Identification and Functional Characterization of A-Kinase Anchoring Proteins

by

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

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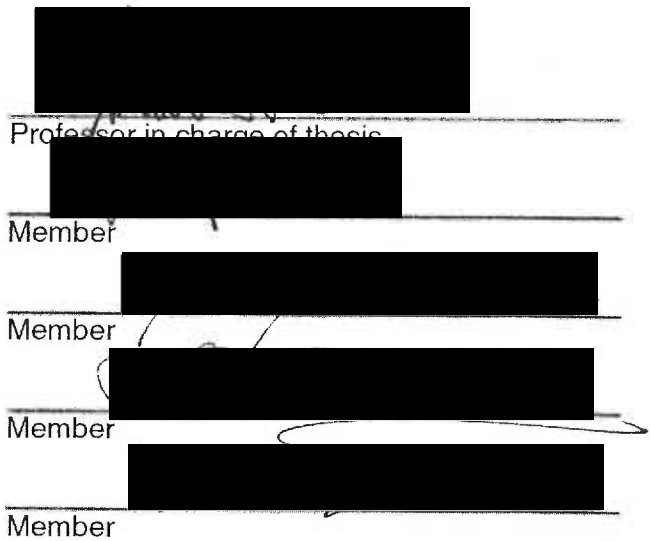

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ABSTRACT

Compartmentalization of signal transduction enzymes is an important mechanism to impart cellular signaling specificity. This occurs through the interaction of enzymes with scaffolding or anchoring proteins. To date, one of the best-studied examples of kinase anchoring is the targeting of PKA to cellular locations through its association with A-kinase anchoring proteins (AKAPs). AKAPs mediate a high affinity interaction with the type II regulatory subunit (RII) of PKA for the purpose of localizing the kinase to pools of cAMP and within proximity of preferred substrates. Furthermore, AKAPs can organize entire signaling complexes made up of kinases, phosphatases, and regulatory proteins.

The role of AKAPs in cAMP signaling has been elucidated using competitive peptide-inhibitors such as the prototypic inhibitor of PKA anchoring termed Ht31. This peptide is derived from the RII binding domain of the naturally occurring AKAP, AKAP-Lbc. The use of Ht31 and similar peptides has proven indispensable for the identification of physiological processes regulated by anchored PKA. Using a computer generated consensus sequence and peptide array modifications, we have developed a highly potent inhibitor of AKAP mediated PKA anchoring. This peptide is called AKAPIS for its design "*in silico*". Results presented here demonstrate that AKAPIS has a greater affinity for RII than any endogenous AKAP studied. Moreover, cellular data suggests that AKAPIS is a more potent inhibitor of PKA anchoring then Ht31.

AKAPs have been identified in a number of multicellular organisms including *C. elegans*, *Drosophila melanogaster*, and many mammalian species. Using the yeast two-hybrid assay we have identified the human protein Rab32, a member the Ras-family of

small G-proteins, as an AKAP. Biochemical and cellular data indicates that Rab32 can bind PKA through a conserved amphipathic helical motif *in vitro* and *in vivo*. Interestingly, Rab32 localizes to the mitochondria where it participates in mitochondrial dynamics. Cellular expression of a putative dominant-negative Rab32 mutant form promotes aberrant accumulation of mitochondria at the microtubule-organizing center. Under these conditions, mitochondria display a highly fused morphology. This implicates endogenous Rab32 as a participant in synchronization of mitochondrial fission events. Together, the data presented here describes the first small G-protein to interact with PKA and the fourth gene family that can regulate mitochondrial dynamics.

CHAPTER ONE

INTRODUCTION

Signal Transduction: The Role of A-Kinase Anchoring Proteins

and the Rab Family of Small G-Proteins

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Topic 1: A-Kinase Anchoring Proteins as Mediators of Cellular Signal Transduction

Introduction

Extracellular signals, such as hormones, neurotransmitters, and growth factors, regulate a wide variety of cellular activities, including ion channel modulation, neuronal excitation, cell growth, and cell differentiation (Sutherland, 1972). Intracellular transduction systems receive these signals via receptors and transmit them quickly and precisely, resulting in the amplification of specific biological responses. Cells often are exposed to several messengers simultaneously; thus maintaining the fidelity of these networks is crucial in eliciting the appropriate physiological response. Doing so requires the accurate selection of effector molecules for activation and deactivation, often by phosphorylation and dephosphorylation events. A principal strategy in achieving this selection of specificity is compartmentalization of signaling enzymes (Colledge and Scott, 1999; Diviani and Scott, 2001; Pawson and Scott, 1997). This chapter focuses on introducing the conceptual advances that have resulted from studying the localization of protein kinase A (PKA) mediated by A-kinase anchoring proteins (AKAPs).

Cyclic-AMP Dependent Protein Kinase

The role of cAMP as a second messenger was first discovered in 1957 (Sutherland and Rall, 1957). Since that time more than 78,000 papers have been published on this subject. From these studies, many of the molecular mechanisms and physiological processes governed by cAMP have been determined. For example, cAMP is generated

following hormonal activation of G-protein-coupled receptors (GPCRs) (Beavo and Brunton, 2002). Hormone binding to a receptor leads to conformational change, followed by the conversion of heterotrimeric G-protein subunit, Gαs, into its active GTP-bound state (Pierce et al., 2002). Activated Gαs is released from the βγ subunits and activates the enzyme adenylyl cyclase which converts ATP to the second messenger cAMP. The primary downstream target of cAMP is the cAMP-dependant protein kinase, PKA (Walsh et al., 1968). However, it should be noted that cAMP has roles independent of PKA including the regulation cAMP activated ion channels and a recently a discovered family of Ras family-Guanine Nucleotide Exchange Factors (GEFs) (de Rooij et al., 1998; Kaupp and Seifert, 2002; Kawasaki et al., 1998).

PKA is a serine/threonine kinase composed of two catalytic (C) subunits that are held in an inactive state by association with a regulatory (R) subunit dimer (Corbin and Keely, 1977; Corbin et al., 1973; Potter et al., 1978; Potter and Taylor, 1979). The catalytic subunits (C) are expressed from three different genes-Cα, Cβ, and Cγ-, whereas the Regulatory subunits (R) are expressed from four different genes -RIα, RIβ, RIIα, and RIIβ (Chrivia et al., 1988; Lee et al., 1983; Scott et al., 1987). The R subunit is a modular protein containing an NH_2-terminal homo-dimerization domain, an autophosphorylation site that serves as a principal contact site for the C subunit, and two cAMP binding sites. The primary activator of PKA is the second messenger cAMP (Su et al., 1995; Su et al., 1993). Binding of cAMP to the R subunits relieves the autoinhibitory contact, allowing the C subunits to dissociate and phosphorylate local substrates (Gibbs et al., 1992; Wang et al., 1991). Two forms of the heterotetrameric PKA holoenzyme exist, type I (RIα and RIβ dimer) and type II (RIIα and RIIβ dimer).

Type I PKA is predominantly cytoplasmic, whereas type II PKA associates with specific cellular structures and organelles (Scott, 1991).

One of the most fascinating and complex features of PKA signaling is that it can regulate a multitude of physiological processes. For example, in many neuronal cell types, PKA can regulate both gene expression in the nucleus and synaptic transmission by modifying the excitation state of ion channels at the synapse. This occurs, in part, because PKA has broad substrate specificity. In the example above, PKA alters gene expression by directly phosphorylating the transcription factor CREB in the nucleus (Gonzalez et al., 1989), and at dendrites PKA can phosphorylate and regulate the activation state of AMPA-type glutamate receptors (Banke et al., 2000). In fact, over 150 physiologically relevant PKA substrates have been identified (Shabb, 2001), however, the mechanism by which PKA distinguishes between substrates to elicit a given biological response continues to be the subject of intense investigation. There are two prevailing theories to explain how this is accomplished. First, it is possible that the cell can create localized gradients of cAMP that only activate a subset of PKA molecules (Beavo and Brunton, 2002). This has been demonstrated experimentally (Zaccolo and Pozzan, 2002) and may require the selective and localized activation of adenylate cyclases and phosphodiesterases, the enymes that catalyze cAMP metabolism. Another tenant of this hypothesis is that PKA must be compartmentalized, creating pools of active enzyme that are spatially restricted within the cell (Zaccolo and Pozzan, 2002). The second prevailing theory is that PKA can be physically coupled to its substrate, thus decreasing the likelihood of spurious phosphorylation events (Colledge and Scott, 1999).

Both of these theories are probably correct, and exciting new evidence suggests that these two types of regulation are not mutually exclusive. These topics will be discussed below.

