF in the central nervous system, secretion may occur in a regulated manner (24).

NGF is isolated from male mouse submaxillary gland as a 7S hexameric complex consisting of two each of α, β and γ peptide chains, the products of separate genes. The β chain, which dimerizes to form a 2.5 S complex of NGF, alone possesses physiological activity, whereas the α and γ subunits are members of the kallikrein family of serine proteases (see section VII.A of this review). Mature murine β NGF is a 13-14 kDa protein which is endoproteolytically processed from 27 and 35 kDa precursors, products of alternatively spliced transcripts (17, 19, 22). Cleavage of the precursor on the C-terminal side of arginine 120, and between arginines 237-238, releases the physiologically active peptide (see figure 2). Evidence for a precursor form of β-NGF was first described by Berger and Shooter (17), who immunoprecipitated a high molecular weight form of β-NGF which was converted to 13 kDa mature β-NGF by the purified γ subunit. Cloning of the pro-β-NGF cDNA revealed a precursor structure with several potential sites for proteolysis at pairs of basic amino acids (Arg and Lys), including the N-terminus and C-terminus of the mature factor (figure 2) (20). In vitro, γ-

NGF can form a complex with and cleave pro-β-NGF (17, 19, 22), whereas α-NGF carries no apparent enzymatic activity.

Metabolic labeling experiments showed expression of the precursor to β-NGF and accumulation of processing intermediates that likely result from cleavage of paired or multiple basic amino acid sites in the pro region (see figure 2) (17, 19). The role of the pro region in NGF expression is not clear; one function may be to impart proper folding of the mature bioactive peptide, yet another function may be to regulate the activities of either the processing enzymes or pro-β-NGF. Removal of a 27 amino acid domain from the pro region results in secretion of unprocessed mutant precursor which is biologically active (25). This study suggests that a discreet segment of the pro region may contribute to cleavage site recognition. Furthermore, since native pro-β-NGF is relatively inactive compared to the mature peptide (26), the pro region may actually prevent complete activation of NGF in the absence of proteolysis.

Other members of the mammalian neurotrophic factor family, including brain-derived neurotrophic factor (BDNF) (27), neurotrophin-3 (NT-3) (28), NT-4 (29) and NT-5 (30) derive from larger precursors which contain conserved basic amino acid residues at their N-terminal cleavage site similar to that in the NGF precursor (see figure 2). While maturation intermediates for these factors have not been detected, presumably they are processed similarly to NGF. Like NGF, the mRNA for NT-3 is widely distributed in brain and peripheral tissue, (28, 31) suggesting that the enzymes responsible for processing its precursor are present in many tissues. Expression of BDNF is more restricted, with its primary site of transcription in the brain. Another member of this family, NT-4 has been detected only in *Xenopus* and viper (29). NT-5, recently cloned from rat and human (30) is found widely distributed in peripheral tissue, and apparently has overlapping function with NT-3 and BDNF. The differential distribution of these neurotrophic factors suggests subtle differences in

their targets and actions, though sequence and structural similarities support their related function in development and maintenance of the nervous system.

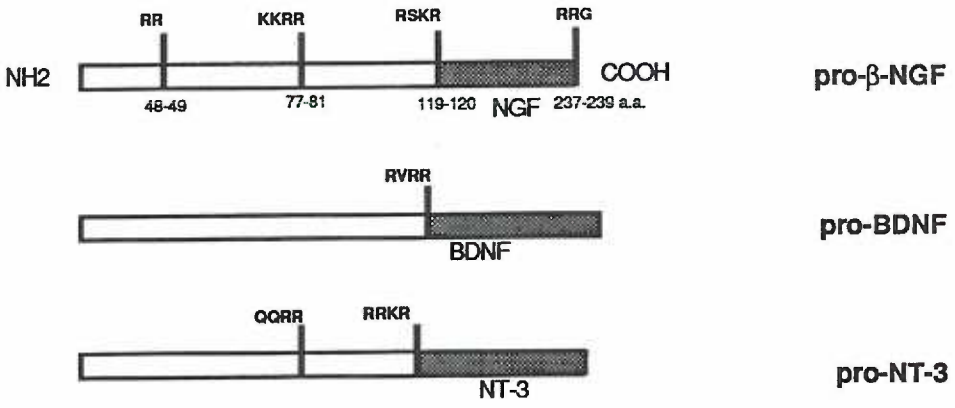


Figure 2. Structural features of some mammalian neurotrophic factor precursors with conserved cleavage sites at the N-terminus of the mature factor. Open bars represent pro region; shaded bars represent mature factor.

III. Other endogenous substrates in mammalian cells which are processed at single or multiple basic amino acids

Diverse compartmentalization of precursors within the biosynthetic transport pathway may occur, possibly in a tissue-dependent manner. As precursors transit through these membrane compartments they can interact with resident enzymes, coupling processing to intracellular trafficking. Many precursors to proteins other than growth factors are proteolytically cleaved during transit through the constitutive secretory pathway. Representing many functionally diverse cell products, precursors to serum proteins, cell adhesion molecules, receptors and viral proproteins are cleaved at single or multiple basic amino acids, presumably by the same enzymes responsible for growth factor processing. These

proproteins all transit through the same compartments in the constitutive secretory pathway, and they are all processed at cleavage sites containing paired or multiple basic amino acids in non-endocrine cell types. Thus any discussion of the enzymes responsible for growth factor processing must consider the evidence for cleavage site specificity provided by these other endogenous proteins which presumably interact with the same endoproteases active in the constitutive secretory pathway.

Initial evidence that sequences other than paired basic amino acids were required for proper processing of substrates in the constitutive secretory pathway came from a report of a mutant blood clotting Factor IX in a hemophilia patient (32). In this patient an abnormal Factor IX with an intact pro region was found in the blood plasma. The normal cleavage site sequence for removal of the pro region is Arg^{-4}-Pro^{-3}-Lys^{-2}-Arg^{-1} (number denotes position relative to the cleavage site toward the N-terminus), whereas the mutant contained a single point mutation changing the arginine at -4 to glutamine. Thus in addition to a requirement for arginine at the -1 position (33), an arginine at the -4 position is critical for proper cleavage of this precursor .

A number of other serum proteins are also produced as protein precursors which require proteolysis at basic amino acids. Protein C is one of several serum proteins which require proteolysis at two steps for proper maturation: intracellular cleavage at a multiple basic amino acid site to produce a mature precursor, and then extracellular cleavage at an unrelated sequence for activation in the serum (34). Normally, Protein C is intracellularly cleaved at a tribasic amino acid site (-Arg^{-3}-Lys^{-2}-Arg^{-1}-) to a different extent in the constitutive secretory pathway of different cell types. Mutation of the intracellular cleavage site to insert an arginine at the -4 position (relative to the cleaved bond) was shown to increase cleavage of this precursor from 30% to greater than 90% efficiency in hamster kidney (BHK) cells (34). The increased cleavage of mutant Protein C in these cells, correlating with their normally efficient cleavage of pro-Factor X at a site containing a -4 arginine (-Arg^{-4}-Val^{-3}-Thr^{-2}-Arg^{-1}-), indicate

that the endogenous BHK endoprotease prefers a cleavage site sequence of -Arg-X-X-Arg-, with arginines in the -1 and -4 positions.

Similarly, the insulin receptor is synthesized as a precursor, and is processed at basic amino acids in the constitutive secretory pathway (20, 35, 36). In a remarkable case of clinical analysis, defective processing of the insulin proreceptor resulting from a single amino acid change was found to be the cause of insulin-resistant diabetes (37). The normal cleavage site sequence of the proreceptor (-Arg^{-4}-Lys^{-3}-Arg^{-2}-Arg^{-1}-) in this patient was mutated at the -1 position to serine. The unprocessed mutant proreceptor was expressed normally at the cell surface, but bound insulin with much lower affinity than the appropriately cleaved receptor. Subsequent engineered mutations of the native cleavage site sequence for insulin proreceptor verified a distinct requirement for arginine in the -1 and -4 positions; mutation of the -2 lysine or -3 arginine to alanine did not affect processing of the precursor when expressed in COS cells (38). The insulin proreceptor is one of many plasma membrane proteins, including insulin-like growth factor receptor, low density lipoprotein receptor-related protein precursor, platelet glycoprotein IIb precursor and the precursor to a murine leukocyte cell adhesion molecule (L-CAM), which require cleavage (at sites with consensus sequence -Arg-X-X-Arg-) during transit through the constitutive secretory pathway.

Several mutants of proalbumin have been identified and indicate some specific substrate requirements for the proalbumin convertase, including a -1 arginine, a -2 arginine and a +1 serine (39). Proalbumin may represent a distinct precursor structure within the constitutive secretory pathway, as it lacks the consensus arginine in the -4 position, but has one in the -6 position, with a pair of basic residues (-Lys-Arg-) in the -1 and -2 positions (see Table I). While isolation of a proalbumin convertase activity (39-41) demonstrates its presence in the appropriate tissue, the general distribution of this activity has not been described. Thus whether a unique enzyme or a more generalized protease activity is responsible for proalbumin processing is not clear. *In vitro* characterization of the proalbumin convertase distinguishes it

from the products of recently cloned subtilisin-like mammalian genes, and will be discussed later in this chapter.

IV. Exogenous bacterial and viral precursors processed at single or multiple basic amino acids by mammalian host proteases

Virally derived glycoproteins recently have recently emerged as models for precursor proteolysis at basic amino acid sequences in the mammalian cell secretory pathway. These coat glycoproteins generally have signal sequences which facilitate transit through the host secretory pathway. The virulence of many viruses depends directly on proteolysis of a coat proprotein at single or multiple basic amino acids, presumably by the same host enzymes which act on endogenous precursors. Cleavage site sequence analysis of variant viral strains supports a role for host enzymes with distinct substrate specificities and the ability to act on viral proproteins. For example, host range is directly related to the number of arginine and lysine residues at the cleavage site of Fo protein from various Newcastle Disease Virus (NDV) strains (42). Apathogenic strains of NDV have two isolated basic amino acids at the cleavage site and are restricted to infectivity of specific cell types, whereas strains carrying two pairs or more of basic residues are pantropic, infecting a wide range of host cell types. Thus, addition of more basic residues to the cleavage site of Fo protein may either enhance its recognition by a number of different enzymes which are expressed in a tissue-restricted manner, or could alter the Fo cleavage site to one recognized by a single ubiquitously expressed enzyme.

Site-directed mutagenesis of the hexabasic cleavage site (with the sequence -Arg^{-6}-Lys^{-5}-Arg^{-4}-Lys^{-3}-Lys^{-2}-Arg^{-1}-) of influenza virus hemagglutinin (HA) in CV-1 cells showed that most of the basic residues are necessary for cleavage (43). In contrast to the endogenous insulin proreceptor, discussed above, the presence of an arginine at positions -1 and -4 to the cleavage was required but not sufficient for processing of HA. Thus factors other than sequence,

such as secondary structure or compartmentalization may contribute to cleavage of this virally derived proprotein.

Evidence for at least two different mammalian proteases with basic amino acid-directed activity comes from analysis of the sequence preference for host proteases able to cleave single or paired basic amino acids in synthetic substrates. In this study chromogenic substrates were designed to mimic the cleavage site sequence of various strains of influenza HA. These substrates were used to detect and inhibit host enzyme activity (44). Crude cell lysates from several cell types, including kidney epithelial cells (BHK, MDCK and MDBK) and chicken chorioalantoic membrane (CAM) cells, were tested for protease activity with the synthetic substrates. All cell types were able to cleave substrates containing paired basic amino acids (-Lys-Arg- or -Arg-Arg-), whereas the cells differed in their ability to cleave substrates with a single arginine, correlating with the pathogenicity of various influenza strains in these cell types. In addition, chloroalkyl ketones containing paired basic peptide sequences were highly effective inhibitors of HA cleavage by cell lysates, while a similar ketone with a tripeptide containing a single arginine did not significantly inhibit HA cleavage (44). These studies suggest not only that alkyl ketones may be effective antiviral agents, but that a single-arginine-directed protease activity is restricted to a limited number of cells. The paired-basic-directed endoprotease activity appears to be more broadly expressed in many cell types.

In another study, the proteolysis of Rous sarcoma virus (RSV) $Pr95^{env}$ in CV-1 cells showed that mutation of the tetrabasic cleavage site at the -2 position (-Arg-Arg-Lys-Arg^{-1}- to -Arg-Arg-Glu-Arg^{-1}-) resulted in poor processing to the gp85 and gp37 products (45). Deacidification of cellular compartments by addition of chloroquine, was able to completely block the minor amount of mutant $Pr95^{env}$ processing but did not alter processing of the wild-type proprotein. These results suggest that a single primary protease is responsible for cleavage of $Pr95^{env}$, though another enzyme can perform processing, perhaps in another subcellular

compartment. Thus viral proproteins may evolve to present chimeric cleavage sites which are recognized by a variety of host proteases, consequently increasing tropism. In addition, targeting of viral proproteins to appropriate cellular compartments may contribute to efficient processing by host enzymes.

Processing at basic amino acids occurs not only intracellularly in the secretory pathway, but can also occur at the cell surface. For example, *Bacillus anthracis* secretes two toxins (composed of three separate proteins) which have been demonstrated to be important virulence factors (46). These toxins share a protein, protective antigen (PA), which binds to an unidentified receptor on the surface of eukaryotic cells (47). While bound to the cell surface, PA undergoes a specific proteolytic cleavage that is required for binding of the other toxin components (47). Cleavage occurs at a tetrabasic sequence (-Arg-Lys-Lys-Arg^{-1}-) similar to that of other precursors discussed above. Thus like the precursor to epidermal growth factor (EGF), which is cleaved at the cell surface at a single arginine site, bacterial toxins may serve as substrates for mammalian cell proteases with trypsin-like specificity.

VI. Patterns of Secondary Structure at Precursor Cleavage Sites

Since not all basic sequences are substrates for intracellular proteases, factors other than cleavage site sequence clearly contribute to precursor processing. Differential compartmentalization of the precursors and enzymes, as discussed above and elsewhere in this volume, may restrict the exposure of a precursor to an enzyme which recognizes flanking sequences or structures. Furthermore, the unique environment of different cellular compartments (i.e. pH, ionic milieu) may affect the structure of the entire precursor or the region containing the cleavage site. Thus the proposed structures for many prohormones cleaved at paired basic amino acids have been analyzed for similarities in secondary structures.

Examination of twenty prohormone protein sequences revealed that of 66 pairs of basic amino acids which flanked the hormone sequence, all of them were predicted to be in or adjacent to β-turns . This study concluded that paired basic residues are critical but not sufficient for cleavage of prohormones and that β-turns may provide a flexible site suitable for cleavage or a recognition signal for precursor-enzyme interaction. Another study suggested that β-turns, which are 3-5 amino acids in length (48), were not accurately represented in prohormone secondary structure (49). This study predicted that Ω loops, which contain up to 16 amino acids and are generally found on the external surface of protein structures, more accurately describe the structure for about a third of the previously predicted β-turn sequences. This reported heterogeneity in cleavage site secondary structure may reflect a conformational recognition of prohormone cleavage sites by other proteins (eg. proteolytic enzymes or proteins involved in sorting) determined by secondary structure. Both of these studies were conducted on prohormones processed in the regulated secretory pathway and may reflect features of processing in this pathway, including the requirement for sorting to regulated secretory vesicles. Similar studies have not been reported for precursors in the constitutive pathway, though a role for secondary structure may be implicated by the requisite for cleavage site accessibility to the protease.

Another precedent for a role in secondary structure and enzyme-substrate interactions was recently set by the discovery of an endopeptidase in Xenopus that recognizes an amphipathic α-helical structure (50). In this study, magaininase, isolated from frog skin and assayed by its ability to cleave magainin peptides on the amino side of a specific lysine, was characterized as a metalloproteinase which recognized an amphipathic, α-helical structure. Peptides which are functionally related as antimicrobial agents but which do not share substantial amino acid sequence identity were all identified as appropriate substrates for magaininase. This study concluded that magaininase is able to cleave the amino-terminal

bond of a lysine in the context of a distinct secondary structure (amphipathic α-helix) present in all its substrates.

VII. Basic Residue-Directed Endoproteases from Mammalian Tissue

A full understanding of the processing of precursor proteins requires identification of all the specific enzymes involved as well as an understanding of the compartmentalization of both precursors and enzymes. Partially purified and purified activities capable of cleaving at single or paired basic residues *in vitro* have been proposed as candidates for authentic precursor processing endoproteases in the regulated and constitutive secretory compartments (17, 39, 51-64). Many prohormone processing activities are expressed in a tissue specific manner by basic residue-directed proteases. Participation of these activities in the processing of non-endocrine precursor proteins cannot be dismissed. However, genetic identification of mammalian proteases has been elusive and thus it has not been possible to authenticate the biochemically isolated activities, either endocrine or non-endocrine, for specific precursors *in vivo*. In addition, factors other than tissue specific processing enzymes are likely to play a role in endocrine hormone expression: expression of cofactors which regulate enzyme-substrate interactions, the requirement for co-sorting of enzymes and precursors to intracellular compartments, and specialized microenvironments of the secretory pathway which may influence proper folding of the precursor or activities of the enzymes. These factors may also play a role in regulating the precursor processing of constitutively secreted proteins.

The host proteases responsible for proteolysis of viral proproteins have not yet been identified, but are presumably the same enzymes which cleave endogenous substrates are involved. As described above, HA molecules from apathogenic strains of influenza virus are cleaved at a single arginine residue in a restricted number of cell types, while HA from pathogenic strains contains multiple basic amino acids at a cleavage site which is recognized in a broad range of host cell types. The endoproteolytic activity responsible for influenza virus

HA cleavage has been shown to be calcium dependent and active at a neutral pH *in vitro* (65, 66).

A. Kallikreins

Kallikreins are a group of enzymes which are implicated in growth factor processing in the constitutive secretory pathway (64, 67, 68). These proteins are trypsin-like serine endoproteases defined by their ability to process kininogen to produce bradykinin. They are abundant in pancreas, kidney and submaxillary gland, the primary tissues from which they are isolated. The kallikreins contain highly conserved amino acid homology around the three residues which form their catalytic triad. They cleave on the carboxyl side of single or paired basic amino acids, and can bind with high specificity to form stable complexes with their substrates.

Kallikreins which bind to both NGF (α-NGF and γ-NGF) (21, 69) and EGF (EGF binding protein) (69, 70) were initially coisolated with the factors, as they bind and form a stable complex. As previously discussed, γ-NGF has been shown to be capable of cleaving pro-β-NGF, the biologically active subunit of the NGF 7S complex , *in vitro*, though only at high molar ratios. Heterologous expression of pro-β-NGF in a variety of cultured cell types led to the observation that many, including those lacking γ-NGF, correctly and efficiently process pro-β-NGF (26, 71). Both L929 (fibroblast) and BSC40 (epithelial-like) cells process and constitutively secrete β-NGF (72). These findings suggest that a ubiquitous enzyme which cleaves pro–β-NGF in the constitutive secretory pathway is distinct from the enzyme or enzymes responsible for processing prohormones in the regulated secretory pathway (73).

Initially proposed to play a role in processing prohormone precursors, kallikreins are highly specific for their substrates and are expressed in a tissue specific manner. However, genomic analysis has revealed a limited number of human genes encoding members of this family of enzymes. The combined features of high specificity with limited diversity argues against kallikreins being responsible for endoproteolysis of the many endocrine prohormones in

human. Thus the physiological functions of each of these isozymes are being reexamined, and while kallikreins may be responsible for cleaving a select set of precursors in a limited number of tissues, they can not be regarded as general processing enzymes.

B. Mammalian Subtilisins: KEX2-like Proteases

Genetic and biochemical studies unequivocally identified the gene in *Saccharomyces cerevisiae* required for excision of α-factor mating hormone from its precursor (74, 75). This locus, *kex2*, encodes a subtilisin-like, membrane-bound, calcium-dependent serine endoprotease (Kex2p or Kex2) specific for cleaving on the carboxyl side of pairs of basic residues (-Lys-Arg- and -Arg-Arg-) (76). Several lines of evidence suggested that mammalian precursor processing endoproteases are functionally, and possibly structurally, similar to Kex2p. The mammalian precursor proalbumin, for example, is efficiently converted *in vitro* to albumin by Kex2p, with cleavage at its paired basic amino acid sequence (-Arg-Arg-) (77). Furthermore, co-expression of the *kex2* gene with mouse proopiomelanocortin (POMC) in BSC-40 cells (a line incapable of processing this peptide precursor) resulted in efficient proteolysis at pairs of basic amino acids producing authentic pituitary peptides, including γ-LPH and β-endorphin 1-31 (78, 79). Kex2p also shows similarities to the insulinoma type I and type II protease activities (55, 80).

Subsequently, three mammalian genes, *fur* (81-84), *PC2* and *PC3* (also called *PC1*) (85-88) have been reported which share significant sequence identity with the *kex2* gene sequence (see figure 3). All three predicted translation products share a high degree of homology with Kex2p, and to a lesser degree with the bacterial subtilisins. Homology is particularly high within the catalytic domain which contains conserved aspartic acid, histidine and serine residues.

RNA hybridization studies find the *fur* gene message expressed in all tissues and cultured cells examined, albeit at different levels (83, 89, 90). Most notably, furin mRNA is found in high levels in fibroblast and epithelial cells incapable of processing endocrine precursors, while relatively low expression of fur is detected in endocrine tissue. In contrast,

expression of *PC2* and *PC3* is apparently restricted to neuroendocrine and endocrine tissues (85-88). PC3 is expressed at relatively high levels in anterior pituitary and bovine adrenal medulla, whereas lower expression is found in pancreatic β-cells and related cell lines. In contrast, PC2 is expressed at high levels in pancreatic β-cells and pituitary intermediate lobe cells . Thus a role for furin is suggested in cleavage of nonendocrine precursors in many different cell types, even those lacking a regulated secretory pathway. Furthermore the restricted expression of PC2 and PC3 suggests a role for these enzymes in processing endocrine precursors in biologically appropriate cells.

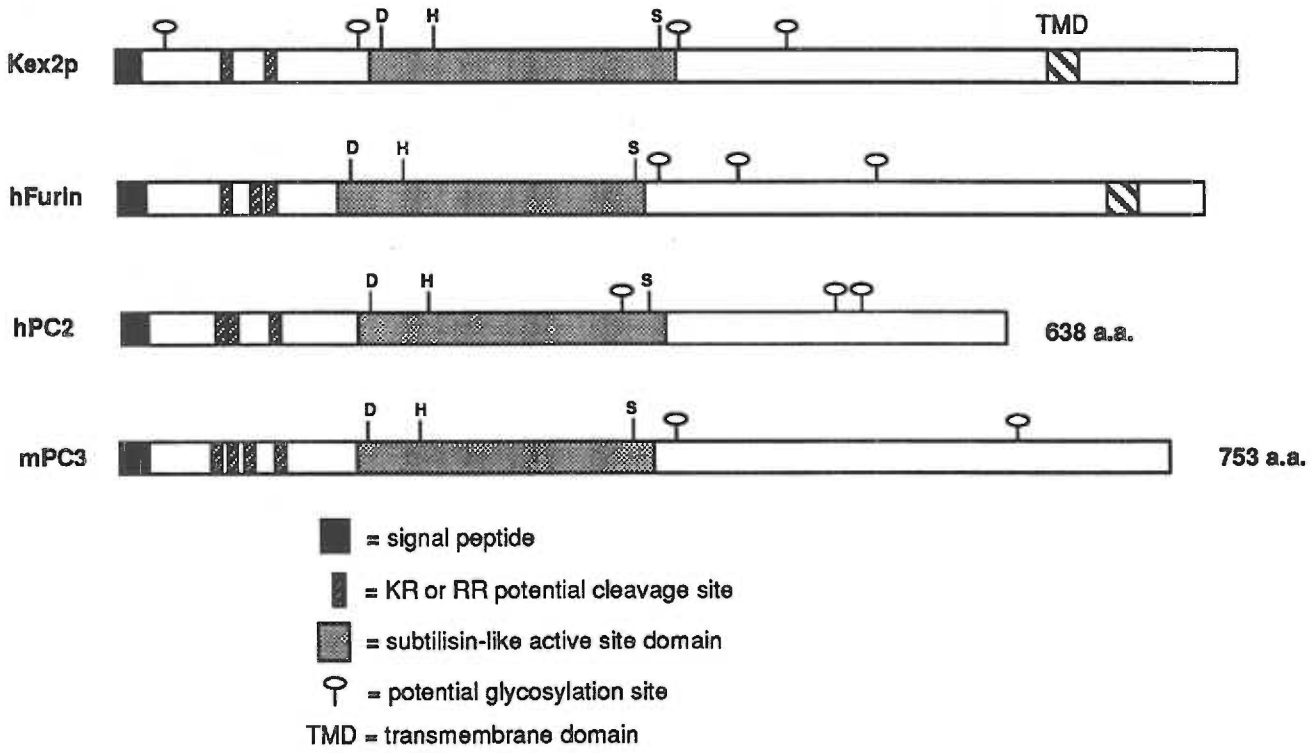


Fig. 3 Structural Features of Kex2p and Mammalian Homologs

C. Cathepsins

Cathepsins are generally considered degradative enzymes which function within the endocytic and/or lysosomal compartments. Representing a diverse group of proteases, not all cathepsins are of the same class: cathepsin D and E are aspartyl proteases, while H, L and B are cysteine proteases. However, all cathepsins appear to be optimally active at acidic pH, an observation consistent with activity in the lysosome compartment. At least some of these enzymes (e.g. cathepsin B) show both exo- and endoproteolytic activities with a variety of substrates, a characteristic serving their degradative function. Studies on the intracellular routing of these enzymes reveal that their pro regions are modified with mannose-6-phosphate (M-6-P) residues which route the inactive zymogens through the trans-Golgi network (TGN) to prelysosomal endosomes and ultimately to the lysosomes by virtue of binding to the M-6-P receptor (14, 91). Many of the cathepsins (B,D,H, L and E) undergo proteolytic activation in acidic compartments during or following transport through the Golgi.

Despite their general role as intracellular degradative enzymes, some of the cathepsins have been implicated in the cleavage of extracellular proteins, including collagen, fibronectin and laminin (92, 93). Furthermore, cathepsin L is secreted from fibroblasts when they are stimulated by PDGF (94) while cathepsin D is constitutively released from mammary tumor cells (95). In both cases, however, the enzymes are primarily secreted in their inactive pro form. It has been suggested that these secreted cathepsins could play a role in wound healing or tissue invasion via disruption of the extracellular matrix.

As described, several types of mammalian proteases may participate in intracellular cleavage at paired or multiple basic amino acid sequences. In order to establish whether these enzymes are primarily active in the constitutive or regulated pathway, or endosomal or lysosomal compartment, requires further characterization of these compartments and their resident enzymes. In addition, none of these enzymes has been demonstrated as absolutely required for processing of a particular mammalian precursor.

VIII. Overview of Thesis and Statement of the Problem.

The work described in this thesis addresses the mechanism of endoproteolysis of precursor proteins in the constitutive secretory pathway. The impetus for this work is based on the observation that many endocrine precursor proteins are processed in cells possessing a regulated pathway, while the precursors to growth factors and many non-endocrine precursors are cleaved at sites similar to the endocrine precursors in many cell types including cells possessing only a constitutive secretory pathway. One model for the tissue-specific proteolysis of endocrine precursors suggests the tissue-specific expression of appropriate endocrine-specific proteases. However, other factors such as colocalization of enzymes and substrates in cellular compartments, cofactors required for appropriate folding or transport of precursors, or specialized compartment conditions which facilitate precursor folding or enzyme activation may also contribute to the tissue specificity endocrine processing.

Until recently, little was known about requirements for proteolysis in the constitutive secretory pathway. The initial work reported here involved establishing an assay for processing in the constitutive secretory pathway by blocking the proteolysis and allowing accumulation of a precursor protein. We chose pro-β-NGF as a model precursor because it carries a cleavage site recognized in all cell types tested including those cells lacking a regulated secretory pathway (71, 83); it can be successfully expressed in a vaccinia virus system and immunoprecipitated from culture medium; and because its processing involves several intramolecular cleavages at basic amino acid sites similar to those observed in many endocrine precursors.

Chapter one describes the assay conditions for determining cleavage of pro-β-NGF. In addition, we obtained a clone for one of the first mammalian homologs to the yeast Kex2p endoprotease, human furin (hfurin), and found its expression in all cell types tested. In order to test the activity of hfurin in vivo, we coexpressed this protease with the β-NGF precursor in

cultured cell lines. The resulting cleavage of pro-β-NGF by furin *in vivo*, along with its ubiquitous tissue distribution suggested a function for furin in the constitutive secretory pathway.

The next segment of this work involves the isolation of furin activity for *in vitro* characterization of its activity. The successful strategy described is based on expression of a truncated form of hfurin, lacking the transmembrane region, and isolation of this protein from a defined culture medium. Both fluorogenic peptide substrates and the purified PA protein from *Bacillus anthracis* were used to characterize truncated hfurin (hfur713t) activity *in vitro*. The inhibitor and pH profiles of this protease, along with the calcium concentration dependence suggest an activity distinct from other protease activities isolated from mammalian tissue. Furthermore, a distinct cleavage site sequence preference for hfurin is demonstrated to include an arginine in the -4 position to the cleaved bond in addition to the paired basic residues at positions -1 (-Arg-X-X-Arg-). These results are consistent with the observed conservation of this -4 arginine in many precursors, including pro-β-NGF, which are cleaved in the constitutive secretory pathway.

Finally, the requirements of cleavage site structure for proteolysis of pro-β-NGF in BSC-40 cells was examined with regards to the arginine in position -4. The cleavage site sequence of pro-β-NGF was altered by in vitro mutagenesis, and the endogenous cleaving activity in BSC-40 cells was compared to cleavage by hfurin. A distinct preference for a cleavage site containing an arginine at position -4 to the cleaved bond was observed for the endogenous cleaving activity in BSC-40 cells, while coexpression of hfurin with pro-β-NGF in these cells resulted in cleavage of native as well as mutated β-NGF precursor. Whether the endogenous cleavage of pro–β-NGF is solely performed by hfurin, or other endoproteases is not clear. However, both the substrate preference of hfurin *in vitro* and the preferred cleavage of native pro-β-NGF over the cleavage site mutants *in vivo* suggest that cleavage of this growth factor precursor is performed by an activity similar to hfurin.

Materials and Methods

Cell Culture. BSC-40, an African Green monkey kidney epithelial cell line and AtT-20, a mouse pituitary corticotroph cell line were cultured in MEM containing 10 % heat-inactivated serum (HyClone Laboratories, Logan, UT). HepG2 cells, a human hepatoma, were cultured in Minimal Essential Medium (MEM) containing 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT) supplemented with 0.1 mM pyruvate and 0.1 mM nonessential amino acids (Gibco Laboratories, Grand Island, N.Y.)

Vaccinia Virus. Marker rescue procedure, as previously described (78) was used to generate a recombinant vaccinia containing a foreign cDNA sequence (see figure 4). In brief, the foreign DNA is first cloned into a plasmid (pVV3 or pZVneo) containing flanking sequences for the vaccinia thymidine kinase gene, and the 7.5 K promoter element from vaccinia (96). Cells are then concurrently infected with wild type virus and transfected with the plasmid. Homologous recombination of the foreign DNA into the thymidine kinase locus results in incorporation of the foreign cDNA into the vaccinia genome.

Through multiple rounds of plaque purification, a crude preparation of the recombinant virus is subsequently used for the infection of cells. For the standard vaccinia stock preparation 5 (150 mm) plates of BSC40 cells are grown to confluency in MEM plus 10% FCS. After counting one plate, the remaining cells are infected at 0.005 plaque forming units (p.f.u.) per cell. Infected cells are harvested after 60-72 hours of infection, before cells detach from plate, by scraping with a rubber policeman and transferring to a 50 ml conical tube. Cells are washed in 10 ml Phosphate Buffered Saline plus magnesium (PBS-M: 2.8 mM KCl, 1.5 mM KH_2PO_4, 1.4 mM NaCl, 8 mM Na_2HPO_4, 1 mM $MgCl_2$), and resuspended in 5 ml of cold 10 mM Tris, pH 9.0 . A cell homogenate is formed, on ice, with 25 strokes (Kontes dounce homogenizer), transferred to a centrifuge tube, and spun at around 2000 x g for 5 minutes at 4^{o}C. The supernatant is reserved, and the cells are homogenized again, spun down, and the second supernatant is pooled

with the first. The vaccinia virus supernatant is then underlaid with a sucrose pad (36% sucrose, 10 mM Tris, pH 9.09) and centrifuged at 18,000 rpm in a Sorvall SW 28 rotor at 4°C. After aspiration of the supernatant and sucrose pad, the vaccinia pellet is resuspended in 1-1.5 ml of 10 mM Tris, pH 9.0 and dounced to a milky consistency. Aliquots of this crude preparation, which should yield around 10^{10} p.f.u./ml, may be stored at -70°C .

Vaccinia virus strain WR was used in these studies and infections were performed at multiplicity of infection (m.o.i.) 2-5 p.f.u. per cell. For standard infection and expression of recombinant virus, cells near confluency were rinsed in PBS-M , then inoculated with PBS-M containing crude virus preparation, which had previously been titered . After incubation for 30 minutes at room temperature, inoculum was replaced with culture medium and cells were returned to 37°C.

To construct the vaccinia recombinant virus expressing the human furin cDNA (VV:hFUR), plasmid pBluescript-PACE (from Chiron Corp., Emeryville, CA) containing the human furin cDNA from which the 3' untranslated region was deleted was digested with Sca I and the insert excised by digestion with Hinc II and Sma I. Bgl II linkers were ligated on the 2.5-kb insert and the fragment was ligated into the vaccinia recombination plasmid, pVV3, which was previously cut with Bgl II (see figure 4). The orientation of the ligated cDNA was verified and the resulting plasmid pVV3:hFUR was used for marker transfer into vaccinia.