A-Kinase Anchoring Proteins

Association with A-kinase Anchoring Proteins (AKAPs) leads to discrete localization of PKA within the cell. The first AKAPs were discovered as contaminants of type II PKA holoenzyme preparations (Rubin et al., 1979; Theurkauf and Vallee, 1982; Vallee et al., 1981) and the family has since grown to include over 20 members **(Table 1.1)**. With only one known exception, AKAPs contain an amphipathic helix that functions to interact with the binding surface formed by the amino termini of the PKA-RII dimer **(Figure 1.1)** (Carr et al., 1991; Diviani et al., 2000; Newlon et al., 1997). Solution structure analysis of the RII binding domain from two different AKAPs indicates that this interaction occurs through hydrophobic contacts (Newlon et al., 2001). While AKAPs share this functionally similar 14 to 18 amino acid domain, they are structurally diverse proteins that are found in various cell types and subcellular locations. All AKAPs contain targeting domains that localize PKA to different organelles and subcellualr structures **(Figure 1.1)** (Colledge and Scott, 1999). This provides a mechanism to control the intracellular localization of PKA. However, it is now clear that AKAPs can coordinate the assembly of signaling complexes by simultaneously tethering enzymes that can directly or indirectly regulate the phosphorylation state of a variety of substrates **(Figure 1.1)** (Colledge and Scott, 1999).

Table 1.1: AKAP Family Members

AKAP	Tissue/ Compartment	Features	Reference *
AKAP15/18	Plasma membrane Basalateral or apical targeting in epithelial cells	Targeted to plasma membrane via fatty acid modifications Modulation of L-type calcium channels Three isoforms differentially targeted basolaterally or apically	1, 2, 3
Ht31 AKAP-Lbc	Isolated from thyroid library cytoplasm	Peptide corresponding to RII binding site disrupts PKA anchoring in cells Contain Rho Gef domain Down stream of Gα12	4, 5, 6
AKAP75/79/150	Plasma membrane Postsynaptic density	Binds PKC and calcineurin (PP2B) Polybasic domains target to plasma membrane and dendrites Directly binds PSD-95 Interacts with beta 2-adrenergic receptor	7, 8, 9, 10
Ezrin	Actin cytoskeleton	Linked to CFTR via EBP50/NHERF	11
TAKAP80	Fibrous sheath of sperm tail	Testis-specific	12
AKAP82	Fibrous sheath of sperm tail	potential role in sperm capacitation Tyrosine phosphorylated	13, 14
AKAP95	Nuclear matrix	Zinc finger motif Role in chromosome condensation Binds AMY-1	15, 16
AKAP-KL Parralemmin	Kidney and lung Actin cytoskeleton Apical membrane of epithelial cells	Multiple splice variants	17, 18
AKAP220	Vesicles	Binds PP1 Binds and regulates GSK-3b	19, 20, 21
Gravin SSECKS	Actin cytoskeleton	Binds PKC interacts with beta 2-adrenergic receptor	22, 23
mAKAP	Cardiac and skeletal muscle and brain Nuclear membrane	Spectrin repeat domains involved in subcellular targeting Developmentally regulated Binds PDE4D3 Interacts with Ryanondine Receptor	24,25,26,27,
yotiao AKAP350	Postsynaptic density Neuromuscular junction Centrosomes-Golgi	Multiple splice variants Targets PKA and PP1 to NMDA receptor Binds hKCNQ1	28, 29, 30, 31,
S-AKAP84/ D-AKAP1 AKAP121/ AKAP149	Outer mitochondrial membrane Endoplasmic Reticulum	Dual specificity AKAP, binds RI and RII Multiple splice variants	32, 33,
D-AKAP2	Wide tissue distribution as assessed by Northern	Dual specificity AKAP, binds RI and RII	34
$AKAP_{CE}$	isolated from C. elegans expression library	binds RI-like C. elegans R subunit RING finger protein	35
DAKAP550 Neurobeachin	Identified by RII overlay of Drosophila embryos	Contains two RII binding sites Regulates membrane trafficking	36, 37,
Rab32	Mitochondria	Regulates mitochondrial dynamics	38
MTG16B, MTG8	Golgi	Found in mylogenic leukemia	39, 40
AKAP97	Found in radial spokes of Chlamydomonas flagella	Regulates flagellar motility	41
Pericentrin	Centrosome	Unique RII binding domain Interacts with dynein	42, 43
Wave1	Actin Cytoskeleton	Binds Arp2/3, Abl, Wrp, Nap125, PIR121 Down Stream of Rac signaling	44, 45, 46

1) (Fraser et al., 1998)
2) (Tibbs et al., 1998)
3) (Trotter et al., 1999)
4) (Carr et al., 1992a)
5) (Diviani et al., 2001)
6) (Rosenmund et al., 1994)
7) (Carr et al., 1992b)
8) (Klauck et al., 1996)
9) (Colledge et al., 2000)
10) (Fraser et al., 2000)
11) (Dransfield et al., 1997)
12) (Mei et al., 1997)
13) (Moss et al., 1999)
14) (Mandal et al., 1999)
15) (Eide et al., 1997)
16) (Furusawa et al., 2002)
17) (Dong et al., 1998)
18) (Hu et al., 2001)
19) (Lester, 1996)
20) (Schillace and Scott, 1999)
21) (Tanji et al., 2002)
22) (Nauert et al., 1997)
23) (Gelman et al., 1998)
24) (Kapiloff et al., 1999)
25) (Dodge et al., 2001)
26) (Marx et al., 2000)
27) (Kapiloff et al., 2001)
28) (Westphal et al., 1999)
29) (Witczak et al., 1999)
30) (Shanks et al., 2002)
31) (Marx et al., 2002)
32) (Huang et al., 1997b)
33) (Steen et al., 2000)
34) (Huang et al., 1997a)
35) (Angelo and Rubin, 1998)
36) (Han et al., 1997)
37) (Gilbert et al., 1999)
38) (Alto et al., 2002)
39) (Schillace et al., 2002)
40) (Fukuyama et al., 2001)
41) (Gaillard et al., 2001)
42) (Diviani et al., 2000)
43) (Purohit et al., 1999)
44) (Westphal et al., 2000)
45) [Soderling, 2002 #2790]
46) (Eden et al., 2002)