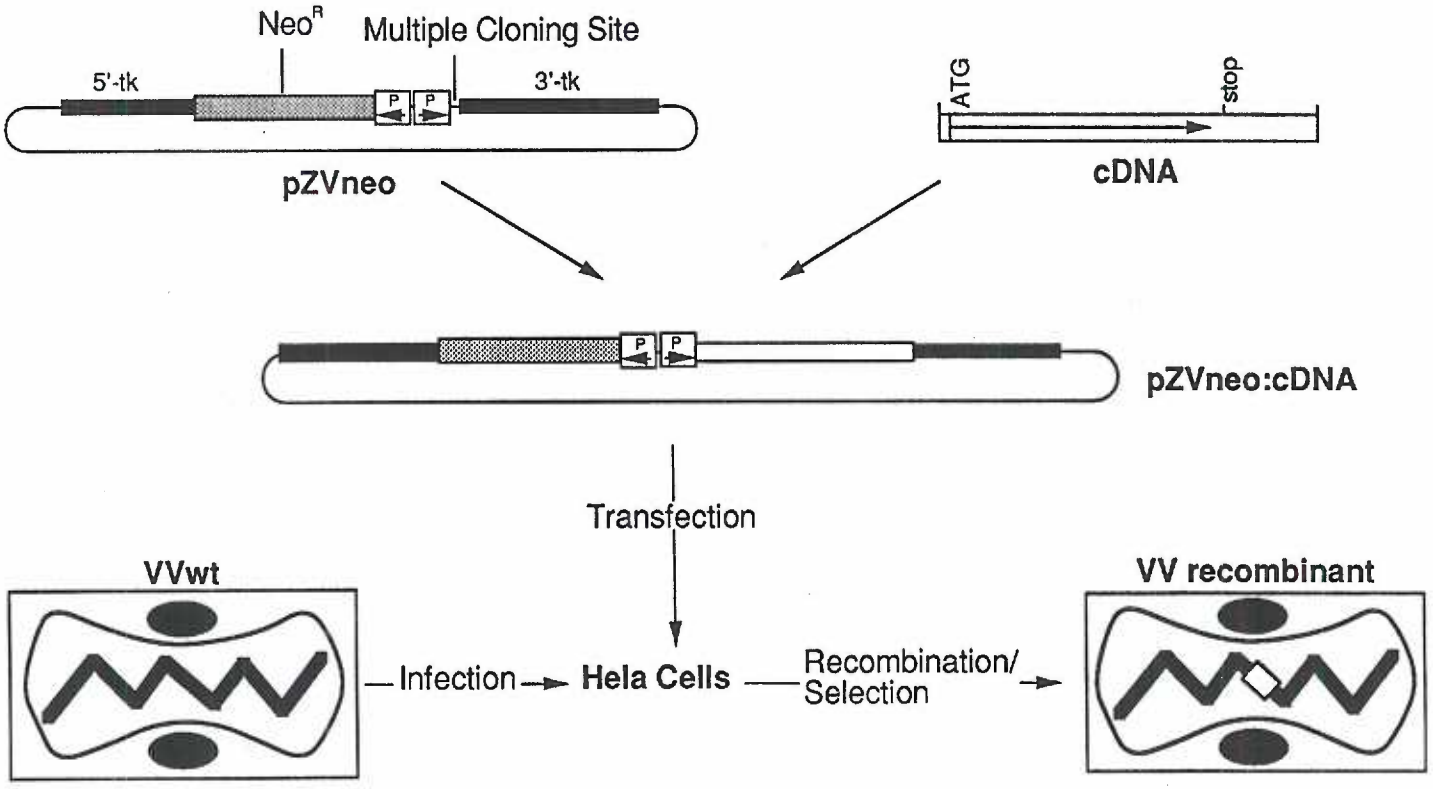


Fig. 4 Construction of Recombinant Vaccinia Virus

A vaccinia recombinant directing expression of the mouse β-nerve growth factor precursor (pro–β-NGF) cDNA (VV:mNGF) was constructed from an original plasmid, pGEM3/ngf (a generous gift of Dr. J. Garrett), containing a full-length mouse β-NGF-cDNA. This plasmid was first digested with Hind III and incubated with Klenow polymerase to generate blunt ends. The linearized, blunt-ended fragment was then digested with Bam HI to yield an 820-bp insert coding for the shorter transcript of β-NGF (using the second initiation codon) as described (97). Plasmid pVV3 was digested with Bgl II, incubated with T4 DNA polymerase to generate blunt ends, and digested with Bam HI. Vector and insert were then ligated and a plasmid containing the mNGF insert (pVV3:mNGF) was used to introduce the mouse NGF cDNA into vaccinia virus by standard marker transfer as described (78).

Immunofluorescence. Cells grown on coverslips were rinsed in PBS-M and fixed for 15 minutes in PBS-M containing 3% paraformaldehyde plus 1 mM $CaCl_2$. After fixation, cells were permeabilized in 0.2% BSA, 0.1% Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ in PBS. Next, the fixed cells were preincubated in 0.1% Triton X-100 and 2% normal goat serum (NGS) to block nonspecific binding of antibodies. Coverslips were rinsed briefly and incubated overnight at 4°C with rabbit anti furin antiserum (anti-PACE, Chiron Corp., 1:50 dilution). The antifurin antibodies were generated against the catalytic domain of furin by expression in Escherichia coli of amino acids 146-372 of furin as a fusion to human superoxide dismutase (84). The furin antiserum was preabsorbed with an acetone precipitate of a wild-type vaccinia-infected BSC-40 cell extract and incubated at room temperature for 4 hours, followed by clarification of the antiserum by centrifugation in a SPIN-X column (Costar, Cambridge, MA). After overnight incubation with the furin antiserum, coverslips were then rinsed in permeablizing solution, incubated with a biotinylated goat anti-rabbit antiserum (1:200 dilution, Vector Laboratories, Inc., Burlingame, CA) for 30 minutes, rinsed and incubated with FITC-conjugated avidin (1:500, Vector Laboratories, Inc.) for 50 minutes. Coverslips were again

rinsed and incubated with wheat germ agglutinin (WGA; diluted 1:50, Molecular Probes Inc., Eugene, OR) for 30 minutes at room temperature, rinsed, mounted on microscope slides in 50% glycerol, and viewed with a Leitz Dialux 22 EB fluorescent microscope (63x objective).

Cell Fractionation and In Vitro Proteolytic Assays. BSC-40 cells were grown to confluency on 100-mm plates in Minimal Essential Medium (MEM) containing 10% fetal calf serum. Parallel plates were infected with vaccinia virus at an m.o.i. of 5, as described above. At 18 hours after infection, cells were chilled on ice, rinsed once in ice-cold PBS-M, harvested by scraping in 0.5 ml of 10 mM HEPES, pH 7.3, and lysed with 20-30 strokes in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) until about 70% total lysis, as determined by trypan blue exclusion, was obtained. Lysates were cleared by centrifugation at low speed (1000x g, 5 min.) to remove cellular debris and the crude extract was transferred to an ultracentrifuge tube. After centrifugation at 100,000 g for 60 minutes, the supernatant fraction was removed and the pellet was resuspended in 200 microliter of 10 mM HEPES, pH 7.3.

For assay, the sample was placed in 200 microliters of standard substrate buffer (100 mM HEPES, pH7.3, 1% Triton X-100, 1 mM $CaCl_2$,) and incubated at 37°C for experiments in Chapter One. Subsequently, the standard assay conditions, as described in chapter two, were in 0.5 ml reaction volume, containing 100 mM HEPES, 0.5% Triton X-100, 1 mM $CaCl_2$, 1 mM 2-mercaptoethanol and 50 μM synthetic substrate, and incubated 30 min. at 37°C unless otherwise noted (e.g., if salt, pH or Calcium were varied). Fluorogenic substrates boc-Leu-Arg-Arg-4-methyl coumeryl-7-amide (MCA), boc-Leu-Gly-Arg-MCA, boc-Gly-Lys-Arg-MCA, boc-Leu-Lys-Arg-MCA, and boc-Gln-Arg-Arg-MCA were from Peninsula Laboratories, Inc., Belmont, CA. Substrate boc-Arg-Val-Arg-Arg-MCA was from Peptides International, Louisville, KY. Substrate boc-Arg-Gln-Arg-Arg MCA was a gift of Dr. H.D. Klenk. To assay, the quantity of liberated amino methyl coumarin was determined by fluorimetry (excitation = 380 nm, emission = 460 nm). Protein determinations were performed with bicinchoninic acid procedure (98) reagent kit from Pierce, Rockford, IL. Purified α1-antitrypsin Pittsburgh was provided by Dr.

P. Barr. All other chemicals and reagents were obtained from either Boehringer-Mannheim Biochemical, or Sigma Chemical Co., St. Louis, MO.

Digestion of anthrax protective antigen (PA) was conducted in a 25-µl reaction volume containing 50 mM HEPES (pH 7.5), 0.25% Triton X-100, 2.5 mM $CaCl_2$, 5 µg of purified PA, and 0.2 µg of total protein from MonoQ fractions containing the peak of proteolytic activity. The reactions were incubated at 30°C for 5 minutes, and terminated by addition of 25 µl of 2X concentrated SDS sample buffer (0.12 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.05% Bromphenol Blue). Aliquots were separated by SDS-PAGE on 10% gels and the cleavage products visualized by staining with Coomassie Brilliant Blue R250.

Western blot. For immunoblotting furin, samples containing equivalent amounts of total protein were loaded onto a 10% polyacrylamide gel and run in SDS buffer as originally described (99). Protein was then transferred under electric current (24 V) for 16 hours at 4°C to nitrocellulose in transfer buffer (39 mM glycine, 48 mM tris, 0.037% SDS, 15% methanol). The blot was preincubated in TBST (10 mM Tris-HCL, pH 8, 150 mM NaCl, 0.05% Triton X-100, 0.02% NaN_3) containing 3% dried nonfat milk for 50 min. at room temperature, followed by incubation with primary antibody (rabbit anti-PACE, diluted 1:8000) for 3 hours. After washing three times with TBST, the blot was incubated with goat anti-rabbit-alkaline phosphatase and developed (Promega Protoblot Immunoblot System, Promega Corp., Madison, WI).

Northern Blot. Isolation of poly A+ RNA, transfer to a nylon membrane and hybridization with labeled DNA fragment were performed as described (100). In brief, total RNA was isolated as described, then poly A+ RNA was isolated by adsorption onto a oligo(dT)-cellulose column and eluted with low salt elution buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.05% SDS). A total of 5 µg of poly A^+ RNA isolated from cultured cells was loaded onto a 1% agarose gel and separated by electrophoresis. The RNA was then transferred

to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL) and hybridized with a randomly-primed radiolabeled insert containing the hfur cDNA sequence.

Metabolic Labeling and Immunoprecipitation. Cells were grown to confluency in MEM containing 10% fetal calf serum in 35-mm, 6-well plates. Prior to label, media was removed and cells were rinsed once with PBS-M. MEM media (-Met, -Cys) was then added to cells, along with 100 μCi of ^{35}S label (NEN EXPRESS, Met and Cys ^{35}S, Dupont Co., Wilmington, DE). Cells were then incubated at 37°C until harvest. Media from VV-infected cells was harvested and centrifuged for 5 min. at 1000 x g in order to remove any nonadherent cells or cellular debris. Samples were then transferred to a fresh tube and centrifuged 10,000 x g for 20 min. in order to remove suspended virions. All subsequent treatment of samples was at 4°C. Samples were then diluted 1:1 with RIPA buffer (1% NP-40, 1% SDS, 1% sodium deoxycholate [DOC], 150 mM NaCl, 50 mM Tris-HCl, pH 8). A pool of four anti-β-NGF rat monoclonal antibodies generated to 2.5S NGF (generously provided by Dr. Rae Nishi) was added to each sample and incubated for 2 hours. A second antibody (rabbit anti-rat IgG + M; Zymed Laboratories, Inc., South San Francisco, CA) was added to each sample and incubated for 1 hour. Finally, protein A-Sepharose (Zymed) was added to each sample and incubated for 1 hour. The sepharose beads were washed two times with RIPA buffer, once with 1M NaCl, 1% NaCl, and then loaded on a 12.5 or 15% Laemmli-type SDS polyacrylamide gel, as stated in figure legends.

Furin Expression and Purification. Confluent monolayer of BSC-40 cells grown in MEM plus 10% fetal calf serum were inoculated with VV:hFUR713t (m.o.i. = 5) and the culture refed with serum-free defined medium, MCDB 202 (101) as previously described (83, 102). After 16-18 hours, the medium was collected and centrifuged at 1000 x g for 5 minutes. The supernatant was transferred to a fresh tube and centrifuged at 10,000 x g for 20 minutes at 4°C, then passed through a 0.2 μm filter. The filtered 10,000 x g supernatant was diluted approximately 1 to 1 with buffer A (20 mM BisTris, pH 7.0, 1 mM 2-mercaptoethanol) and applied to a MonoQ FPLC anion exchange column (1 ml bed volume, Pharmacia) at a flow rate of 1 ml/min. at 4°C.

Proteins were eluted with a gradient of 0-750 mM NaCl in buffer A over 10 min. at a flow rate of 1 ml/min. Fractions of 0.25 ml were collected and analyzed for protein using the bicinchoninic acid assay as above, and assayed for protease activity as described.

Site-directed Mutagenesis of Pro-β-NGF. Mutagenesis of Pro-β-NGF was performed with the Bio-Rad Mutagene kit (Bio-Rad Laboratories, Richmond, CA), by the method originally described by Kunkel (103). A cDNA fragment coding for pro-β-NGF was cut from pGEM3/ngf with Bam HI, Hind III restriction enzymes, and cloned into M13mp19 (Boehringer Mannheim) cut with the same enzymes. The M13/NGF was then transfected into bacteria CJ236, a *dut,ung* mutant which incorporates uracil instead of thymidine during DNA replication. Single stranded M13 phage were then isolated by standard method (100) and annealed to oligomers which carried replacement nucleotides for the native NGF sequence corresponding to the proprotein cleavage site. Oligomers for NGF Mutants (NM 1-4) were obtained from Oligos Etc. (Wilsonville, OR), with sequences as follows (bases which differ from native sequence are underlined): PB1 (NM1), 5'-GGACTCACAAGAGCAAGCGC-3'; PB2 (NM2), 5'-GGACTCACATGAGCAAGCGC-3'; PB3 (NM3), 5'-GGACTCACCAAAGCAAGCGC-3'; PB4 (NM4), GGACTCACCATAGCAAGCGC-3'. Second strand synthesis and ligation were performed as described (103) (Biorad mutagen kit, Biorad) and reaction mix was transfected into bacteria strain MV1190, with an intact uracil N-glycosylase activity which degrades the uracil-containing template strand. Bacteria from this transformation were plated on standard LB plates, plaques picked and mini-preparations of single stranded DNA were prepared for sequencing. Once the mutant sequence was verified, the entire cDNA fragment coding for pro–β-NGF was subcloned into plasmid pGem7. Subcloning was performed either by cutting inserts directly out of double-stranded (RF) preparations of M13 DNA with Bam HI, Hind III sites (NM1, NM3), or by performing "runoff" reactions on single stranded M13. "Runoff" reactions were performed as a second strand synthesis, above. All sequences were verified after subcloning into pGEM7 as described below.

DNA sequencing. Double-stranded and single-stranded DNA sequencing was performed by incorporation of a radiolabeled dideoxynucleotide in the DNA chain termination reaction as described by Tabor and Richardson (104), with reagents and protocol as described in Sequenase Kit (U.S. Biochemical, Cleveland, OH). Briefly, DNA synthesis was initiated either on single-stranded or double-stranded template at the site where an oligonucleotide primer anneals. For sequencing through the mutation site sequence of pro-β-NGF, oligo PB5 was used as a primer, with sequence 5'-CAGCCTCCACCCACC-3', which occurs about 40 bases 5' to the site of mutagenesis. The synthesis reaction was terminated by incorporation of dideoxy nucleotide analogs which cause chain elongation to terminate. A radiolabeled nucleotide (in this case deoxyadenosine triphospate-P^{32}, NEN, Dupont) was included to label the terminated chain fragments. When four reactions are run, each with one of the four nuclotide analogs (G, A,T or C), and the products were separated on a 6% polyacrylamide sequencing gel, a complete template sequence was obtained.

Transfection and Expression of pro-β-NGF and Mutants with T7 Polymerase. In order to express pro–β-NGF and NGF mutants in culture cells we took advantage of the T7 transcription promoter on the pGem7 plasmid (104, 105, 106). cDNA fragments for NGF or the NGF mutants (NM 1-4) were cloned into pGem7, in the proper orientation for transcription from the T7 promoter. Purified plasmid was then transfected into cells which were infected with vaccinia virus expressing the T7 polymerase [VV:VTF7, obtained from Dr. Bernie Moss (105, 106). Cells were grown to 50-75% confluency in MEM plus 10% FCS, and either infected with VV:VTF7 alone, or coinfected with VV:VTF7 and vaccinia expressing either hfur, *kex2*, PC2 or PC3, at m.o.i. of 5 p.f.u. for each virus. After removal of inoculum, cells were refed with MEM alone (no FCS). A mixture of purified plasmid DNA (5 μg), brought to 50 μl volume in water, and 20 μl of lipofectin reagent (BRL) with 30 μl of water was incubated for 20-30 minutes at room temperature in a polystyrene tube. The DNA/lipofectin mixture was then added dropwise to the infected cells, and the cells returned to the incubator at 37°C. After 3 hours, FCS was

added to the cells to a final concentration of 10%, and the cells were incubated and metabolically labeled as described before. However, for metabolic labeling with T7 expression, the pulse label period was extended to 8-10 hours because of lower expression from this system. Immunoprecipitation of labeled media was as described.

Manual Radiolabeled Sequencing of Peptides by Edman Degradation. Procedure used was described by Matsudaira (107). Peptides for sequencing were metabolically labeled and immunmoprecipitated as before and run on SDS-PAGE. Proteins were then transferred to Polyvinylfluoridine (PVDF) Immobilon-P transfer membrane (Millipore Corp., Bedford, MA) in carbonate transfer buffer (108). The membrane was washed with two changes of transfer buffer and stained briefly with Ponceau S (100) to detect protein bands molecular weight markers. After washing with three changes of distilled water, the membrane was air-dried and exposed to film to detect labeled bands. The 14 kDa band of processed NGF was cut out from the membrane used for Edman degradation sequencing.

In summary, sequencing by Edman degradation involves three steps: coupling of phenyl isothiocyanate (PITC) to the N-terminus, cleaving the PITC-coupled amino acid from the peptide, and extracting the converted amino acid derivative into an organic phase for subsequent counting in a scintillation detector. Sample was placed in an acid-washed glass tube; 80 µl of water and 80 µl of sequencing grade PITC (Pierce Chemical Co., Rockford, IL) were added, the tube was flushed with Nitrogen gas and stoppered. After vortexing briefly, the sample was incubated for 45 minutes at 45^{o}C. Next the sample was dried completely in a vacuum centrifuge, 80 µl of Trifluoroacetic acid (TFA) was added, the tube flushed with N_2 and restoppered. Sample was incubated again for 15 minutes at 45^{o}C, then dried completely as before. For extraction of the cleaved amino acid, 200 µl of water was added to tube and vortexed. Then 300 µl of ethyl acetate (sequencing grade, Pierce Chemical Co.) was added and vortexed well. After spinning at low speed (1000 x g) in a centrifuge, the organic (top) layer was removed. the aqueous phase was extracted twice more, the organic layers were pooled, and

added to scintillation cocktail (Ecolume, ICN Biomedicals, Inc., Irvine, CA.) for counting. The rest of the sample (aqueous phase) was dried completely in preparation for another cycle.

Chapter One

Human *fur* Gene Encodes a Yeast Kex2-like Endoprotease That Cleaves Pro–β-NGF *in Vivo*

Introduction

As one of the first growth factor precursors described (17), the proteolysis of pro-β-NGF at paired basic amino acids serves as a model for this processing in the constitutive secretory pathway. Early studies of this precursor demonstrated the ability of γ-NGF, a member of the kallikrein family of proteases, to process pro-β-NGF immunoprecipitated from murine submaxillary gland (17). Thus it was proposed that γ-NGF was the specific enzyme responsible for pro-β-NGF processing in this tissue. These results do not explain the processing of pro-β-NGF in many cell types which have no detectable γ-NGF. Furthermore, no equivalent to γ-NGF is found in human, though human β-NGF is highly homologous to murine β-NGF, including a conserved cleavage site sequence and conservation of all cysteine residues in the bioactive region of the protein (109), and similarly requires endoproteolytic cleavage for biological activity. Recent evidence suggests that a more generally distributed protease is responsible for cleaving pro-β-NGF and other precursors in the constitutive secretory pathway of mammalian cells.

Many candidate proteases for precursor processing at paired or multiple basic amino acids have been biochemically isolated (19, 40, 52-64, 67). However, the significance of these candidate activities *in vivo* for processing a specific precursor protein has never been demonstrated. In contrast, genetic (74, 75) and biochemical (76, 81) studies unequivocally identified the gene required for excision of the peptide mating hormone (α-factor) from its precursor in the yeast *Saccharomyces cerevisiae*. This locus, the *kex2* gene, encodes a unique subtilisin-like, membrane-bound, calcium-dependent, serine endoprotease (Kex2p) specific for cleaving on the carboxyl side of pairs of basic residues

(-K-R-/ -R-R) (76).

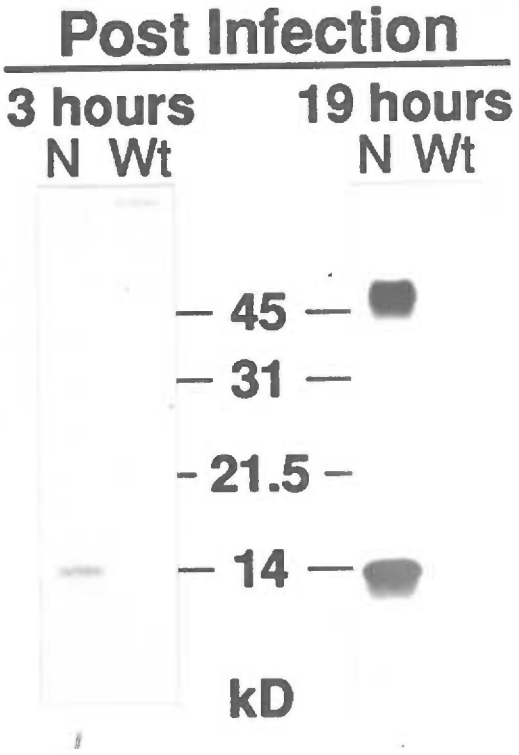
The sequence homology and shared topology (see figure 3) suggest that the furin cDNA encodes a Kex2p-like protease that could be a functional human homolog of Kex2p. Furthermore, the wide tissue distribution found for furin (89) (figure 8) compared to relatively restricted distribution for PC2 (85, 87) suggested a general role for furin in proteolysis of widely distributed precursors such as pro-β-NGF.

Results and Discussion

To express pro–β-NGF and a functional furin translation product in mammalian cells the cDNA was inserted into a vaccinia virus expression vector as described in methods. Construction of a recombinant vaccinia virus to express Kex2p enzyme was as described (78, 79). A vaccinia virus recombinant that directs expression of the mouse pro-β-NGF cDNA (VV:mNGF) was constructed to study the processing of this precursor protein by Kex2p and furin in the mammalian cell secretory pathway. BSC-40 cells were selected since this cell line possesses only the constitutive secretory pathway (102) and is amenable to infection with multiple vaccinia recombinants (78).

In order to establish an assay for pro-β-NGF processing we needed to determine whether processing of the precursor could be blocked. Replicate plates of BSC-40 cells were infected with either VV:WT or VV:mNGF or both, and at increasing times after virus infection each culture was metabolically labeled for 3 h with [^{35}S] methionine. The secreted products were immunoprecipitated with pooled monoclonal antibodies directed against β-NGF and resolved by SDS gel electrophoresis. In agreement with earlier studies (71), metabolic labeling of BSC-40 cells at short times after infection (3 hours) resulted in expression of pro-β-NGF and efficient processing of the prohormone to mature 13-kDa β-NGF peptide, presumably by the endogenous enzyme present (figure 5). However, when labeling was initiated at a later time (19 hours postinfection), the rate of pro-β-NGF synthesis was significantly higher and the major

secreted product was unprocessed precursor (figure 5). This observation provided a means to examine the effect of elevated furin protease activity on the efficiency of processing of pro-β-NGF.


Post Infection
3 hours
N Wt
19 hours
N Wt
45
31
21.5
14
kD

In addition to BSC-40 epithelial cells, many other cell lines were tested for pro-β-NGF processing, including human carcinoma (HeLa), canine kidney (MDCK), mouse fibroblast (L929), mouse pituitary corticotroph (AtT-20), as well as primary cultures of bovine adrenal medullary chromaffin cells. In all cases a 13 kDa form of β-NGF was immunoprecipitated, though expression was generally lower than that detected in BSC-40 cells (data not shown).

The cDNA for human furin was obtained from Dr. Phil Barr (Chiron Corp.). The furin cDNA was isolated from a hepatoma (HepG2) cDNA library (84). In order to express the gene product we constructed a recombinant vaccinia virus (VV:hFUR) as described in methods. Plates of BSC-40 cells were infected either with wild-type vaccinia virus (VV:WT), with a vaccinia recombinant containing the yeast *kex2* gene (VV:KEX2), or with a vaccinia recombinant containing the VV:hFUR. Cells were harvested 20 hours after infection and crude membrane fractions were prepared as described. Each sample was analyzed for proteolytic activity capable of cleaving fluorogenic peptide substrates containing a pair of basic amino acids. Endoproteolytic activity capable of cleaving substrates containing Lys-Arg (Table I) increased 4.9- and 2.3-fold, respectively, in cells infected with VV:KEX2 or VV:hFUR. The increased proteolytic activity observed in either VV:KEX2 or VV:hFUR infected cells was quantitatively associated with the 100,000 x g membrane pellet. This selective partitioning to cellular membranes is consistent with earlier observations for Kex2p in yeast (62, 76, 81) and for Kex2p expressed in BSC-40 cells (79).

Table I. Expression of Kex2p and Furin Activity in Mammalian Cells

	Virus	2 mM Ca^{2+}	+3 mM EGTA	2 mM Ca^{2+} + 1 mM PMSF
HSS	WT	12	32	17
	KEX2	56	15	29
	FUR	18	35	24
HSP	WT	109	131	45
	KEX2	533	137	295
	FUR	250	110	64
Media	WT	16	27	21
	KEX2	25	22	24
	FUR	21	23	23

Parallel plates of BSC-40 cells were infected with either VV:WT, VV:KEX2, or VV:hFUR (m.o.i.=5). At 18 hours after infection crude membrane fractions were prepared as described in methods, and proteolytic activity was determined using a fluorogenic substrate (boc-G-K-R-MCA). Values are reported as enzyme units per milligram protein. One enzyme unit equals 1 mol of substrate converted per minute. Values shown are from a single experiment, but qualitatively similar results wre obtained in three independent trials. Abbreviations: HSS, high speed supernatant; HSP, high speed pellet; WT, wild-type.

In addition to its apparent substrate specificity, furin shares other enzymic properties with Kex2p. Kex2p is a serine protease that is active at neutral pH, strictly calcium dependent, and inhibited by only high concentrations of PMSF (62, 76) (Table I). Similarly, the furin-dependent activity was inhibited by EGTA (a calcium chelator) and was active a pH 7.3 (Table I). Unlike the yeast prohormone endoprotease, however, furin activity appeared to be much more sensitive to PMSF. Neither Kex2p nor furin activity were detected in the culture medium (Table I).

Aliquots of the supernatant and pellet fractions from the samples described in Table I, in addition to control samples from mock-infected cells, were resolved by one-dimensional SDS gel electrophoresis and furin translation products were identified by immunoblotting with specific antibodies (figure 6). A doublet of proteins with the apparent sizes of 90 and 96 kDa,

respectively, was detected and was found only in the membrane fraction from VV:hFUR infected BSC-40 cells (figure 6, lane 6). These apparent molecular masses are in good agreement with the 87-kDa translation product predicted by the furin cDNA. No furin was detectable by this method in the soluble fractions from these cells or in samples from either mock- or VV:WT-infected BSC-40 cells (figure 6, lanes 1-5).

To establish whether furin is tightly associated with membranes, the 100,000 x g pellet prepared in the presence of 1 mM PMSF and 2 μg/ml leupeptin from BSC-40 cells infected with VV:hFUR was extracted with 0.1 M sodium carbonate (pH 11) and resedimented at 100,000 x g. Western analysis demonstrated that the furin doublet was retained in the pellet fraction (figure 6, compare lanes 7 and 8), as expected for the behavior of an integral membrane protein.

The Western blot analysis revealed two prominent furin translation products (90 and 96 kDa) present in crude membrane fractions of VV:hFUR-infected BSC-40 cells. The furin cDNA sequence predicts a small cluster of paired basic amino acids at nearly the identical position as found in Kex2p. Cleavage at either of these sequences would generate a protein ~6 kDa smaller than the primary translation product (the same size difference detected in figure 6). It has recently been demonstrated that mutagenesis of Arg_{107} (located -1 in position from the cleavage site) or Arg_{104} (located -4 in position from the cleavage site) in furin results in the production of only the 96 kDa furin protein (R. Leduc, S.S. Molloy, B.A. Thorne, G. Thomas, in press). Also in this study, *in vitro* activity was associated with the 90 kDa form and not the 96 kDa form of furin. Thus removal of the N-terminal pro-region is required for furin activation. These results are consistent with the requirement for autoproteolytic removal of the entire amino terminal extension for activation of the bacterial subtilisin precursor, which is structurally related to furin (110, 111). Similarly, the precursor of Kex2p protease undergoes removal of an NH_2-terminal propeptide, probably by an autoproteolytic mechanism (112).

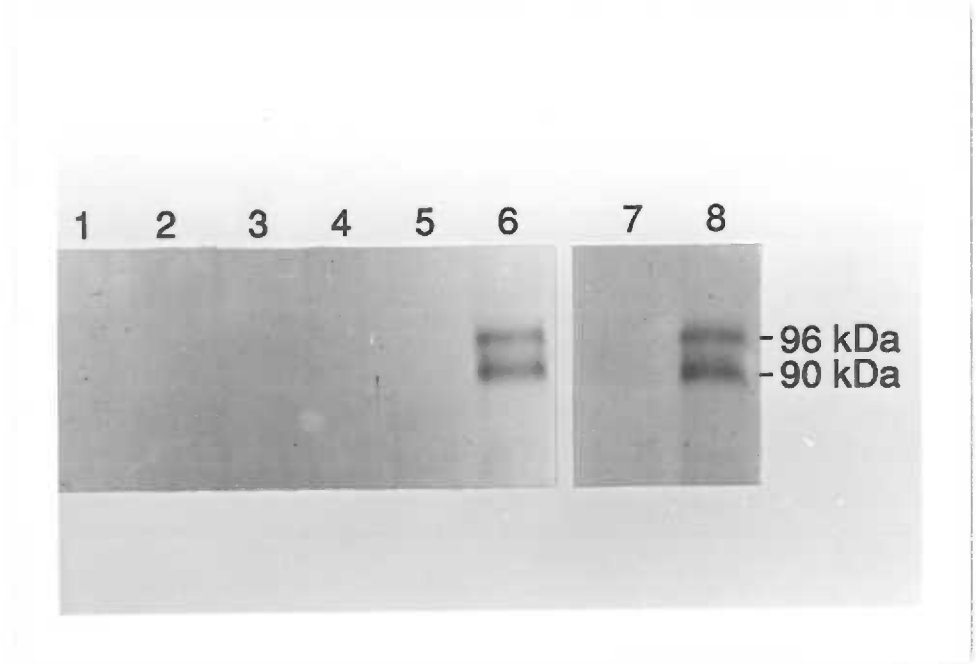
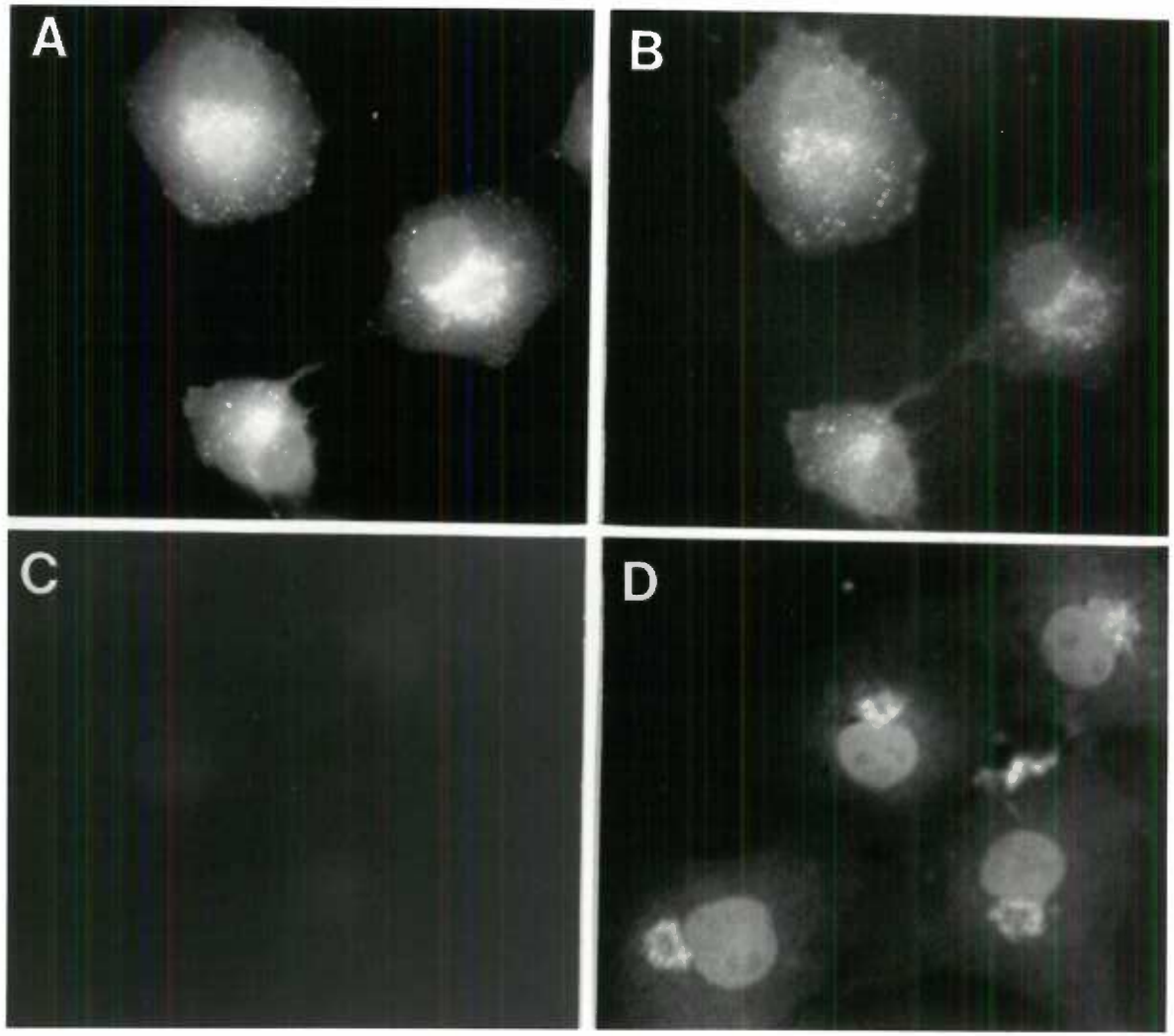