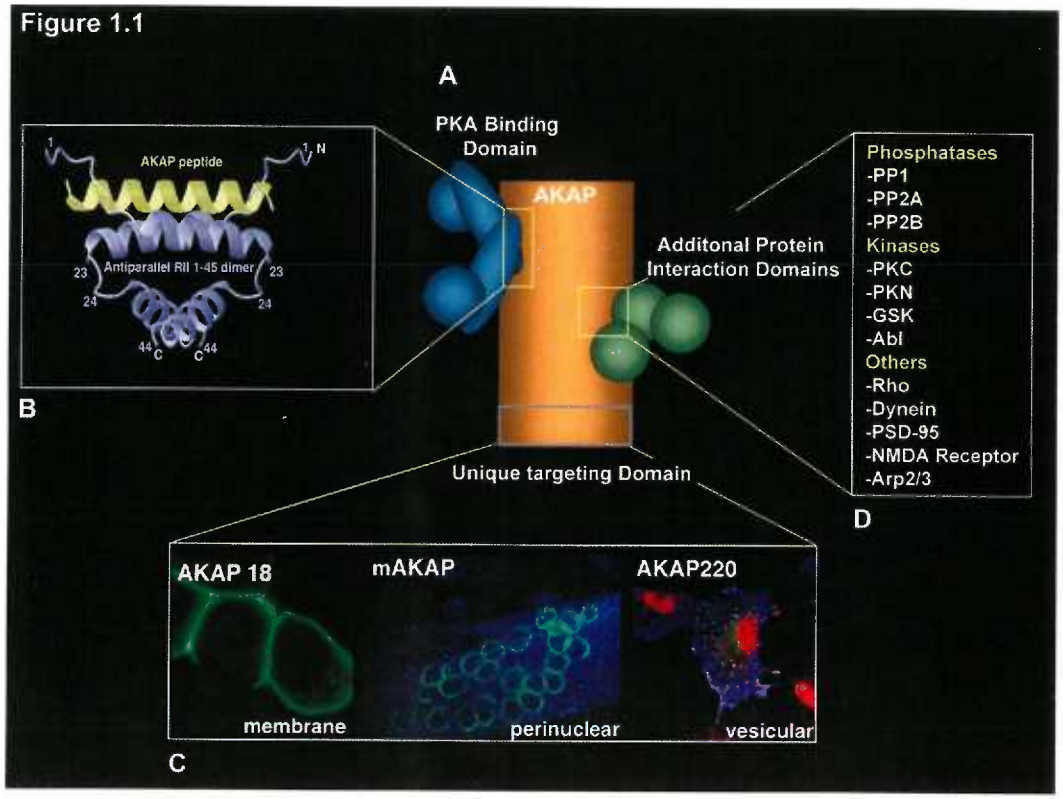

Figure 1.1: Functional domains of AKAPs

A) At least three distinct functional domains are found on all AKAPs. These include an RII binding domain that mediates high affinity interaction with the PKA holoenzyme, a unique intracellular targeting domain, and one or more protein-protein interaction motifs.
B) The RII binding domain of AKAPs is formed by a 14-18 amino acid region that folds into a well ordered amphipathic α-helical structure (Hausken et al., Newlon et al.). The amphipathic helix directly interacts with the dimerization domain of the type-II regulatory subunit (RII) of PKA. This high affinity interaction is mediated by hydrophobic contacts along the surface of both proteins. This image is a modification of that found in Newlon et al.
C) AKAPs have a unique targeting domain that is required for the localization of PKA to distinct regions of the cell. For example, AKAP18 is targeted to membranes through N-terminal lipid modifications (Fraser et al.), mAKP is localized to the perinuclear region of the cell through spectrin repeat domains (Kapiloff et al.), and A KAP220 is found vesicularly through a yet unidentified targeting motif (Shillace et al.) Images presented here were adapted from those found in College et al.
D) AKAPs are modular proteins that contain many protein-protein interaction motifs. Phosphatases are localized to AKAPs for the regulation of many bi-directional phospho-transfer reactions. Phosphodiesterases may control the levels of local cAMP gradients. Additional kinases are found associated with AKAPs for the purpose of integrating the cAMP second messenger system with other second messenger systems. Additional proteins are found associated with AKAPs bringing together diverse cell biological processes and cAMP signaling. Please see Table 1.1 for references and details of these types of interactions.

PKA Anchoring and Cellular Physiology

Insights into the spatial and temporal regulation of PKA has spurred the acceptance of the "anchoring hypothesis" to explain the multiple roles of this kinase in physiology. One tenant of the anchoring hypothesis is that PKA signaling specificity is achieved through AKAP-mediated compartmentalization of the kinase. An early demonstration the importance of PKA anchoring came from studies of the AMPA-type glutamate receptor in hippocampal neurons. PKA can directly phosphorylate the AMPA-channel at a defined serine, serine 845, leading to a potentiation of the channel (Blackstone et al., 1994; Roche et al., 1996). Rosenmund et al. demonstrated that this type of regulation requires AKAP mediated targeting of PKA to the AMPA channel (Rosenmund et al., 1994). They found that disrupting anchored PKA with a peptide that competitively displaces the kinase from AKAPs leads to dephosphorylation of serine 845 and subsequent time-dependant decrease in peak current amplitude. Since this initial study, other investigators have performed experiments to suggest that AKAP79 is the endogenous AKAP that is responsible for anchoring PKA and the phosphatase PP2B, to the AMPA-receptor (Colledge et al., 2000; Tavalin et al., 2002). These studies have implicated AKAP79 in such processes as Long Term Depression (LTD) (Tavalin et al., 2002), a state of synaptic plasticity that serves as a model for studying the formation and storage of memories in the human brain.

Since the initial use of Ht31 as an inhibitor of PKA-AKAP interactions *in vivo*, analogous experiments have been performed to implicate AKAPs in numerous physiological processes. For example, PKA anchoring is required for normal contractility of cardiac myocytes upon stimulation of the β-adrenergic receptor (Fink et

al., 2001). In addition, PKA anchoring is necessary for the cell surface expression of the water channel, aquaporin-2, in renal collecting duct principal cells (Klussmann et al., 1999). Finally, the role of AKAPs in animal behavioral models is now being tested. Moita et al. infused a cell permeable Ht31 into the amygdala of adult rats prior to a fear conditioning protocol. They found AKAP-mediated anchoring in the lateral amygdala is necessary for the consolidation, but not acquisition, of conditioned fear (Moita et al., 2002). Together, these examples highlight the importance of using AKAP derived reagents to further define the mechanisms that regulate cAMP signaling events in cellular and animal models.

AKAPs Localize PKA to cAMP Gradients.

While it is clear that AKAP mediated PKA anchoring is a physiologically important process, the consequences of anchoring at the molecular level are only now beginning to be appreciated. For example, AKAPs can directly link PKA to upstream or down stream regulatory proteins (Dodge et al., 2001; Fraser et al., 2000). A connection between PKA and the cAMP degrading enzyme, phosphodiesterase, can be made by various AKAPs (Dodge et al., 2001; Tasken et al., 2001). This could ensure that the kinase is transiently exposed to cAMP gradients and may provides a mechanism to favor the efficient catalytic activation of the kinase. Interestingly, this type of regulation has been elegantly described for two different AKAPs, and in a unique study using chimeric proteins. Dodge et al. found that the muscle-selective mAKAP directly binds PKA and a splice variant of the cAMP-specific, type 4 phosphodiesterase, PDE4D3 (Dodge et al., 2001). Subsequently, Tasken et al. reported the interaction of PDE4D3 with AKAP450, a large

centrosomal AKAP found in Sertoli cells (Tasken et al., 2001). Both studies suggest that the role of PDE4D3 within these complexes is to depress cAMP levels within the vicinity of anchored PKA. At rest, PDE4D3 inhibits basal PKA activity associated with mAKAP, possibly acting to dampen noise and increase gain in the system. Furthermore, PKA phosphorylation is known to up-regulate PDE4D3 activity two- to threefold, establishing a negative feedback loop that rapidly terminates the cAMP signal.

Recently, Zaccolo and Pozzan demonstrated that pools of cAMP generated through a specific G-protein couple receptor could selectively activate anchored PKA (Zaccolo and Pozzan, 2002). This group developed a chimeric cAMP reporter system by which the catalytic subunit of PKA is fused to yellow fluorescent protein (YFP) and the Type II regulatory subunit is fused to cyan fluorescent protein (CFP). Under basal conditions, these chimeras undergo Fluorescent Resonance Energy Transfer (FRET). FRET occurs upon excitation of the donor CFP with 440nm wavelength of light, part of the excitation energy is transferred to the acceptor YFP, and the YFP emits 545nm light. Detection of the excited YFP indicates that the donor and acceptor molecules are with in 50nm of each other, and in the case of PKA, the C subunit and R subunit form the inactive heterotetramer. Expression of the cAMP reporter chimera in cardiac myocytes, a cell type that is highly regulated by cAMP, resulted in FRET detection throughout the T-tubule system. This indicates that the chimeric proteins are anchored to the T-tubules and they form the heterotetrameric holoenzyme complex. Upon activation of the $G\alpha_s$ coupled β-adrenergic receptor (β-AR), a loss in FRET signal, thus PKA activation, was observed. The crux of these experiments came when the investigators deleted the AKAP binding domain of the RII-CFP chimera, effectively creating a PKA molecule that could not

target to the t-tubules via AKAPs. They found no detectable loss of FRET upon β -AR stimulation under these conditions. Together, these data suggest that β-AR stimulation in cardiac myocytes leads to a compartmentalized increase of cAMP at the vacinity of the t-tubules, thus allowing localized activation of PKA.

AKAPs directly couple signal transduction complexes to down stream effectors.

A newly recognized duty for AKAPs is to coordinate signaling complexes by recruiting multiple signaling enzymes near potential substrates. This effectively bridges the gap between upstream activators and downstream targets. AKAP79, AKAP220, and yotiao have already been shown to function in this capacity (Colledge et al., 2000; Fraser et al., 2000; Tanji et al., 2002; Westphal et al., 1999). As discussed above, AKAP79 links PKA directly to the AMPA-type glutamae receptor. Recently it was demonstrated that AKAP220 interacts with GSK-3beta and promotes its phosphorylation by PKA. Finally, in neurons, yotiao targets both PKA and the phosphatase PP1 to the C1 exon-containing NR1 subunit of the NMDA receptor (Lin et al., 1998; Westphal et al., 1999). The localized, active, PP1 maintains the NMDA receptor in a depressed state, and upon cAMP elevation and subsequent PKA activation, the NMDA receptor current is increased by 55%. These examples highlight a growing theme in signal transduction: multiple kinases, phosphatases, and signal transduction enzymes can be directly tethered to downstream targets (see Figure 1.1).

Directly tethering kinases to their substrates may impart both spatial and temporal specificity to a signal transduction system. This idea was tested using a variation of the FRET technology described above. Instead of developing a cAMP reporter, Zhang et al.

designed a PKA phosphorylation indicator (Zhang et al., 2001). They built a chimeric protein consisting of a CFP molecule followed by the phospho-amino acid binding region of 14-3-3, a PKA substrate peptide, and a YFP molecule. PKA phosphorylation of the substrate peptide results in an intramolecular interaction between the 14-3-3 module and the phospho-amino acid. This brings the CFP and YFP moieties within a distance to detect a FRET signal. In vivo, direct activation of PKA leads to a rapid and reversible increase in FRET. Interestingly, if PKA is targeted to this chimera by introducing an RII-binding domain from a known AKAP, the time course for phosphorylation of the reporter is significantly shortened. This occurs as a direct result of coupling PKA to the substrate within the chimera. Together, these results suggest that AKAPs may not only allow spatial restriction of the enzyme, but may impart temporal control of the system.