Figure 6. Immunoblot of furin expressed by VV:FUR in BSC-40 cells. High speed supernatant (lanes 1,3,and 5) and pellet (lanes 2,4 and 6) fractions from BSC-40 cells either mock-infected (lanes 1 and 2) or infected with VV:WT (lanes 3 and 4) or VV:hFUR (lanes 5 and 6) were prepared as described in methods. In a separate experiment the high speed pellet prepared from VV:hFUR-infected BSC-40 cells in the presence of 1 mM PMSF and 2 μg/ml leupeptin was resuspended by Dounce homogenization in 0.1 M sodium carbonate (pH 11) followed by recentrifugation at 100,000 x g. Proteins from the high speed carbonate supernatant (lane 7) and pellet (lane 8) fractions were precipitated with TCA, resolved by gel electrophoresis, and furin protein was identified by immunoblot as described in methods.

Because Kex2p-dependent precursor cleavage appears to occur in the Golgi apparatus in yeast (75), immunofluorescence microscopy was used to determine the subcellular localization of furin in mammalian cells. Plates of BSC-40 cells were infected with either VV:WT or VV:hFUR and the fixed cells were stained with antifurin antiserum. Cells infected with VV:hFUR revealed intense perinuclear staining (figure 7A). No detectable staining was evident in control cells infected with VV:WT (figure 7C). The fixed cells were also incubated with rhodamine-coupled wheat germ agglutinin (WGA), a specific Golgi compartment marker (113, 114). As shown in figure 7B, the pattern of staining by WGA was superimposable with the localization of furin in BSC-40 cells. Thus, as predicted for Kex2p in yeast, furin appears to be a Golgi compartment-associated enzyme in mammalian cells.

The preferential localization of furin to the Golgi apparatus of BSC-40 cells (Figure7) suggests conserved mechanisms for intracellular targeting of processing proteases between yeast and humans. Intracellular retention of Kex2p in the Golgi apparatus of *Saccharomyces cerevisiae* is required for efficient maturation of α-mating factor. Yeast mutants lacking clathrin heavy chain are unable to retain Kex2p intracellularly, resulting in deficient processing of the pro-α-mating pheromone (81). The observation that both Kex2p and furin share a highly charged cytoplasmic tail, including conserved tyrosine residues (81, 115), suggests that the furin cytoplasmic tail may also be required for retention of this protease in the Golgi compartment. Golgi localization of furin is unlikely to be an artifact of over-production of the protein because several other proteins of known subcellular localization reach their proper destination when produced from vaccinia expression vectors. For example, in polarized epithelial cells, VSV-G protein and influenza virus HA antigen are correctly targeted to the basolateral and apical membranes, respectively, when expressed by vaccinia virus recombinants (116).


A
B
C
D

Likewise, addition of the KDEL sequence responsible for retention in the endoplasmic reticulum onto a soluble form of CD4 expressed from vaccinia confers retention of this protein to the ER (117). The actual compartment in which furin-directed proteolysis occurs remains to be determined. However, the apparent localization to the Golgi compartment and substrate specificity of this endoprotease correlates with the reported endoproteolysis of several retroviral envelope proteins, including RSV Pr95 (45) and HIV gp160 (118), which apparently requires a -LysArg- directed endoprotease and takes place in the Golgi apparatus.

The structural and enzymic properties shared by the yeast KEX2 endoprotease and furin suggested that this novel human endoprotease may be responsible for processing precursors containing paired basic sites to produce mature bioactive products in the secretory pathway. However, a wide variety of precursor proteins, synthesized in a large number of endocrine and non-endocrine tissues, require endoproteolysis at cleavage sites composed of a doublet of basic residues. To facilitate identification of authentic furin substrates, we examined the cell type specificity of furin gene expression. Our initial screening of cell lines indicated detectable levels of furin transcript in all cell types examined including AtT-20 (mouse pituitary corticotroph), BSC-40 (African Green monkey kidney epithelial), GH4C1 (rat pituitary somato/lactotroph), HeLa (human cervical epithelial), HepG2 (human hepatoma), L(mouse fibroblast), NG108-15 (rat glioma X mouse neuroblastoma hybrid), and Rin m5F (rat insulinoma). Figure 8 shows a comparison of relative furin transcript levels in three of these cell lines, including the cell line used as a source for the cloned cDNA (HepG2) and two cell lines (AtT-20 and BSC-40) previously characterized for endogenous precursor protein processing capability (78, 79, 102, 119, 120). As expected, a 4.5-kb furin transcript was detected in poly A+ RNA from HepG2 cells, the human hepatoma cell line from which the furin cDNA was isolated (84) (Figure 8, lane C). This cell line endogenously expresses and processes proalbumin at an -ArgArg- site (121, 122). Only very low levels of furin transcript were detected in the mouse pituitary corticotroph line, AtT-20 (Figure 8, lane A). These cells express high levels of

POMC and efficiently process this precursor in the regulated secretory pathway (119) at several sites composed of paired basic amino acids (123). Note that a second 3.3-kb RNA band, detected only with the AtT-20 RNA, hybridized to the radiolabeled furin probe. Interestingly, this species is approximately the size of PC2 mRNA (85). Whether this band represents PC2, a different furin transcript, or yet another KEX2 homologue expressed in endocrine cells remains to be determined. The highest levels of furin transcript were found in BSC-40 cells, a monkey kidney epithelial line (figure 8, lane B). These cells possess only a constitutive secretory pathway (102) and are incapable of processing certain neuroendocrine peptide precursors, including POMC and proenkephalin. However, BSC-40 cells endogenously express and process the transforming growth factor β precursor (pro-TGF-β) at a pair of basic amino acids (124). Furthermore, when expressed in BSC-40 cells, the nerve growth factor precursor (pro–β-NGF) was cleaved at a basic residue doublet (Lys_{119}-Arg_{120}, figure 9A) to produce mature β-NGF (71). One potential role, therefore of the furin endoprotease in mammalian cells is to process precursor to growth factors and other constitutively secreted proteins (as opposed to neuropeptide precursors.)

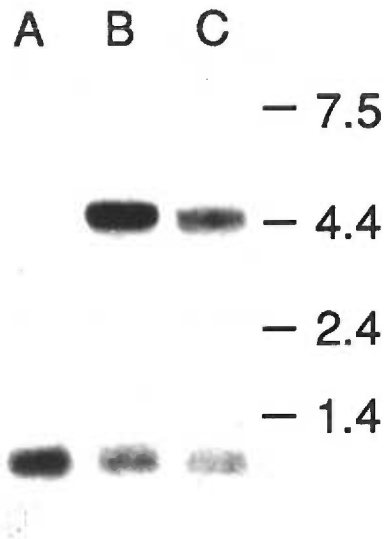
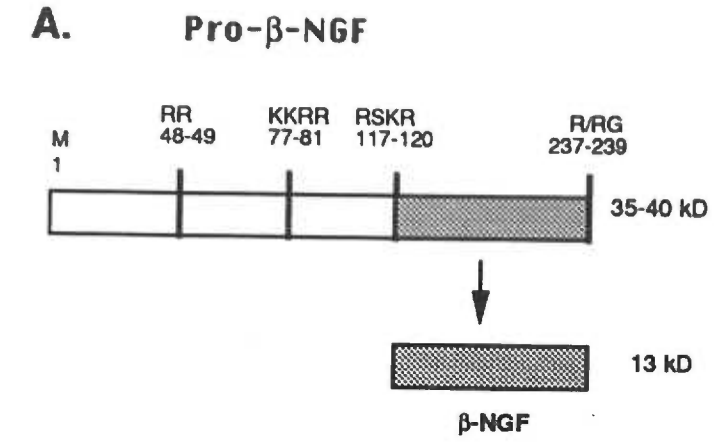
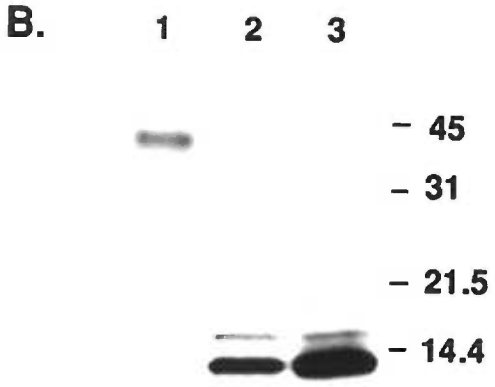
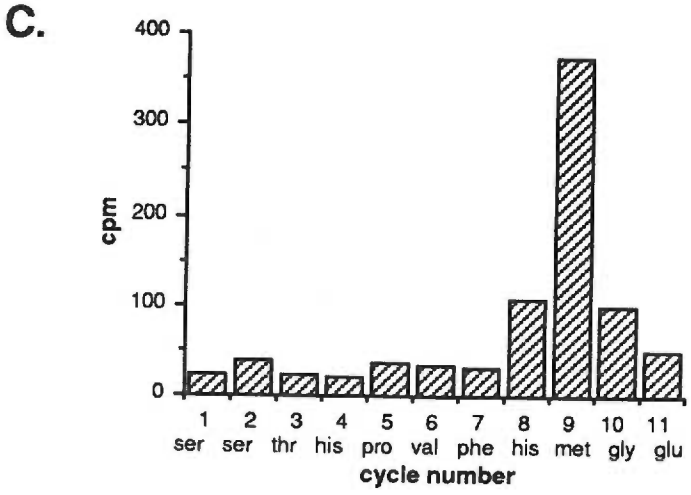


Figure 8. Northern blot of hFUR expression in different cell types. 5 μg of poly A^+ RNA isolated from AtT-20 (A), BSC-40 (B) and HepG2 (C) cells was denatured with formaldehyde, subjected to electrophoresis in a 1% agarose gel, transferred to a nylon membrane as described in methods. After exposure to x-ray film, the blot was stripped and rehybridized with a random-primed ^{32}P-labeled probe of the 1B15 (cyclophilin) sequence to control for relative amounts of RNA loaded per each lane. hfurin mRNA migrates at 4.5 kb. 1B15 migrates at 1 kb.


A.
Pro-β-NGF
M
1
RR
48-49
KKRR
77-81
RSKR
117-120
R/RG
237-239
35-40 kD
13 kD
β-NGF


B.
1
2
3
45
31
21.5
14.4


C.
400
300
200
100
0
cpm
1 2 3 4 5 6 7 8 9 10 11
ser ser thr his pro val phe his met gly glu
cycle number

Either Kex2p or furin was coexpressed with pro–β-NGF in BSC-40 cells. 18 hours after infection, each sample was incubated for 3 hours with [^{35}S]-Met,Cys and β-NGF-containing peptides were immunoprecipitated from the culture media as described in methods. Under these conditions, coexpression of Kex2p together with pro-β-NGF markedly increased processing of pro-β-NGF to mature (13 kDa) β-NGF peptide (figure 9B, lane 2). The sequence of the 13 kDa peptide was verified by Edman degradation sequencing, where a peak of activity in cycle 9 correlated with the predicted labeling of Methionine 9 in the mature peptide (figure 9C).

Two peptides of 18 and 20 kDa were also immunoprecipitated from the medium of VV:mNGF-infected cells (figure 9). These peptides most likely represent processing intermediates resulting from cleavage within the proregion of the precursor (51). Thus Kex 2p, which can efficiently process mouse POMC in mammalian cells (79) also can process pro-β-NGF to β-NGF peptide. Strikingly, coexpression of human furin and pro-β-NGF resulted in complete conversion of pro-β-NGF to smaller peptides, primarily 13-kDa β-NGF (figure 9B, lane 3). As a control to ensure that the apparent pro–β-NGF processing was not occurring in the medium after secretion, labeled pro-β-NGF from the culture medium of VV:mNGF-infected cells was transferred to plates of either VV:WT- or VV:hFUR-infected cells. Analysis of this material after 3 hours of incubation demonstrated that pro-β-NGF was completely stable in the culture medium.

Comparison of the 13-kDa β-NGF to 35-D pro–β-NGF by densitometric analysis revealed that in the sample from cells coinfected with VV:mNGF and VV:WT, only 23% of the radioactivity was present as 13-kDa β-NGF; in marked contrast in cells coinfected with VV:mNGF and VV:KEX2 or VV:hFUR, the fraction of radioactivity present as 13-kDa β-NGF was 79 and over 98%, respectively. The processed NGF in the medium from cells coinfected with VV:mNGF and VV:hFUR was biologically active as determined by its ability to induce neurite outgrowth on chick dorsal root ganglia, whereas medium from cells infected with

VV:WT alone was relatively negative for this biologic activity (data not shown). Because the vaccinia-produced furin activity measured by in vitro assay of the infected cells was substantially lower than vaccinia produced Kex2p activity (Table I), yet pro-β-NGF conversion was complete in the furin producing cells but not in the KEX2p-producing cells, the human endoprotease appears to be a more efficient processing enzyme for this precursor in BSC-40 cells.

The results described in this chapter demonstrate that the human furin cDNA isolated from HepG2 cells encodes a Golgi compartment-localized membrane-associated. In agreement with the reported structural homology between furin and the yeast Kex2p prohormone endoprotease (81, 115), both enzymes share significant functional homology in vitro (Table I) and in vivo (figure 9).

While both prohormone (e.g. POMC, proinsulin) and other precursors (e.g. pro-β-NGF) require proteolysis at paired basic amino acids to liberate biologically active polypeptides, the dependence of prohormone processing on localization to the regulated secretory pathway suggests a specific mechanism for maturation of this class of substrate. This restriction may arise from: (a) structural differences in the precursor molecules, including sequences in the vicinity of the processing site or higher order structural properties (e.g., folding); (b) differential compartmentalization of the substrate and the processing enzyme(s); or, perhaps, (c) expression of distinct processing enzymes in the regulated pathway. One notable structural similarity shared by several "nonhormone" substrates, including the precursors for Factor IX (32), insulin receptor (37) , endothelin (125), TGF-β (126), EGF (109), β-NGF (17), brain-derived neurotrophic factor (BDNF) (27), neurotrophin 3 (28), and HIV gp160 (127), is an arginine located four residues amino-terminal to the paired basic cleavage site (-Arg-X-Lys/Arg-Arg-). The lack of processing of a mutant pro-Factor IX (Arg-Pro-Lys-Arg- to -Gln-Pro-Lys-Arg-) suggests that this residue is required (32). In contrast, the neuroendocrine prohormone, prosomatostatin, is not processed in fibroblasts (128) even though it possesses an arginine residue four amino acids amino-terminal to the paired basic cleavage site. However, the

apparent processing site on prosomatostatin is an -ArgLys- sequence, not -LysArg- or -ArgArg-, typically found in nonhormone precursors. Mutation of this -ArgLys- site in prosomatostatin to -ArgArg- or -LysArg- results in a partial processing of this prohormone in fibroblast cells (129), suggesting that -ArgArg- or -LysArg- are indeed the preferred sites for the processing enzyme in the constitutive pathway, presumably furin.

Consistent with this view, using pro-β-NGF as a model substrate, we demonstrated that furin expressed in a recombinant vaccinia vector efficiently processed a mammalian precursor protein in the constitutive secretory pathway of BSC-40 cells. Taken together, the results presented here unequivocally identify furin as the initial member of a unique family of mammalian proteases capable of processing precursor proteins in the secretory pathway.

Chapter Two

Isolation and Characterization of Human Furin: A Calcium-dependent Serine Endoprotease That Recognizes the Sequence Arg-X-X-Arg

Introduction

Purification of furin activity and its characterization *in vitro* would allow comparison of its activity to previously reported paired basic-directed proteases which have not yet been identified at the genetic level. In addition the substrate specificity of furin, as determined *in vitro*, would aid the assignment of its biological role *in vivo*. Initial attempts to isolate furin activity from cell extracts were modeled on reported methods by Davidson et al., for the insulin processing activities (55) and Brennan and Peach for the hepatic proalbumin convertase (40). While some success was obtained, yields from an initial DEAE column were low, and we designed another approach.

Because native furin activity is associated with the membrane component of cell extracts, its purification would be facilitated by construction and expression of a soluble secreted form of the endoprotease. The structurally related yeast protease, Kex2p, has been expressed in a truncated form and shown to retain protease activity (112). Therefore we prepared a vaccinia recombinant containing a construct of human furin that lacked both the putative transmembrane and cytoplasmic domains (VV:hFUR713t, figure 10).

In order to characterize furin activity *in vitro*, we also wanted to optimize the assay for enzyme activity using synthetic substrates. Using the boc-Gln-Lys-Arg-MCA or boc-Gln-Arg-Arg-MCA substrate reported in our previous work yielded only around 2.5-fold enhancement of protease activity over VV:WT cell extracts under all conditions tested. These results suggested either that the protease was not highly active under the test conditions, or that the synthetic substrate did not contain all the structural features necessary for optimal furin activity. We thus obtained a series of synthetic fluorogenic substrates and compared

furin activity among them in a variety of conditions. One substrate, boc-Arg-Val-Arg-Arg- was found to give optimal furin activity *in vitro* and was subsequently used for further characterization of the protease.

An additional protein substrate for furin was obtained in the form of protective antigen protein (PA) from *Bacillus anthracis*. The two secreted toxins from this bacteria are distinct trimeric complexes, though PA is a component of each. PA serves to bind an unknown receptor on the surface of eukaryotic cells and is critical for *anthracis* virulence (47). While bound to the cell surface, PA undergoes a specific proteolytic cleavage which is obligatory for binding of the other toxin components and their subsequent entry into the cell (130). Cleavage of PA occurs at a sequence (-Arg-Lys-Lys-Arg-) similar to that in many precursor proteins, including those suggested substrates for furin. We thus obtained a series of PA mutants with amino acid substitutions around the cleavage site from Dr. Kurt Klimpel, and used these proteins to test the cleavage site specificity of furin for an authentic biological protein *in vitro*.

Results and Discussion

A vaccinia virus expressing the truncated form of furin (figure 10) was constructed as described in methods. BSC-40 cells were infected with either VV:WT or VV:hFUR713t and then cultured in a serum-free defined medium, MCDB 202 (101). Anion exchange chromatography of the VV:hFUR713t culture supernatant showed a single major peak of endoproteolytic activity, capable of cleaving boc-Arg-Val-Arg-Arg-MCA (figure 11, top, fractions 22-27). No activity was detected in parallel samples from VV:WT infected cells (data not shown). The fractions with the most endoproteolytic activity showed two closely spaced bands which reacted with anti-furin antiserum by Western blot (figure 11, middle). The smaller size of the immunoreactive doublet relative to native furin (90 kDa) is consistent with a translation product lacking the C-terminal 81 amino acids. Coomassie stained gels demonstrated that this protein comprised approximately 80% of the total protein in column

fractions containing the peaks of both proteolytic activity and immunoreactivity (figure 11, bottom). The observations that the 81 kDa bands were exclusively immunoreactive, that they co-eluted with the peak of endoproteolytic activity and that they were the appropriate predicted size indicated that this was the secreted truncate form of furin (713t). These peak fractions were used for the characterization of furin's catalytic properties.

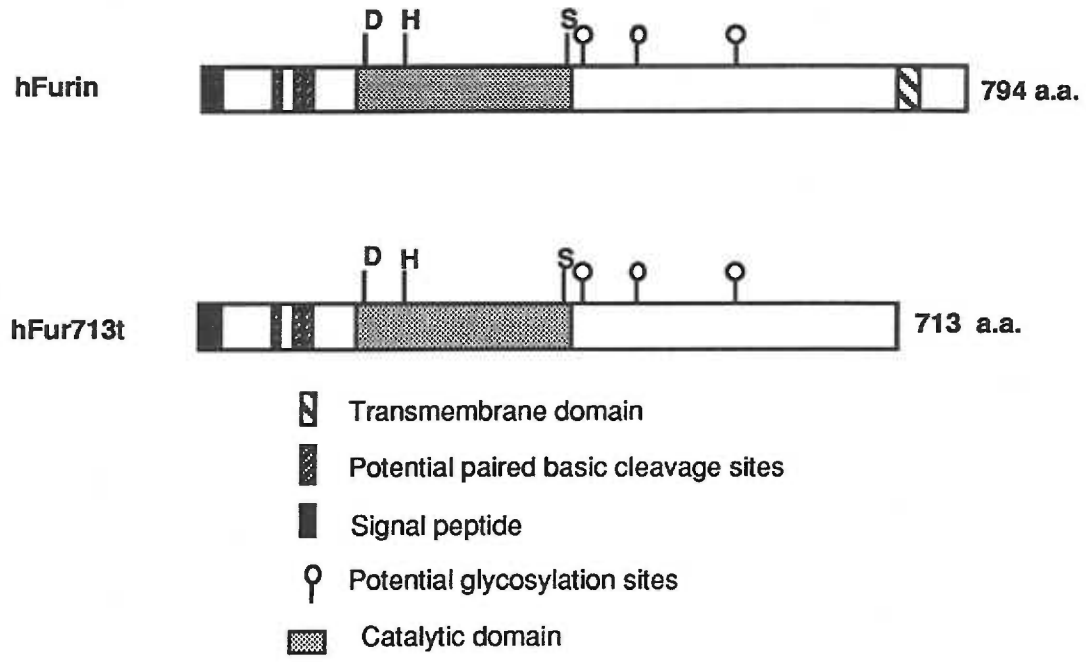
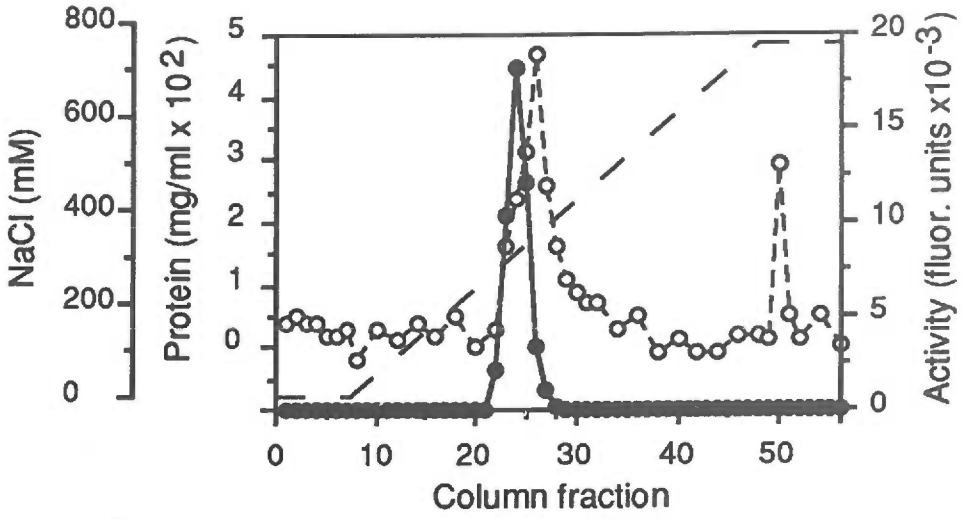
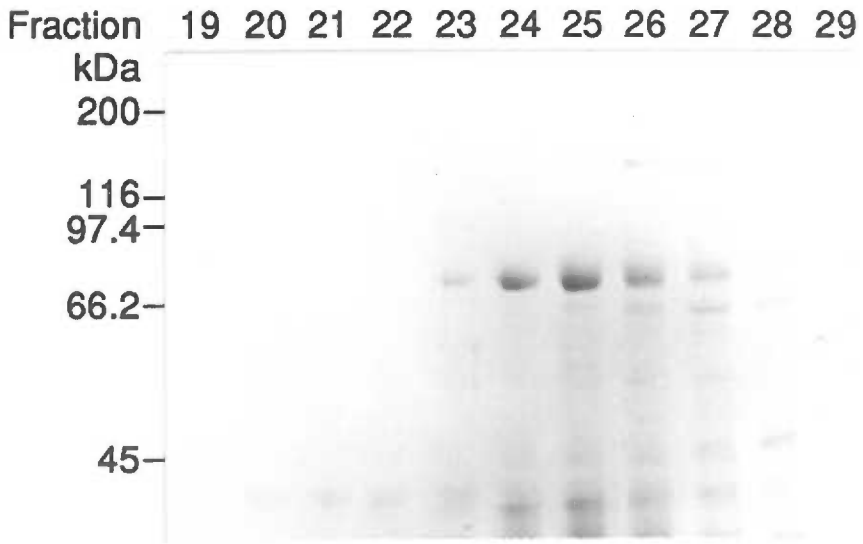


Figure 10. Structural Features of hFurin and truncated form (hFur713t)


NaCl (mM)
800
600
400
200
0
Protein (mg/ml x 10^2)
5
4
3
2
1
0
Activity (fluor. units x10^-3)
20
15
10
5
0
0
10
20
30
40
50
Column fraction


kDa
200
116
97.4
66.2
45


Fraction 19 20 21 22 23 24 25 26 27 28 29
kDa
200
116
97.4
66.2
45

A series of fluorogenic tri- and tetrapeptides possessing a pair of basic amino acids were screened for their potential as substrates for furin, first with crude cell extracts and then with isolated furin activity. The data for the purified furin activity only is shown in Table II. At equal concentrations (50 μM) only tetrapeptides were cleaved at a significant rate (1 or 10 nmol/min/mg of total protein for boc-Arg-Gln-Arg-Arg-MCA and boc-Arg-Val-Arg-Arg-MCA respectively). In contrast, several tripeptides, including boc-Gln-Arg-Arg-MCA and boc-Gln-Lys-Arg-MCA, which are efficiently cleaved by Kex2p (62, 76) were poorly processed by furin (<0.2 nmol/min/mg of total protein). In light of these results, boc-Arg-Val-Arg-Arg-MCA was the substrate of choice for further characterization of furin activity.

Table II. Substrate Preference for Furin Activity

Substrate	Percent Maximal Activity
boc-R-V-R-R-MCA	100
boc-R-Q-R-R-MCA	18
boc-Q-R-R-MCA	1.9
boc-L-K-R-MCA	1.7
boc-G-K-R-MCA	2.2
boc-L-G-R-MCA	1.5
boc-L-R-R-MCA	1.5
blank	0.7

The sensitivity of furin activity to a variety of inhibitors was also tested in order to compare the enzyme to previously described proteases (Table III). The chelators EDTA and EGTA effectively suppressed furin activity, presumably by removing the free calcium required for activation. Indeed, increased calcium, but not barium or magnesium, reversed inhibition by EGTA. Interestingly, an equimolar concentration of $MnCl_2$ had no effect in the absence of $CaCl_2$, and EGTA has a 10-fold higher affinity for Mn^{2+} than Ca^{2+} (131), we presume Mn^{2+} indirectly stimulated furin activity by competing Ca^{2+} from the chelator. Although $MnCl_2$

alone did not stimulate activity, it did attenuate stimulation by calcium, suggesting that Mn^{2+} may bind to, but not activate furin. The relative ineffectiveness of phenanthroline, a heavy metal chelator, also indicates that EGTA and EDTA inhibit furin by binding calcium, and not by removal of other cationic cofactors.

Table III. Inhibition of Furin Activity *in vitro*.§

Additive	Concentration	% Activity	Additive	Concentration	% Activity
BSA	1 mg/ml	88	Iodoacetamide	3.0 mM	103
STI	0.5 mg/ml	93		10.0mM	68
Aprotinin	0.1 mg/ml	95	DTT	1.0 mM	14
Pepstatin	1.0 mM	100	$ZnCl_2$	1.0 mM	0
Leupeptin	0.1 mM	84	$HgCl_2$	1.0 mM	
	1.0 mM	67	o-Phenanthroline	1.0 mM	82
E-64	1.0 mM	96	EDTA	2.0 mM	0
TLCK	1.0 mM	104	EGTA	2.0 mM	0
	5.0 mM	29	EGTA/$BaCl_2$	2.0 mM/2.0 mM	1
PMSF	3.0 mM	97	EGTA/$MgCl_2$	2.0 mM/2.0 mM	0
	10.0 mM	19	EGTA/$MnCl_2$	2.0 mM/2.0 mM	70
	20.0 mM	0	EGTA/$CaCl_2$	2.0 mM/2.0 mM	80
pA-PMSF	3.0 mM	99	$MnCl_2$ (no Ca^{2+})	1.0 mM	0
	10.0 mM	37			
DIFP	1.0 mM	99	α1-antitrypsin-P	0.5 mg/ml	103
	10.0 mM	77			
	30.0 mM	0			

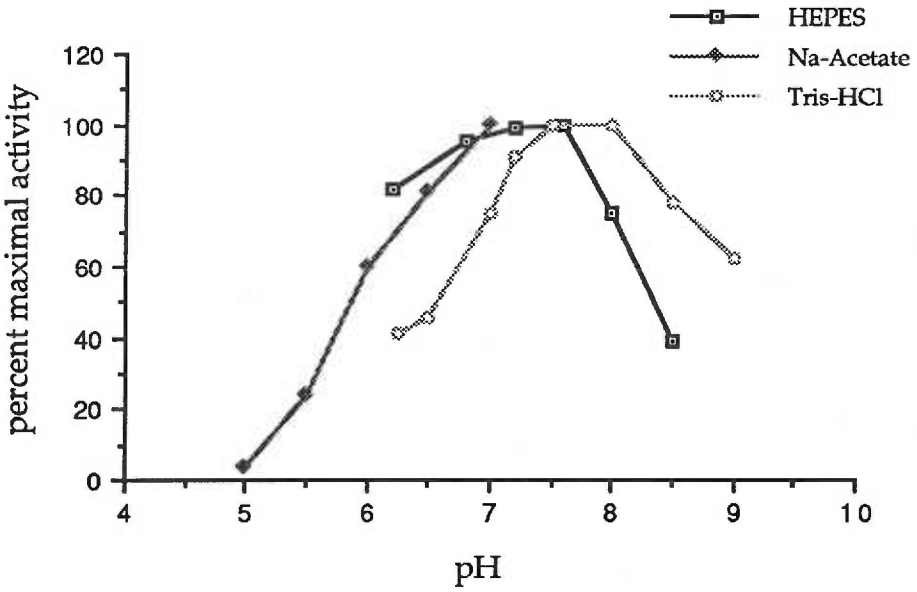
§. Fluorometric enzyme assays were conducted as described in Methods. Activity remaining in the presence of additives is given as percent of that obtained for matched controls containing the appropriate concentration of carrier (if any). The values reported are averages of at least 2 separate determinations performed in duplicate. Free Ca^{2+} was calculated to be 12 or 22 nM in the presence of 2 mM EDTA and EGTA respectively. Abbreviations: BSA, bovine serum albumin; STI, soybean trypsin inhibitor; TLCK, L-1-chloro-3(4-tosylamido)-7-amino-2-heptanone; PMSF, phenylmethane-sulfonyl fluoride; pA-PMSF, (4-amidino-phenyl)-methane-sulfonyl fluoride; DIFP, diisopropyl fluorophosphate; α1-antitrypsin-P, α1-antitrypsin Pittsburgh.

The heavy metal ions Zn^{2+} and Hg^{2+}, and the reducing agent DTT were also effective inhibitors of furin. These results are consistent with those previously obtained for Kex2p (62, 76), and suggest that the enzyme is sensitive to modification of a reactive thiol group. Sequence alignment has identified a conserved cysteine, Cys_{198}, near the active site His_{194}, and it may be this residue which is the site of action of these inhibitory metal ions. The fact that furin, like Kex2p, was not sensitive to the thiol protease inhibitor E-64, nor to moderate concentrations of the thiol reagent iodoacetamide, argues against any essential role of cysteine in catalysis.