Conclusion:

It is now clear that AKAPs can localize PKA in close proximity to physiological substrates and to areas where PKA can be modulated by localized cAMP pools. Furthermore, recruitment of phosphatases is critical for the bidirectional phosphotransfer reactions on substrates. In addition to phosphatase binding, AKAPs can interact with enzymes that are not necessarily linked to cAMP signaling. This suggests that AKAPs provide a platform to integrate multiple signal transduction inputs to coordinate a physiological response. Many challenges still remain for the AKAP field. First, it is imperative that all AKAPs are identified. This is a difficult problem because there has not yet been a way to search computer databases for novel AKAPs. However, with more advanced bioinformatic software and the ability to screen entire genomes, these

approaches may yet be possible. Second, it will be important to distinguish PKA signaling events that are anchoring-dependent versus those that are not. This will include the identification of PKA anchoring-dependent substrates and determining the physiological processes mediated by anchored PKA. Third, identifying the full complement of AKAP binding proteins will be indispensable for piecing together the integration of signal transduction pathways mediated by AKAPs. For these types of experiments, the existence of gene knockouts and specific inhibitory reagents will be useful.

Topic 2:

Rab proteins as mediators of Intracellular Membrane Dynamics

Introduction

The Ras-superfamily of small GTP binding proteins (small G-proteins) are a unique class of signal transduction enzymes. As their name implies, small G-proteins can alternately bind guanosine diphosphate (GDP) and guanosine triphosphate (GTP) and hydrolyze GTP to GDP (Takai et al., 2001). They are small in size compared to other G-proteins and range from about 20 to 40 kilo-daltons. The Ras-superfamily consists of a large number of proteins with more then 100 members identified in eukaryotic cells (Bock et al., 2001). Each member is categorized into one of five distinct families that include the Ras, Rho , Ran, Arf/Sar, and the Rab families. Classification of each small G-protein is based on sequence homology and in most cases family members regulate a similar biological process. For example, members of the Rho-family participate in the assembly and disassembly of the actin cytoskeleton and are involved in cell motility (Hall, 1998), while Ran family members regulate nucleo/cytoplasmic trafficking (Melchior and Gerace, 1998; Moore and Blobel, 1993).

The Rab family (Ras genes from rat brain (Touchot et al., 1987)) constitutes the most numerous branch of small G-proteins (Bock et al., 2001). They are found in all eukaryotic organisms; however, their total number varies significantly depending on the evolutionary complexity of each genome. There are 11 Yeast, 29 Drosophila, and 60 human Rabs identified to date (Bock et al., 2001; Pereira-Leal and Seabra, 2001). Research on these proteins has steadily increased over the past decade and now there are

over one thousand entries in the PubMed online publications database. The majority of these studies suggest that Rabs are key regulators of intracellular membrane trafficking events **(Table 1.1)**. They have roles in vesicle docking and fusion, membrane budding, and cytoskeletal trafficking (Novick and Zerial, 1997). Surprisingly, very few members of the mammalian Rab family have been characterized. Rather, intensive research is focused on elucidating the functions of a few Rab proteins such as Rab1, Rab3, Rab5, Rab6, and Rab9. Importantly, these studies have revealed many aspects of general Rab function and now serve as models by which all newly studied members are compared.

Structural Features of Rabs

Members of the Rab family share structural features that classify them as small G-proteins (Pereira-Leal and Seabra, 2000). All small G-proteins share a similar structure with an arrangement of six central strands of β-sheet and five α-helical regions (Ostermeier and Brunger, 1999). They are approximately 200 amino acids in length and have a similar domain organization. Each contains five guanine nucleotide-binding loops, a GTPase domain, effector loops, and C-terminal cysteines that are sites of lipid modification **(Figure 1.2A)**. These common domains are the basis by which the proteins have biological activity. For instance, they can transiently associate with either GDP or GTP (Novick and Zerial, 1997). The GDP bound state is considered to be inactive and the exchange of GDP for GTP is the activation trigger. All Rabs have a GTPase domain that catalyzes the hydrolysis of bound GTP to GDP. This enzymatic reaction results in the inactivation of Rabs. While these general features are required for the activity of all small G-proteins, Rabs contain unique regions that distinguish them from the other

Table 1.3: Rab Family Members

Rab	Membrane Localization	Features	References *
Rab1a, b, c	Endoplasmic reticulum	ER to Golgi Trafficking Interacts with p115 and GM130	1, 2, 3
Rab2	Endoplasmic reticulum	ER to Golgi Traffic Interacts with GRASP55	4, 5
Rab3a, b, c, d	Synaptic vesicles, Granules	Exocytosis of vesicles	4
Rab4	Early endosomes	Endosomal Trafficking	4
Rab5 a, b, c	Endosomes	Endocytosis, Best characterized Rab protein to date GAP, GEF, and Effectors identified	4, 6
Rab6	Golgi	Retrograde trafficking golgi to ER Intra-golgi trafficking Interact with Rabkinesin-6 for Cytoskeletal traffic	7, 8, 9
Rab7	Early endosome	Endocytic trafficking to late endosome Endosome to Golgi trafficking	4
Rab8	Trans-golgi network	Exocytic transport from trans-golgi Interacts with Fip-2 linked to Huntingtin	4, 10
Rab9	Late endosomes	Endosome to Trans-golgi network trafficking M6PR trafficking from endosome to golgi, Recruits TIP47	11,12
Rab10	Perinuclear membranes	Unknown, Localization of expression only	13
Rab11	Recycling endosome	Recycling through recycling endosome Many effector proteins	14, 15
Rab12	Secretory vesicles	Found in atrial myocytes	16
Rab13	Tight Junctions of Epithelial cells	Remodels tight junctions	17
Rab15	Early endosome	Regulates early endosome Trafficking	18
Rab17	Recycling endosome	Epithelial cell specific	4
Rab18	Apical and basal localized endosomes	Endosomal Trafficking	18
Rab19	Unknown	Unknown	19
Rab20	Apical localized, endosomes	Endosomal Trafficking	19
Rab21	Endoplasmic Reticulum	Found in Caco-2 cells	20
Rab22	Endosome	Endosome trafficking Interacts with EEA-1	21, 22
Rab23	Unknown	Negative regulator of sonic hedgehog signaling	23, 24
Rab24	Late Endosome	Unknown	4
Rab25	Recycling endosomes	Apical recycling in epithelial cells	25, 26
Rab26	Secretory granules	Unknown	27
Rab27a, b	Pigment granules, melanosomes	Recruits Myosin Va to melanosomes Melanophilin linker protein	28, 29, 30
Rab32	Mitochondria	Regulates mitochondrial fission Interacts with PKA	31
Rab36	Golgi	Unknown	32
Rab37	Secretory granules	Mast cell specific	33

* Corresponding references found on next page

Table 1.3: Corresponding References

1) (Wilson et al., 1994)
2) (Alvarez et al., 1999)
3) (Moyer et al., 2001)
4) (Takai et al., 2001)
5) (Bacskai et al., 1993)
6) (Gorvel et al., 1991)
7) (Martinez et al., 1994)
8) (Martinez et al., 1997)
9) (Echard et al., 1998)
10) (Hattula and Peranen, 2000)
11) (Barbero et al., 2001)
12) (Carroll et al., 2001)
13) (Chen et al., 1993)
14) (Ullrich et al., 1996)
15) (Hales et al., 2001)
16) (Iida et al., 1996)
17) (Marzesco et al., 2002)
18) (Zuk and Elferink, 1999)
19) (Lutcke et al., 1994)
20) (Opdam et al., 2000)
21) (Mesa et al., 2001)
22) (Kauppi et al., 2002)
23) (Olkkonen et al., 1994)
24) (Eggenschwiler et al., 2001)
25) (Goldenring et al., 1993)
26) (Goldenring et al., 2001)
27) (Yoshie et al., 2000)
28) (Hume et al., 2001)
29) (Seabra et al., 2002)
30) (Strom et al., 2002)
31) (Alto et al., 2002)
32) (Mori et al., 1999)
33) (Masuda et al., 2000)