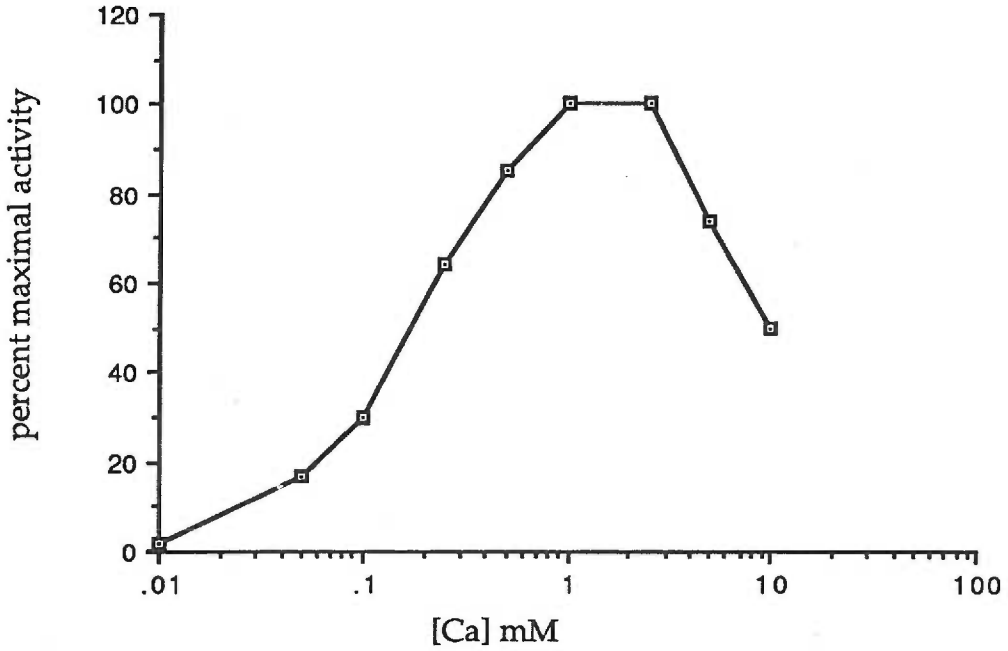
Similar to that observed for Kex2p (62, 76), PC2 (132), proalbumin convertase activity isolated from liver Golgi membrane (39, 62) and islet cell secretory granules (55), the serine protease inhibitors PMSF, pAPMSF, TLCK, and DIFP all suppressed furin activity, but only at high concentrations (between 5 and 30 mM). Inhibitors of trypsin-like serine proteases (STI and leupeptin) were relatively ineffective. α_1-antitrypsin Pittsburgh (a variant of the natural serpin α1-antitrypsin), which inhibits activity of both thrombin and Kex2p (39, 62, 77) had no detectable effect on furin activity (Table III). These data show that the inhibitor sensitivity of furin is similar to, yet distinct from, the previously described subtilisin-like proteases.

Several buffers previously shown effective in assaying Kex2p activity (62, 76) were used to determine the pH requirements of furin. The normalized activity measurements obtained in HEPES, sodium acetate, and Tris-HCl are shown in figure 12A. The curves indicate that furin has a broad pH optimum in the neutral range, with greater than 50% of maximal activity obtained between pH 6.0 and pH 8.5. the calcium dependence of furin was determined using HEPES buffer at pH 7.5. Relatively high concentrations of calcium were required to fully stimulate the protease (1-2- mM, see figure 12B) with half maximal activation ($K_{0.5}$) at approximately 200 μM. Higher concentrations of calcium (5-10 mM) inhibited the enzyme.

A.

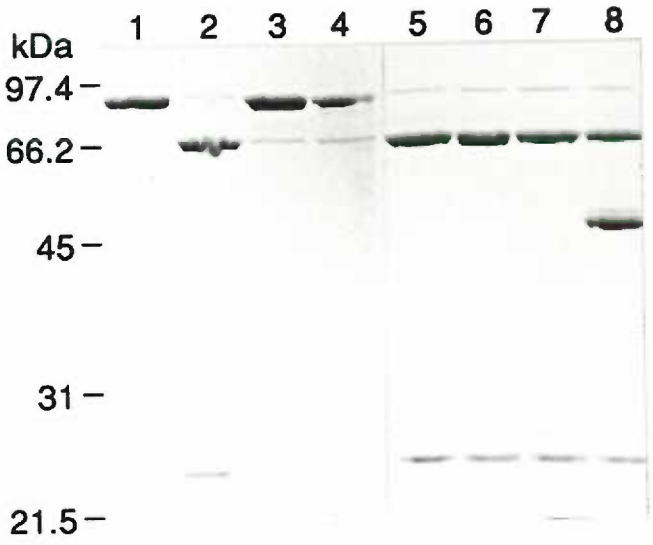

HEPES
Na-Acetate
Tris-HCl
percent maximal activity
120
100
80
60
40
20
0
4
5
6
7
8
9
10
pH

B.

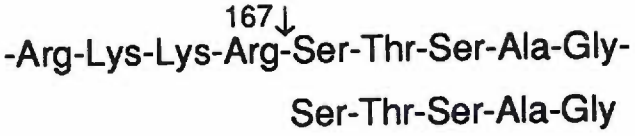

percent maximal activity
120
100
80
60
40
20
0
.01
.1
1
10
100
[Ca] mM

Purified protective antigen (PA) was obtained from Dr. Kurt Klimpel. The cell surface binding component of anthrax toxin, this protein was used to examine the sequence specificity of furin within the context of an intact protein. PA contains a tetrabasic cleavage site, -Arg-Lys-Lys-Arg167- (see figure 13B) similar to those present in proposed biological substrates of furin which are processed in the secretory pathway. Native PA was rapidly and efficiently cleaved to its 63- and 20- kDa products during a brief incubation with furin (figure 13A, lane 2). Nearly complete conversion of the precursor was observed within 15 minutes, with the same final enzyme concentration used in the fluorogenic assays. Correct cleavage of native PA at the C-terminal side of Arg_{167} (-Arg-Lys-Lys-Arg $_{167}$ ↓) by furin was confirmed by N-terminal sequencing of the 63 kDa cleavage product (figure 13B). The inhibitor sensitivity of cleavage of PA by furin was identical to that obtained using the fluorogenic peptide (figure 13A, lanes 3-8), indicating that these characteristics were not substrate specific. Lineweaver-Burk analysis, using ^{125}I-labeled PA at various substrate concentrations, revealed a Km for PA of 2.0 ± 0.3 μM, which was approximately 10 fold lower than that for the synthetic peptide. The K_m for PA with furin is nearly identical to that obtained with Kex2p using a n acylated pentapeptide (Ac-Pro-Met-Tyr-Lys-Arg-MCA) representing cleavage sites within pro-α-factor (112). A maximum rate for cleavage of PA by furin was determined empirically, as described for the synthetic peptide, using the same enzyme preparation. The cleavage rate obtained, 8 μmol/min/mg of total protein, was strikingly higher (approximately 400-fold) than that observed with fluorogenic peptide, and approaches that reported for trypsin cleavage of insulin β-chain (133). This cleavage rate translates to a K_{cat} of approximately 11 sec^{-1}. Furin, therefore, efficiently processed a model protein substrate *in vitro* at the proposed concensus site, -Arg-x-Lys/Arg-Arg-.

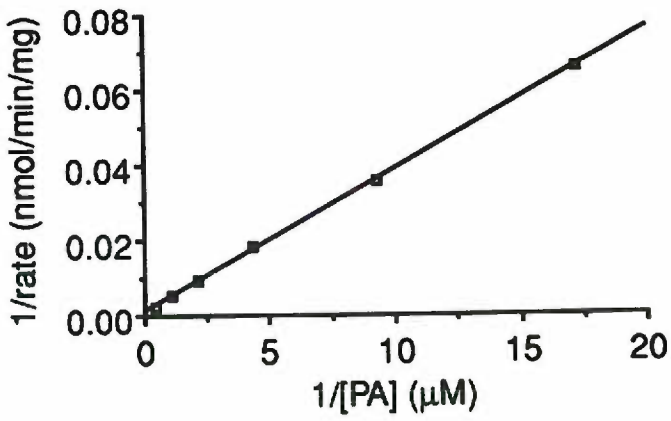
A


kDa
1
2
3
4
5
6
7
8
97.4
66.2
45
31
21.5

B

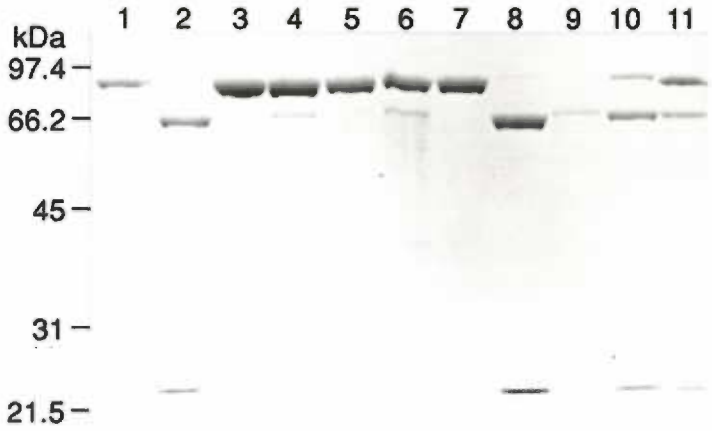

167↓
-Arg-Lys-Lys-Arg-Ser-Thr-Ser-Ala-Gly-
Ser-Thr-Ser-Ala-Gly

C


1/rate (nmol/min/mg)
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
1/[PA] (μM)

Site-directed mutagenesis of PA which substituted the arginine at position 167 (-1 to the cleavage site) with alanine greatly inhibited cleavage (figure 14, lane 6). Substitution of Ala for Arg at position 164 (-4 to the cleavage site) also greatly inhibited processing by furin (figure 14, lane 4). In contrast, substitution of the Lys residues located at positions 165 and 166 with Ala had no detectable effect on cleavage (figure 14, lane 8). These results indicate that the Arg residues at the -1 and -4 positions may be essential for efficient cleavage site recognition, and furthermore, that the basic residues typically located at the -2 position do not contribute as much to efficient cleavage of PA protein. Whether the -4 Arg in the cleavage site contributes to substrate affinity or catalytic rate of conversion cannot be determined by this assay.

The higher rate of proteolysis seen with native PA compared to the synthetic peptides suggests that there are other factors in addition to the primary sequence of the recognition site, such as secondary structure, which contribute to efficient processing by furin.


1 2 3 4 5 6 7 8 9 10 11
kDa
97.4
66.2
45
31
21.5

The results in this chapter demonstrate that furin is a calcium-dependent subtilisin-like serine endoprotease that efficiently cleaves substrates on the carboxyl side of the consensus sequence -Arg-X-X-Arg-. The enzymatic characteristics of truncated human furin are similar to its structural homolog in yeast, Kex2p (62, 76), as well as previously reported calcium dependent precursor protein endoprotease activities (40, 62, 132).

Furin has a broad, neutral pH optimum and, like Kex2p, is strictly calcium dependent (figure 11 A,B). In contrast, more acidic pH optima have been observed in other calcium-dependent proteases such as those from liver Golgi membrane (39, 40, 41), insulin granule types I and II (55) and the neuroendocrine-specific endoprotease PC2 (132). The calcium concentrations required for half maximal activation of most of these proteases differ markedly from that observed for furin. The $K_{0.5}$ is approximately 0.01 mM for Kex2p, 0.1 mM for the β-cell granule type II enzyme, 0.2 mM for furin (Fig 11B), and 1-2 mM for the β–cell type I secretory granule and liver Golgi activities.

The inhibitor profile of furin (Table III) also demonstrates both similarities and differences when compared to other activities. Like Kex2p, furin activity was insensitive to acid (pepstatin) and thiol (E-64) protease inhibitors, whereas serine protease inhibitors (PMSF, pAPMSF, TLCK, DIFP) were effective only at high concentrations. The concentrations of serine protease inhibitors required to affect furin activity were similar to those reported for Kex2p when assayed at neutral pH (62); Kex2p assayed at acidic pH is reported to be more sensitive to these inhibitors. Furin is apparently more sensitive to PMSF than either the secretory granule or liver Golgi activities. Furin is also more sensitive to TLCK and Zn^{2+} than the liver Golgi activity, and more sensitive to DTT than either the liver activity or PC2. Unlike Kex2p and the liver Golgi and secretory granule activities, furin is insensitive to the mutant serpin α_1-antitrypsin-Pittsburgh (Table III). Thus, the activation characteristics and inhibitor sensitivities reported here distinguish furin from previously described endoproteases.

The relative effectiveness of fluorogenic peptides boc-Arg-Val-Arg-Arg-MCA and boc-Arg-Gln-Arg-Arg-MCA (which mimic prothrombin and Newcastle disease virus F_o coat protein cleavage sites respectively) as substrates is consistent with the importance of an Arg at the -4 position for efficient cleavage by furin (Table II). Processing of Factor IX, a proposed furin substrate, *in vivo* is abolished when the -4 arginine is replaced with glutamine (32). Site-directed mutagenesis has demonstrated that human protein C precursor (34) and the human insulin receptor precursor (38) both require an arginine at the -4 position for efficient cleavage. these observations are in agreement with our results demonstrating that the -4 arginine is important for efficient cleavage of PA.

Results in chapter one demonstrated that co-expression of furin and pro–β-NGF in BSC-40 cells resulted in the efficient processing of this precursor to 13-kDa NGF peptide by endoproteolysis at residues Arg-Ser-Lys-Arg (83)(chapter one). Since furin can properly cleave a precursor protein which fits the consensus sequence Arg-X-X-Arg when coexpressed *in vivo*, and furin appears to be expressed in all cells, furin may be involved in the natural processing of proteins containing this consensus cleavage site. Such proteins include other neurotrophic factors, growth factors, serum proteins, proreceptors and viral envelope proteins.

Chapter Three

Mutagenesis of Pro-β-NGF for Characterization of its Proteolysis *in Vivo*

Introduction

Our previous investigation of the human furin protease revealed a widespread distribution in many cell types, localization primarily in the Golgi membrane, and a strong preference for a cleavage site sequence containing an arginine in positions -1 and -4 relative to the cleaved bond. Coexpression of furin with pro-β-NGF (83); (chapter one) or pro-von Willebrand factor (84)*in vivo* resulted in proper cleavage of these precursors at their appropriate cleavage site with sequence -R-S-K-R^{-1}- . In addition, mutation of the native prorenin cleavage site sequence (-F-T-K-R^{-1}-) to a sequence containing an arginine in the -4 position (-R-T-K-R^{-1}-) resulted in cleavage of the mutant precursor but not native prorenin in Chinese hamster ovary (CHO) cells which were co-transfected with furin (134).

In order to characterize the requirements for pro-β-NGF cleavage *in vivo*, we wanted to mutate the cleavage site responsible for the formation of the N-terminus of the mature factor and evaluate the effects of a mutant cleavage site on processing. The endogenous proteolytic activity of BSC-40 cells toward these mutants could then be compared to that of cells coexpressing furin from a recombinant vaccinia expression vector, as described in chapter one. Since the arginine at position -4 was critical for cleavage by furin, this residue was initially mutated to one each of four different residues: lysine, methionine, glutamine or histidine. The rationale for these substitutions was based on previous sequence analysis and mutagenesis studies of cleavage sites in POMC (135), and on evolutionary substitution analysis (136). Like arginine, lysine is the most commonly found residue at paired or multiple basic cleavage sites, though arginine at the -1 position is clearly favored (135). Previous work showed that methionine may substitute for lysine in the -2 position of the ACTH/βLPH cleavage site in POMC (native sequence -L-E-K-R- mutated to -L-E-M-R), possibly due to similar conformation

of their side chains. Glutamine was chosen because it has been reported to be substituted for arginine at the -4 position of the naturally occuring, uncleaved mutant Factor IX (32), with native sequence -R-P-K-R-^{1}-. Substitution of methionine for arginine at the same site in pro-β-NGF and subsequently blocked cleavage, would indicate a similar enzyme specificity for the two precursors. Finally, as a basic amino acid, histidine carries a like charge as arginine at an acidic pH, which could similarly contribute to charge-charge interactions in the enzyme binding pocket within an acidic compartment of the secretory pathway such as the trans-Golgi (137, 138). Analysis of proteins related by evolution led Harlow and Lane to report the observed occurrence of an amino acid substitution as a relative measure of naturally accepted amino acid changes (136). In this analysis the occurrence for an R to K substitution was 43% ; 11% for R to Q ; 9.3% for R to H; and 1.5% for R to M. Thus the selected mutations represented a range of acceptable mutations which naturally occur in evolution. Progress in construction and expression of the pro-β-NGF mutants (NM's1-4) will be discussed.

Table IV.

	Cleavage site Sequence
	-4 3 -2 -1
Native pro–β-NGF	-R-S-K-R-
NM1	-K-S-K-R-
NM2	-M-S-K-R-
NM3	-Q-S-K-R-
NM4	-H-S-K-R-

One step toward demonstrating that furin is responsible for pro-β-NGF processing *in vivo* is to compare the substrate specificity of the endogenous protease activity in BSC-40 cells and furin. For comparison of endogenous BSC-40 proteolysis and furin activity *in vivo*, we wanted to demonstrate a direct interaction of furin with pro-β-NGF and mutants *in vitro*. This experiment required isolation of the β-NGF precursor after metabolically labeling with ^{35}S

Met, Cys in BSC-40 cells as described in chapter one. Several methods of isolating this precursor were attempted, including fractionation of labeled media from VV:NGF-infected cells by HPLC using a reverse phase chromatography column (as per POMC isolation, (102)), by a molecular sieving column (Superose 12, Pharmacia) on FPLC, and by generation of pro–β–NGF through *in vitro* transcription and translation. All attempts to obtain labeled precursor failed. Thus an alternate method for demonstrating pro-β-NGF conversion by furin *in vitro* was initiated, with preliminary success. The procedure involves direct immunoprecipitation of the precursor and incubation of the antibody-bound precursor with purified furin activity.