Figure 1.2

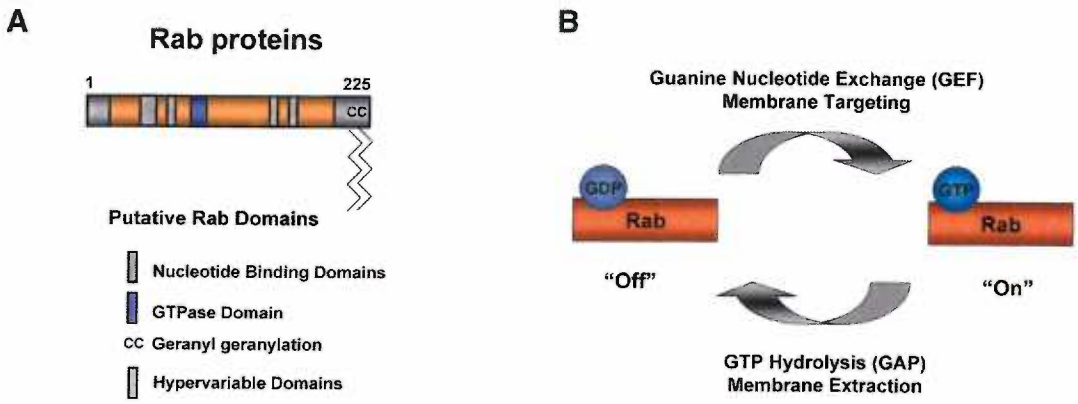

Figure 1.2: Rab small G-proteins and the Rab cycle

A) Rab small G-proteins are 180-230 amino acid proteins that share many conserved domains. Each Rab protein has four nucleotide binding domains that coordinate the interaction with one molecule of GDP or GTP. They have a conserved GTPase domain made up of five amino acids. Two C-terminal cysteines can be lipid modified, typically by geranyl-geranlylation, and mediate the retention of each Rab protein to the membrane of a specific organelle. In addition, each Rab protein has a non -conserved N- an C-terminal hypervariable domain that serves an unknown function.

B) The Rab cycle: Functionality of each Rab protein is mediated by the cycle of nucleotide exchange and hydrolysis and the accompanying membrane targeting and extraction. Rabs are found in there inactive ("off"), GDP bound form in the cytosol. Activation occurs upon nucleotide exchange mediated by a cofactor guanine nucleotide exchange factor (GEF). The nucleotide exchange reaction is accompanied by specific membrane targeting of the Rab protein. At the membrane, GTP bound Rabs are considered "on" and can recruit effector protein complexes, thus eliciting a biological response. Inactivation of Rabs occurs upon GTP hydrolysis stimulated by a GTPase activating protein (GAP). GTP is hydrolyzed to GDP and the subsequent GDP bound Rab is extracted from the membrane and returned to the cytosol.

families. First, they often have two rather that one C-terminal cysteine (Takai et al., 2001). This allows a specific Rab geranyl-geranyltransferase to prenylate the Rab proteins (Seabra, 1998). In addition, Pereira and Seabra identified five unique domains using a statistical algorithm to predict consensus sequences. They found five conserved motifs that are unique to the Rab family and have used these as a criterion to place new members into the family (Pereira-Leal and Seabra, 2000). Although these domains help identify candidate family members, their functions are still unknown. Interestingly, all Rab family members seem to have N-terminal and C-terminal hypervariable domain of 10 to 20 amino acids. The C-terminal domain is thought to be necessary for the correct targeting of the Rab proteins to their receptor membrane (Chavrier et al., 1991). The combination of conserved and unique domains is required to generate functional specificity for each Rab protein.

Function of Rab proteins:

Rab proteins regulate many aspects of organelle function through their ability to mediate membrane dynamic events. At least five general events are ascribed to Rab regulation. Rabs may be involved in vesicle formation. It was shown that Rab1 is required for the formation of Endoplasmic Reticulum (ER) derived vesicles for ER to golgi transport (Chavrier et al., 1991). They can participate in membrane remodeling, mainly through the regulation of phospholipid interactions or kinases that modify specific lipids (Christoforidis et al., 1999b; Simonsen et al., 1998; Wurmser and Emr, 1998). Recent data suggests that Rabs are necessary for vesicle motility. For example, Rab6 can directly interact with a kinesin-motor protein family member (Echard et al., 1998) and

Rab27a regulates melanasome transport along the actin cytoskeleton in melanocytes (Strom et al., 2002). Finally, Rabs have been implicated in both vesicle docking and fusion reactions. Two good examples exist for the role of Rabs in vesicle docking. First, Rab1 directly recruits the golgi tethering factor p115 to CopII vesicles (Allan et al., 2000). Second, Rab5 recruits the tethering factor EEA1 to early endosomes for homotypic fusion of this organelle (Christoforidis et al., 1999a). In addition, Rabs can recruit SNARE proteins for vesicle fusion. The mechanisms by which this occurs is controversial, however it is clear that formation of proteins complexes involved in fusion are regulated by the Rab proteins (McBride et al., 1999).

The Rab Cycle

Three major processes govern the function of Rab proteins: 1) Rabs fluctuate between their GTP- and GDP-bound forms, 2) Rabs cycle between a cytosolic and membrane localization, and 3) Rabs recruit effector proteins to the membranes at which they are localized (Novick and Zerial, 1997). Each of these activities is temporally arranged in what is known as the Rab cycle **(Figure 1.2B)**. Not surprisingly, accessory proteins that stimulate each reaction regulate each step of the Rab cycle. The following sections will elaborate on each step of the Rab cycle: activation, effector protein binding, and inactivation.

The Rab Cycle: Activation

According to current models, three steps are necessary for the activation of Rab proteins (Novick and Zerial, 1997). In the cytosol, Rabs are present in a GDP-bound

complex with an accessory protein known as GTPase dissociation inhibitor protein (GDI) (Luan et al., 2000). The function of this complex is to maintain Rab proteins in their GDP-bound state and to sequester them in the cytoplasm by masking the C-terminal lipid moieties. The first step of activation occurs upon relieving Rab inhibition through the displacement of GDI. This may occur through its interaction with a membrane bound GDI dissociation factor (GDF) (Dirac-Svejstrup et al., 1997). Second, a guanine nucleotide exchange factor (GEF) stimulates the exchange of GDP for GTP. The GTP-bound Rabs seem to be active or in the "on" form. Surprisingly, there are only two identified GEF for mammalian Rab proteins (Dirac-Svejstrup et al., 1997; Horiuchi et al., 1997) and thus it is not clear if all Rabs use a small number of GEFs for their activation or if many GEFs remain to be identified. Third, Rabs must be targeted to a specific membrane compartments. The mechanism by which Rabs are recruited to these compartments is unknown, however they are tethered to the cytosolic side of a membrane through two lipid moieties (Seabra, 1998). Thus, Rab activation occurs upon release of GDI in the cytoplasm, GEF stimulated nucleotide exchange, and subsequent membrane targeting. The exact order of events is not clear, however each step is crucial for activation of Rab proteins.

The Rab Cycle: Interaction with effector proteins

Rabs can transmit signals to downstream effectors in a GTP-dependent manner, activating and recruiting effectors to sites of action. Upon the analysis of these effector proteins, two emerging themes are being realized. Recent data suggests that a single activated Rab protein can selectively bind to a multitude of effector proteins to facilitate

discrete steps in membrane transport (Takai et al., 2001). In the most extreme example, Rab5 has been shown bind 22 proteins in its GTP bound form (Christoforidis and Zerial, 2000). Secondly, although each Rab protein is highly conserved, the effector sequences for individual Rabs are unrelated (Takai et al., 2001). This suggests that each Rab protein regulates an aspect of membrane dynamics through the recruitment of unique effector proteins to those membranes.

Rab effector proteins identified thus far display an amazing array of form and function. They range from proteins with enzymatic activity such as kinases and motor proteins to non-catalytic proteins involved in vesicle docking and fusion (Allan et al., 2000; Christoforidis et al., 1999b; Echard et al., 1998; McBride et al., 1999; Ren et al., 1996). One common thread is that each effector plays a unique role in a membrane dynamic event. This is best illustrated for the Rab5-mediated assembly of molecules that regulates homotypic fusion of endosomes (Bucci et al., 1992). At the early endosome, active Rab5 recruits the tethering factor EEA1 from the cytosol to the membrane (Christoforidis et al., 1999a). In addition to recruiting EEA1, Rab5 also recruits a lipid kinase known as PI3K to these sites (Christoforidis et al., 1999b). Interestingly, EEA1 is stabilized at the endosome through a unique phospho-lipid interaction that is instigated by the activity of localized PI3K (Simonsen et al., 1998). Thus, it is likely that PI3K is recruited to the endosome for membrane remodeling, in turn, allowing phospholipids to stabalize EEA1. Down stream of Rab5 interaction, EEA1 can recruit SNARE proteins, specifically syntaxin 13, and the priming factor NSF to the protein complex (McBride et al., 1999). In this example, Rab5 recruits two unique effector proteins to the endosomal

membrane, which initiates the formation of a multi-protein complex with all of the factors required for tethering and fusion.

Other examples exist that illustrate the diversity of Rab function through the recruitment of unique effector proteins. Rab proteins can regulate vesicle motility by coupling motor proteins to specific organelles. This was first described for Rab6 in which a kinesin-like protein (Rabkinesin-6) was identified as a direct effector protein (Echard et al., 1998). Rab6 regulates retrograde trafficking of vesicles from the Golgi to the endoplasmic reticulum (White et al., 1999). These authors hypothesized that Rab6 recruits the motor proteins kinesin to the Golgi-derived vesicles and thus stimulated their retrograde trafficking. Another well-characterized example of Rabs coupling vesicles to cytoskeletal transport is the recruitment of myosin-Va to melanosomes. Myosin-Va is an actin directed motor protein that transports pigment granules from melanocytes to keritinocytes, a cell type that makes up the majority of skin and hair (Hume et al., 2001). This transport is bidirectional upon microtubules, and peripheral distribution of the melanosome is instigated by a Myosin-Va dependent capture. It was found that mutations in the Rab27a gene in both *ashen* mice and humans with Griscelli disease result in a perinuclear distribution of melanocytes (Schuster et al., 2001; Wilson et al., 2000). From this, researchers have determined that Rab27a is a melanosome membrane protein that acts as a myosin-Va receptor. Together, these results suggest that recruitment of effector proteins by Rabs is a physiologically important process for membrane trafficking events.

The Rab Cycle: Inactivation

The inactivation of Rabs occurs through both GTP hydrolysis and membrane extraction of the Rab protein. Under steady state conditions, Rabs have very low intrinsic GTPase activity that can be stimulated by a specific GTPase activating protein (GAP) (Novick and Zerial, 1997). GAP stimulated nucleotide hydrolysis allows the release of effector proteins and marks the end of a membrane dynamic event. The full Rab cycle is completed when GDP-bound Rabs are extracted from membranes and returned to the cytosol by a soluble GDI protein.

The GTP turnover of Rabs is quite important for the termination of membrane fusion events. This has been demonstrated for multiple Rab proteins whereby cellular expression of GTPase deficient mutants leads to a perturbation in organelle size or function. Expression of GTPase dead Rab5 mutants causes an increase in endosomal fusion and subsequent growth of the organelle (Vitale et al., 1995). Furthermore, analogous Rab6 mutants increase the rate of ER to golgi trafficking, suggesting that Rab6 plays a direct role in this process (Wilson et al., 1994). It is important to note that GTP hydrolysis may not always lead to the membrane extraction and inactivation of Rab proteins. In fact, it is possible that Rabs undergo multiple rounds of nucleotide exchange and hydrolysis throughout the course of a membrane dynamic event (Christoforidis et al., 1999a; Christoforidis et al., 1999b). This may allow the recruitment of multiple effector proteins for the assembly of fusion machinery. Thus, as with Rab activation and effector binding, Rab inactivation is a very important aspect of the Rab cycle and is a tightly regulated process.

Conclusion:

Clearly, there has been groundbreaking work performed on Rab proteins. The structural analysis and comparisons have aided in identifying numerous Rab family member genes and defining key regulatory elements that are required for their functions *in vivo*. Furthermore, it is now clear the cycle of GDP/GTP exchange, membrane targeting, effector protein recruitment, and GTP hydrolysis is a highly regulated process that is essential for membrane trafficking events. There are many challenges that remain for the Rab field. For the well described Rabs, a major challenge is to determine how these membrane trafficking events are coordinated with cellular events such as cell growth and differentiation, cytoskeletal rearrangement, gene regulation etc. Furthermore, it remains to be determined if extracellular signal transduction pathways feed into Rab regulation. For example, how are GEFs and GAPs activated and what effect might this have on Rab regulated membrane trafficking. Additionally, are there second messenger systems that simulate their activity. Another major challenge is to describe the cellular roles of the uncharacterized Rab proteins. Less then half of the Rab proteins in the human genome have been characterized. Surely each Rab is going to have unique features that are specific to the process that it regulates. Because of the models that have been presented for a few Rabs, the tools are now available to determine the function of these orphan Rab proteins.

CHAPTER TWO

Bioinformatic design of AKAP "*in-silico*": A potent and selective peptide antagonist of type II Protein Kinase A Anchoring.

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Abstract

Compartmentalization of the cAMP dependent protein kinase (PKA) is coordinated through association with A-kinase anchoring proteins (AKAPs). A defining characteristic of most AKAPs is a 14-18 amino acid sequence that binds to the regulatory subunits (RI or RII) of the kinase. Cellular delivery of peptides to these regions disrupt PKA anchoring, and have been used to delineate a physiological role for AKAPs in the facilitation of certain cAMP responsive events. Here we describe a bioinformatic approach that yielded an RII selective peptide, called AKAP "*in silico*" (AKAP-*IS)* that binds RII with a KD of 0.4 nM and binds RI with a KD of 277 nM. AKAP-*IS* associates with the type II PKA holoenzyme inside cells and will displace the kinase from natural anchoring sites. Electrophysiological recordings indicate that perfusion of AKAP-*IS* evokes a more rapid and complete attenuation of AMPA receptor currents than previously described anchoring inhibitor peptides. Thus, computer based and high-throughput screening approaches have generated a novel reagent that binds PKA with higher affinity than previously described AKAP peptides.

Introduction.

The intracellular transduction of signals from the plasma membrane to cellular compartments evokes a variety of physiological responses. Perhaps the most rigorously studied signaling pathway utilizes the ubiquitous second messenger cAMP (Sutherland, 1972). Engagement of heptahelical receptors and the recruitment of intermediatory G-proteins activate adenylyl cyclases on the inner face of the plasma membrane (Lefkowitz, 1998; Smith and Scott, 2002; Taussig and Gilman, 1995). This molecular chain of events triggers an elevation of cAMP in certain intracellular compartments where it activates molecules such as cyclic-nucleotide gated ion-channels, guanine nucleotide exchange factors (EPACs), cyclic-nucleotide phosphodiesterases and cAMP-dependent protein kinases (PKA). These cAMP responsive enzymes propagate disparate intracellular events including, the excitation of olfactory neurons, control of certain MAP kinase cascades and a plethora of phosphorylation events catalyzed by PKA (de Rooij et al., 1998; Kaupp and Seifert, 2002; Pearson and Cobb, 2002; Shabb, 2001).

PKA is the predominant intracellular receptor for cAMP. In its dormant form the PKA holoenzyme consists of two catalytic (C) subunits held in an inactive conformation by a regulatory (R) subunit dimer (Taylor et al., 1990). Multiple C subunits (Cα, Cß and Cγ) and R subunits (RIα, RIß, RIIα, and RIIß) have been identified (Scott, 1991). Binding of cAMP to the R subunits causes the dissociation of the C subunits and the concomitant phosphorylation of target substrates within the vicinity of the kinase. Several regulatory mechanisms control the spatial and temporal activation of PKA. Elegant fluorescent imaging techniques have detected intracellular gradients and nano-

compartments of cAMP, formed by the opposing actions of adenylyl cyclases and phosphodiesterases (Barsony and Marks, 1990; Zaccolo and Pozzan, 2002; Zhang et al., 2001). These local fluctuations in cAMP influence where and when the kinase becomes active. Furthermore, spatial restriction of PKA is achieved through association with A-kinase anchoring proteins (AKAPs). AKAPs represent a group of functionally related proteins, classified by their ability to interact with PKA inside cells (Colledge and Scott, 1999). Early on, most AKAPs were identified by a solid-phase overlay procedure and thought to interact exclusively with RII (Bregman et al., 1989; Carr et al., 1993; Carr et al., 1992a; Carr et al., 1992b; Lohmann et al., 1984). More recently, two-hybrid screening and affinity purification techniques have identified dual function anchoring proteins that can interact with RI or RII (Huang et al., 1997a; Huang et al., 1997b; Reinton et al., 2000). In a few instances, RI selective AKAPs have been reported (Angelo and Rubin, 1998; Kussel-Andermann et al., 2000; Li et al., 2001).