Results and Discussion

Mutagenesis of pro–β-NGF was performed as described in methods. The resulting cleavage site sequence of the four mutants constructed as well as the native pro-β-NGF sequence is listed in Table IV. Rather than construct recombinant vaccinia viruses for these mutants we chose to use the T7 expression system, as described in methods (105, 106). Briefly, the pro–β-NGF cDNAs (native and NM's1-4) were cloned into plasmid pGem7 (Promega), with orientation 5' to 3' along with the T7 promoter on this plasmid. This plasmid was then transfected into cells which were infected with a recombinant vaccinia virus expressing T7 polymerase which drives transcription from the T7 promoter. Cells were metabolically labeled and the media immunoprecipitated as before (see methods). However, poor transfection efficiency leads to lower (about 10-fold less) expression in the T7 system than from the recombinant vaccinia virus (data not shown). Pulse times were increased to 6-10 hours in order to allow accumulation of detectable levels of labeled protein in the media.

As shown in figure 15, expression of native pro-β-NGF (lane 1) and two of the mutants (NM1 and 2, lanes 2 and 3 respectively) showed a marked difference in their processing by the endogenous BSC-40 activity. No expression was observed for mutants NM3 and NM4. In multiple experiments, similar results were observed. Conversion of native pro-β–NGF was

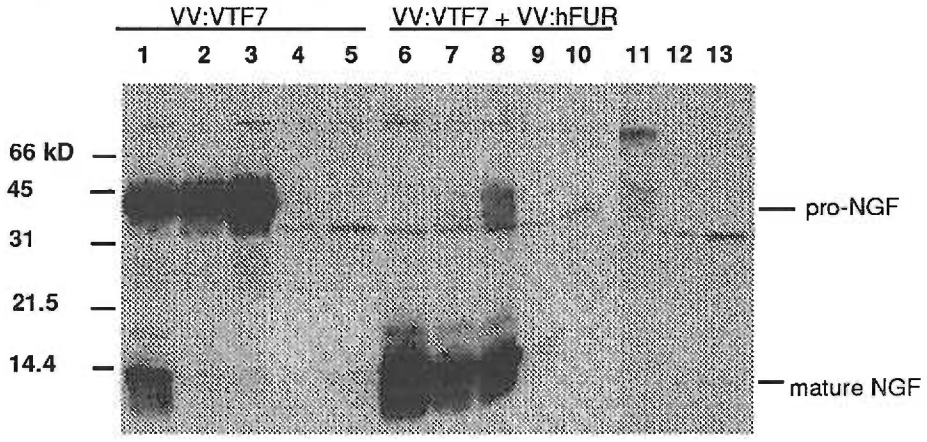
much greater than that of either NM1 or NM2. For coexpression of furin, cells were coinfected with VV:hFUR and VV:VTF7, then transfected with the appropriate plasmid. Coexpression of furin for native pro-β-NGF as well as NM1 and NM2 resulted in conversion of the precursor to the 13 kDa form of β-NGF. However, a residual amount of the NM2 mutant precursor was always observed, indicating incomplete conversion of this precursor. Densitometric analysis was performed on the film from this gel on a BIO-RAD Model 620 Video Densitometer (Bio-Rad,), with results shown in Table V. This analysis further demonstrates the preferential conversion of native pro-β-NGF compared to NM1 and NM2 by both the endogenous BSC-40 activity and ectopically expressed furin, and suggests they are the same or closely related enzymes, with preference for cleavage sites containing a -4 arginine.

Table V.

Densitometric analysis of 35 kDa and 13 kDa peaks from Pro-NGF Processing

Sample	35 kDa	14 kDa	Percentage of total signal, 14 kDa band
NGF, native	21	10.1	33
NM1	20	0.58	3
NM2	30	0.33	1
NGF + furin	0.67	34	98
NM1 + furin	0.45	17	97
NM2 + furin	6.8	19	74

Values are reported are calculated peak areas based on a trace of optical density (O.D.) vs. mobility in mm.

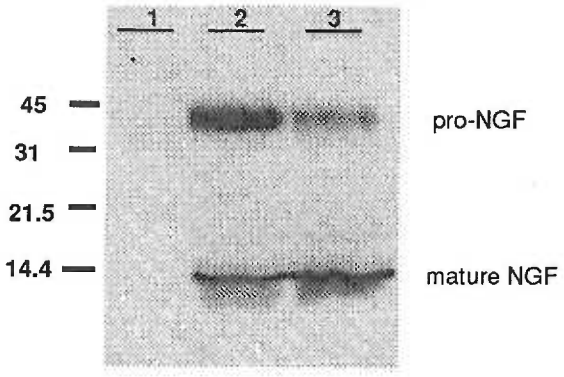

VV:VTF7
VV:VTF7 + VV:hFUR
1 2 3 4 5 6 7 8 9 10 11 12 13
66 kD
45
31
21.5
14.4
pro-NGF
mature NGF

Expression of pro-β-NGF mutants NM3 and NM4 was not observed on several experiments. The sequence around the cleavage site was established as correct for these mutants, with no detectable additions or deletions which could cause a frame shift. Furthermore, the restriction enzyme digests for these plasmids were as predicted. The lack of expression could be due to mutations in the plasmid other than at the cleavage site. Sequencing through the entire pro-β-NGF sequence, from the T7 promoter would establish that the correct sequence was intact. Another possibility is that the single amino acid change could result in intracellular degradation of these mutant proproteins. A recent study in which sequential deletions of the pro region of pro-β-NGF demonstrated that the intact pro region is essential for optimal expression in COS cells (25). Fusion of the mature β-NGF sequence to the signal sequence eliminated all detectable expression. In addition, deletion of only the six amino acids at positions -3 to -8 in the pro region (including the -4 arginine) resulted in diminished expression and blocked cleavage of the precursor (25). Thus it is possible that disruption of the secondary structure by a single amino acid change in this region around the -4 arginine could inhibit expression below detectable levels. A more sensitive test of NGF expression in cells transfected with NM3 and NM4 could be biological assay by dorsal root ganglion neurite outgrowth.

Interestingly, coexpression of exogenous furin with NM1 and NM2 resulted in nearly complete conversion of these mutants as well as native pro-β–NGF, though a clear preference for the native precursor was observed (figure 15 and Table VI). These results may reflect a high enzyme/precursor ratio upon concentration of both in the secretory pathway, presumably the Golgi. The most radical change in cleavage site sequence, -4 R to M, is the least favored substrate for furin *in vivo*, which results in the highest levels of remaining precursor (figure 15, Table V). Thus the kinetics of furin at physiologic levels of expression may contribute to its activity *in vivo*, and the nearly complete conversion of the mutant pro-β-NGF precursors may not accurately represent the relative affinity of furin for native and mutant precursors.

Under the vaccinia expression system, furin is expressed at levels much higher than endogenous levels, as evidenced in figure 6 (chapter one). Immunoblots of furin in BSC-40 cells showed no endogenous enzyme, though the message was clearly present (figure 8, chapter one). In addition, immunolocalization detects furin concentrated in a membrane compartment consistent with Golgi localization. Immunoprecipitation of cell extracts indicated very low levels of intracellular β-NGF, either in precursor or processed form, in agreement with the rapid transit of constitutively secreted proteins through the secretory pathway. Thus the high levels of furin expression under vaccinia virus vector, coupled with the rapid transit of the precursors through the intracellular compartment containing furin may create conditions for effectively large excess of enzyme compared to precursor concentration. Under these conditions the relative affinity of the enzyme for each precursor would not be represented in the ratio of precursor to product as detected by our immunoprecipitation assay. Characterization of furin activity *in vitro* in the presence of limiting amounts of enzyme may more accurately reflect the relative affinity of the enzyme toward these mutant substrates.

In order to test the interaction of native pro-β-NGF and mutants in vitro, multiple attempts were made to isolate precursor. Based on a method originally reported by Berger and Shooter to demonstrate cleavage of the β-NGF precursor by purified γ-NGF *in vitro* (17), purified furin was incubated with an anti-β-NGF immunoprecipitate from metabolically labeled medium prepared as described in chapter one. Isolation of furin activity was as described in chapter two, except that the preparation (S. Molloy, 2/92) was frozen at -20^{o}C in 20% glycerol for a month. Purified furin enzyme was added to one sample of immunoprecipitate, while a control sample was incubated under the same conditions in the absence of enzyme. Figure 16 shows enhanced conversion of the 35 kDa pro-β-NGF in that sample with enzyme (lane 3) compared to the control (lane 2). This experiment provides evidence for the direct interaction of hfurin and murine pro-β-NGF *in vitro*, and could be used for further testing of mutant precursors.


1
2
3
45
31
21.5
14.4
pro-NGF
mature NGF

Summary and Future Directions

Much progress has been made in the last few years toward identifying the sequence specificity of cellular proteases directed toward paired or multiple basic amino acids. Several enzymes which may be responsible for these activities have now been isolated at the genetic level. Thus unequivocal identification of the enzymes responsible for proteolysis of specific precursors in the secretory pathway is now at hand. Protein precursors cleaved in the constitutive secretory pathway may be substrates for a distinct set of enzymes from those involved in cleaving prohormones in the regulated secretory pathway. Evidence for a separate mechanism for endoproteolysis in the constitutive secretory pathway includes the ability of cells incapable of processing prohormone precursors to process precursor to other proteins which are constitutively secreted, and a unique precursor cleavage site sequence, most often including an arginine in the -4 position. Whether the enzymes which cleave constitutively secreted proteins serve any regulatory function is not clear. Further studies are needed to characterize the regulation and tissue distribution of the enzymes, once they have been associated with processing a specific precursor.

The work presented in this thesis is aimed at characterizing endoproteolysis at basic amino acid sequences in the constitutive secretory pathway, using pro-β-NGF as a model substrate. A critical tool for these studies has been use of the vaccinia virus expression vector. Initial evaluation of pro-β-NGF expression with a recombinant vaccinia virus (VV:NGF) showed that the precursor form of the protein was predominantly expressed later in viral expression. With this result, an assay for reconstituting pro-β-NGF cleavage was established as a means of evaluating potential processing enzymes in the constitutive secretory pathway of BSC-40 epithelial cells.

The most likely candidate for the enzyme responsible for endoproteolysis at basic amino acid residues in the constitutive pathway is the recently cloned product of the *fur* gene,

furin. Based on homology to the yeast *kex2* gene, *fur* cDNA was isolated from a human hepatoma (HepG2) cDNA library (139). Using the human PACE (for Paired Basic Cleaving Enzyme, or furin) cDNA as a probe a signal for the *fur* gene product was found in all cell types tested, including both endocrine and non-endocrine. Subsequent expression with a recombinant vaccinia virus allowed evaluation of furin localization and activity *in vitro* and *in vivo* in BSC-40 cells. As reported in chapter one, we found a membrane-associated, Golgi-localized protein, with calcium-dependent activity *in vitro*. Upon coexpression of furin with pro-β-NGF in BSC-40 cells, the precursor was correctly cleaved to mature β-NGF. From this study came the question of whether furin was the protease responsible for pro–β-NGF processing (and other precursors with similar cleavage site sequences) outside the regulated secretory pathway in *vivo*.

Results presented in chapter two demonstrate the isolation of furin activity by construction of a truncated form of the enzyme, eliminating the transmembrane domain and carboxyl terminus. Furin activity expressed from a recombinant vaccinia virus was isolated from the serum-free defined medium of VV:hFUR infected cells by separation on a Mono-Q ion exchange column. Purity and enzyme activity were evaluated by immunoblot and Coomassie staining of sequential column fractions which were run on SDS polyacrylamide gels. A pH activity profile, and inhibitor profile were performed using fluorogenic peptide substrates. These results showed a calcium-dependent activity consistent with a serine protease and which was active over a broad spectrum of pH. Substrate specificity was examined in two ways: fluorogenic peptide substrates established a clear preference for tetrapeptides over tripeptides, both with paired basic residues (-R-R-). Cleavage of both native and mutant forms of PA protein from Bacillus anthracis demonstrated a clear preference for substrates with arginine in the -1 and -4 positions relative to the cleavage site. This specificity is consistent with a role for furin in cleaving substrates in the constitutive pathway, as discussed above.

Finally, we wanted to determine the specific sequence requirements for pro–β-NGF cleavage in vivo. Chapter three reports the construction and expression of mutant pro-β-NGF molecules and their relative processing by both endogenous protease(s) in BSC-40 cells, and by ectopically expressed furin. Both activities displayed a preference for the native pro-β–NGF sequence, though mutation of the -4 arginine to lysine gave nearly comparable conversion. When expressed at high levels by the recombinant vaccinia virus, furin was able to facilitate the conversion of two mutants, with -4 arginine replaced by either lysine or methionine. Due to the potentially high levels of enzyme to substrate in the intracellular processing compartment, these results may not accurately reflect the relative affinity or preference of furin for the different substrates. Two pro-β-NGF mutants were not expressed, either because of anomalies in the expression plasmid introduced during construction, because of low levels of expression or because of intracellular degradation of the mutant protein.

The intracellular compartments for proteolysis in the constitutive pathway have not been determined, though both trans-Golgi and cis-Golgi compartments have been suggested for processing of viral proproteins by host proteases. For many growth factors and serum proteins which are intracellularly processed, the site of proteolysis is difficult to establish because of their rapid transit through the constitutive secretory pathway and extremely low levels of intracellular precursor. Localization of the enzymes involved in their proteolysis will help identify the compartment in which these factors are cleaved. Furthermore, the ability to block expression of the processing enzymes, either through gene disruption, targeting the mRNA with antisense oligonucleotides, or coexpression of specific protease inhibitors will allow accumulation of precursor proteins for further study *in vitro*.

Characterization both *in vitro* and *in vivo* of the endoproteases responsible for cleavage at single and multiple basic amino acids is still a primary focus in this field, though much progress has been made in the last decade. The results reported in this thesis are directed toward establishing the authenticity of a mammalian protease by establishing it

ability to cleave pro-β-NGF *in vivo* in cells which lack a regulated secretory pathway. Whether furin is active in the regulated secretory pathway, and is responsible for cleaving pro-β-NGF there is not addressed. Whether sorting to the regulated secretory pathway occurs by a positive signal or by an exclusionary process (negative signal) is not known. However, pro-β-NGF is capable of being secreted in a regulated as well as constitutive manner from AtT-20 cells (26). In addition, both mRNA and protein levels for β-NGF are rapidly decreased in hippocampal neurons as well as septal neurons in response to muscimol or diazepam, antagonists of γ-aminobutyric [GABA] (24). Since the septal neurons rely on uptake of secreted β-NGF from hippocampal neurons, the rapid decrease in their intracellular β-NGF suggests inhibition of release, or regulated secretion from hippocampal neurons (24).

For further demonstration of the role of furin in processing pro–β-NGF, as well as other pro-proteins in the constitutive secretory pathway, direct inhibition of endogenous furin activity may be attempted. The two approaches most likely to succeed are inhibition of furin expression by culturing cells with oligomers which code for anti-sense sequences to the furin message. This approach has been successfully used for inhibition of prohormone converting enzymes *in vivo* (140). The criteria for designing effective antisense oligomers are not clearly established, so that several different sequences may be used.

Another approach to directly inhibiting furin may be to design a mutant serpin containing a specific sequence correlating to the preferred furin substrate (-R-X-X-R-. Serpins are naturally occurring serine protease inhibitors. Our results demonstrate that the mutant serpin α1-antitrypsin Pittsburgh is not effective for inhibition of furin activity. The primary effect of α1-antitrypsin is to inhibit elastase activity. The mutant α1-antitrypsin Pittsburgh effectively inhibits thrombin, rather than elastase. Both serpins are thought to act by "baiting" the binding pocket of their target enzymes with the relevant substrate consensus sequence and blocking cleavage by remaining bound to the enzyme. By inserting the consensus sequence for furin substrates (-R-S-K-R-, as in pro-β-NGF, or -R-V-R-R-, a useful synthetic

peptide substrate used studies in chapter two) into the α1 antitrypsin sequence, we could effectively target furin activity. Certainly the results reported in this thesis provide the tools for such investigations. The purified furin activity can now be tested for specific inhibition *in vitro*. Furthermore, *in vivo* conversion of pro-β-NGF can be tested with coexpression of a furin-directed α1-antitrypsin. Specific inhibition of furin activity in these studies could be important for multiple reasons. First, disrupting furin activity could authenticate furin as the enzyme responsible for processing pro-β-NGF and other non-endocrine precursors, *in vivo*. Secondly, specific inhibition of furin activity could be used to distinguish this activity from other biochemically isolated activities, such as proalbumin convertase and influenza HA-converting activity.

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