A defining characteristic of most AKAPs is a 14-18 amino acid sequence that binds to the R subunit dimer (Carr et al., 1992a; Carr et al., 1991; Glantz et al., 1993; Hausken et al., 1996). Peptides encompassing this region are effective antagonists of PKA anchoring inside cells and are routinely used to demonstrate a role for AKAPs in the coordination of cAMP responsive events (Fink et al., 2001; Klussmann et al., 1999; Lester et al., 1997; Moita et al., 2002; Rosenmund et al., 1994; Vijayaraghavan et al., 1997; Westphal et al., 1999). Structural studies on two such AKAP peptides indicate that this region folds to form an amphipathic helix that slots into a binding pocket formed by the amino terminal regions of each RII protomer (Newlon et al., 2001; Newlon et al.,

1999). Nonetheless, individual AKAPs bind RII with dissociation constants (K_D) ranging from 2 to 90 nM, which reflects the diversity of sequences that form these "PKA anchoring-regions" (Carr et al., 1992a; Feliciello et al., 1999; Herberg et al., 2000; Nauert et al., 1997). Therefore, we initiated a comprehensive analysis of multiple AKAP sequences in an attempt to define a consensus PKA anchoring motif. Through a combination of bioinformatics, high-throughput screening of peptides arrays and the RII overlay procedure we have designed a high affinity RII-selective binding peptide that we have named AKAP-"*in silico*" (AKAP-*IS*).

Experimental Procedures

Autospot peptide array: Peptide arrays were synthesized on cellulose paper using an Auto-Spot Robot ASP222 (ABiMED) as previously described (Frank, 1992).

RII overlay: Proteins were seperated on SDS-PAGE and electro-transferred to a nitrocellulose membrane. Membranes were blocked in 1% Blotto (5% nonfat milk, 1% BSA). RII overlays were conducted as previously described using [^{32}P]-labeled recombinant murine RIIα [Hausken, 1998 #1673].

MEME Software: MEME software was used for consensus sequence generation, and is a free application found on the world wide web at http://www.meme.sdsc.edu (Grundy et al., 1997). MEME setting included one motif per sequence and a motif length of 17 was specified.

Fluorescence Polarization: FITC-Labeled peptides (Cell Essentials) were used for fluorescence polarization studies: AKAP-*IS* (AMAQIEYLAKQIVDNAIQQAKA), scrambled peptide (AMAQDVEIQLKAAYNQKLIAIA) and Ht31 (AADLIEEAASRIVDAVIEQVKA). Peptides (1 nM for RI experiments and 0.1 nM for RII experiments) were suspended to working dilutions in Phosphate Buffered Saline (PBS), containing 5ug/ul of BSA pH 7.0. Increasing concentrations of recombinant bovine RIα (provided by Dr Kjetil Tasken) or recombinant murine RII-α were added to a PBS solution and mixed with each FITC labeled peptide. Each sample was incubated for 10 minutes. Fluorescence polarization was measured on Beacon 2000 (Panvera)

following manufacturers instructions. Saturation binding curves were generated with Prism graphing software.

Molecular Modeling of Peptides: Predictions of the intrinsic α-helical content of the Ht31 and AKAP-*IS* peptides were performed with the latest version of the predictive algorithm, AGADIR (Fisinger et al., 2001). The solution structure of the Ht31-RIIα was used as a template for comparative modeling of the structure of the AKAP-*IS*-RIIα complex (Newlon et al., 2001). The structural model was refined using the Swiss-Model and Swiss-PDB Viewer (Glaxo Wellcome) programs.

Recombinant DNA constructs:

Oligonucleotides with the following sequence were annealed and directionally cloned into the pcDNA3.1 V5/His TOPO vector (Clontech). The GFP coding region was cloned into plasmid containing AKAP-*IS* and Scramble sequences using *Hind*III and *Kpn*I sites. All chimeric cDNA constructs were verified by sequencing.

IS(+)5'CACCATGGCACAAATCGAATACTTAGCAAAACAAATCGTAGACAACG

CAATCCAACAAGCAAAAGCA3'

IS(-)

5'TGCTTTTGCTTGTTGGATTGCGTTGTCTACGATTTGTTTTGCTAAGTATTCGA

TTTGTGCCATGGTG3'

Sramble (+)

5'CACCATGGCACAAGACGTAGAAATCCAACTCAAAGCAGCATACAACCAAA

AATTAATCGCAATCGCA3'

Scramble(-)

5'TGCGATTGCGATTAATTTTTGGTTGTATGCTGCTTTGAGTTGGATTTCTACGT

CTTGTGCCAGGGTG3'

Co-immunoprecipitation and PKA activity assay: Cells at 50-80% confluency were transfected using Fugene (Roche) following manufacturer instructions. 5υg of plasmid DNA (GFP-IS-V5His, GFP-Scramble-V5His, GFP-Ht31, or GFP-Ht31PP) were transfected into HEK-293 cells. Cells were lysed 24 hours later in 20mM HEPES pH7.5, 150mM NaCl, 1mM EDTA, and 1% Triton-X 100. Co-immunoprecipitation of AKAP-*IS* or the scrambled peptide complexes were performed with an anti-V5 monoclonal antibody (Invitrogen). Co-precipitation of RIIα, RIIβ, RI, and C-subunit were detected with isoform specific polyclonal antibodies (Santa Cruz). PKA kinase assays were performed by the filter paper assay (Corbin and Reimann, 1974). The PKI 5-24 peptide was used as a specific inhibitor of the kinase (Scott et al., 1986)

Confocal Microscopy: Cells were seeded on glass coverslips and incubated overnight at 37°C under 5% CO_2. AKAP-*IS* or Scrambled was detected by intrinsic GFP fluorescence. Immunocytochemistry was performed as previously described (Westphal et al., 2000). Texas Red-conjugated secondary antibodies (Jackson Labs) were used to detect the primary RII-antibody.

Electrophysiology: Human embryonic kidney 293 (HEK293) cells were maintained in DMEM with 10% FBS. Cells were transfected by Fugene (Roche) with one microgram of plasmids, which contains $GluR1_{flip}$ in pRK5 vector and pEGFP-N1 vector at a ratio of 5:1. One day after transfection, cells were replated onto 35 mm dishes at a low density. Recordings were made at room temperature two days after transfection. GFP positive cells were visually selected for recording by the fluorescence. Whole-cell recordings were made with an Axopatch200B amplifier (Axon Instruments, Foster City, CA). Patch pipettes (2-4 MΩ) contained (in mM): 140 Cs methanesulfonate, 5 adenosine triphosphate, 5 $MgCl_2$, 0.2 $CaCl_2$, 1 BAPTA and 10 HEPES, pH7.4. Extracellular solution contained (in mM) 150 NaCl, 5 KCl, 1.8 $CaCl_2$, 10 glucose, 0.1 cyclothiazide and 10 HEPES, pH 7.4. Solution exchanges were accomplished through a two-barrel pipe controlled by a solution stimulus delivery device, SF-77B (Warner Instruments, Hamden, CT). GluR1 receptor currents were evoked by 500 m sec application of 1 mM glutamate at 30 sec intervals. Data were acquired and analyzed using pClamp software (version 7.0). Currents were digitized at 5kHz and filtered at 1 kHz.

Results

Design of a consensus RII binding sequence.

Solid-phase peptide arrays of overlapping 20 mer peptides (offset every three amino acids) that encompassed the PKA anchoring regions of 10 AKAPs were screened by the RII overlay assay (Figure 2.1A). Binding of ^{32}P-radiolabelled RII was detected by autoradiography. The sequence and source of each peptide is included in the supplementary data. Peptides from AKAP-Lbc, AKAP-KL, AKAP18, AKAP79 and mAKAP consistently bound RII with a higher apparent affinity than peptides from the other anchoring proteins (Figure 2.1B). The most favorable RII binding sequence from each "high affinity" AKAP were aligned using MEME software (Figure 2.1C, upper panel). The MEME algorithm generated a position-dependent scoring matrix (PDSM) by systematically calculating the probability that a side-chain would be found at a given position. For example, isoleucine was selected to occupy position 9 (Figure 2.1C, lower panel) although leucine or threonine occupy the corresponding position in some high affinity AKAPs (Figure 2.1C, upper panel). The end result was a computer generated PDSM consensus sequence of seventeen amino acids (Figure 2.1D) that exhibited a higher apparent RII binding affinity than peptides from the five "high affinity" AKAPs (Figure 2.1E).

Figure 2.1

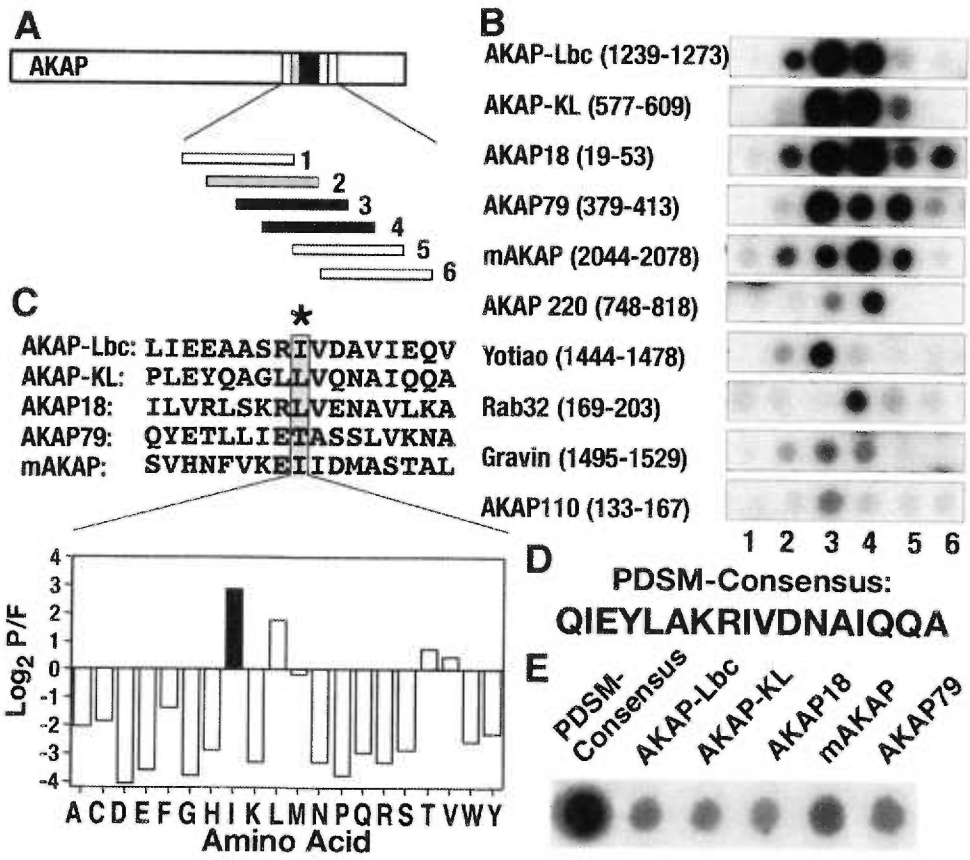

A
AKAP
1
2
3
4
5
6
B
AKAP-Lbc (1239-1273)
AKAP-KL (577-609)
AKAP18 (19-53)
AKAP79 (379-413)
mAKAP (2044-2078)
AKAP 220 (748-818)
Yotiao (1444-1478)
Rab32 (169-203)
Gravin (1495-1529)
AKAP110 (133-167)
1 2 3 4 5 6
C
AKAP-Lbc: LIEEAASRIVDAVIEQV
AKAP-KL: PLEYQAGLLVQNAIQQA
AKAP18: ILVRLSKRLVENAVLKA
AKAP79: QYETLLIETASSLVKNA
mAKAP: SVHNFVKEIIDMASTAL
Log2 P/F
4
3
2
1
0
-1
-2
-3
-4
A C D E F G H I K L M N P Q R S T V W Y
Amino Acid
D
PDSM-Consensus:
QIEYLAKRIVDNAIQQA
E
PDSM-Consensus
AKAP-Lbc
AKAP-KL
AKAP18
mAKAP
AKAP79

Figure 2.1: Derivation of PDSM consensus sequence

A) Schematic representation of how the RII-binding sequences within AKAPs were defined. Peptide arrays of 20-mer peptides (each offset by three residues) from 10 individual AKAPs were screened for RII binding by the overlay procedure. **B)** Autradiographs of RII binding peptides from 10 anchoring proteins. The name of each AKAP and segment of sequence analyzed are indicated. **C)** (**Top panel**) Alignment of the five highest affinity RII-binding sequences using the MEME software. An AKAP specific Position Dependedant Scoring Matrix (PDSM) was calculated by the log (base 2) of the probability that an amino acid is found at a given position in the alignment divided by the frequency that this amino acid is found in the non-redundant protein database (P/F). (**Bottom panel**) Values derived for position 9 in the PDSM-sequence represented as a graphical output. Isoleucine (**black bar**) is the highest scoring amino acid. Amino acids are indicated by their single letter code. **D)** The MEME derived PDSM-consensus sequence. **E)** Overlay assay of a peptide array containing the PDSM-consensus sequence and five "high affinity" AKAPs (indicated above each lane). RII binding was assessed by autoradiography.

Optimization of the PDSM Sequence

Optimization included screening a two dimensional array of 320 peptides where each residue between position 2 and 17 of the PDSM consensus sequence was systematically replaced with every possible side-chain (Figure 2.2A). Solid-phase binding of ^{32}P-labelled RII was assessed by the overlay assay and detected by autoradiography. The relative binding affinity of each modified peptide was compared to the signal strength of internal control peptides (the original PDSM consensus sequence) distributed throughout the array (Figure 2.2A, white circles). Analysis of this data confirmed and extended our previous findings in three ways. First of all, substitution of polar side-chains at positions 6, 9, 10, 13 and 14 generally decreased RII binding (Figure 2.2A, yellow columns). This is consistent with our NMR data suggesting that non-polar side chains at these positions form a hydrophobic face that contacts the RII dimer (Newlon et al., 2001). Secondly, the substitution of proline anywhere within the core of the PDSM consensus sequence abolished RII binding (Figure 2.2A, boxed region). Site-directed mutagenesis experiments on several AKAPs have shown that the introduction of helix breaking amino acids into PKA anchoring sites prevents RII binding (Alto et al., 2002; Carr et al., 1991; Carr et al., 1992b). Thirdly, substitutions at certain positions increased RII binding as evidenced by changing tyrosine 4 for isoleucine (Figure 2.2A, green square) or replacing arginine 8 with serine, methionine, lysine or glutamine (Figure 2.2A, purple squares). In independent experiments, the relative binding affinity of these peptide derivatives was measured by RII overlay (Figure 2.2B, upper panel) followed by densitometry analysis of the autoradiographs (Figure 2.2B, lower panel). The original

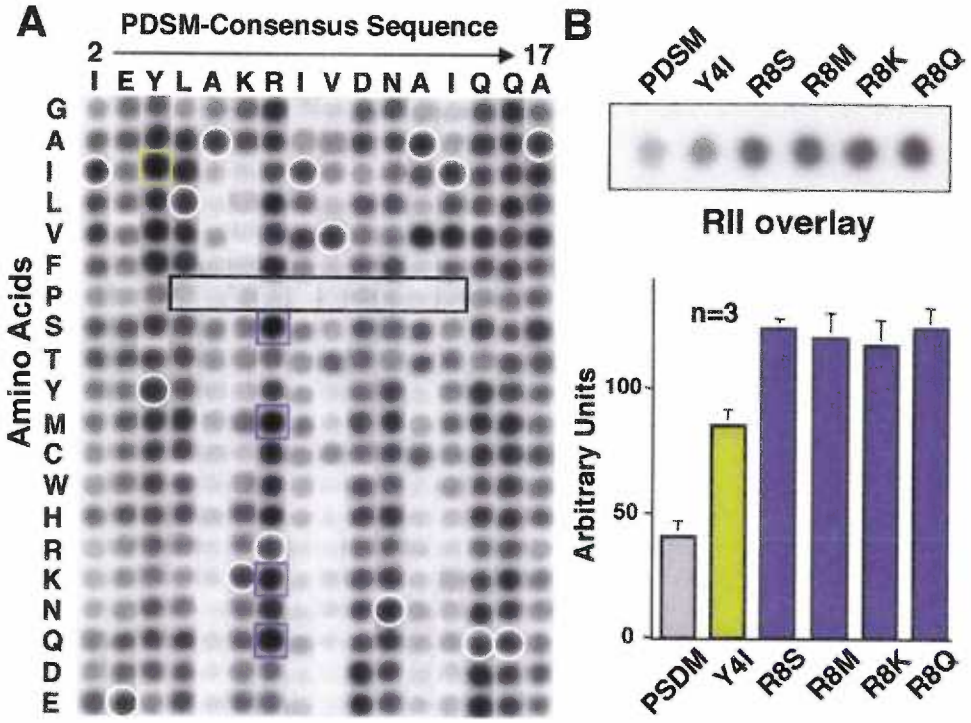
PDSM generated consensus sequence bound at a level of 43 ± 3 arbitrary units (n=3), the Y4I substituted peptide was measured at 85 ± 8 (n=3) and the arginine 8 substituted peptides ranged from 117 ± 8 (n=3) for the R8K peptide to 124 ± 13 (n=3) for the R8Q peptide. Therefore we incorporated neutral amino acid Glutamine at position 8 (Figure 2.2C). Dual substitutions at positions 4 and 8 did not increase the relative RII binding affinity of the peptides further (data not shown). We named the optimized peptide "AKAP-in silico" (AKAP-*IS*) to acknowledge the computer-aided design of this new reagent (Figure 2.2C).

***In vitro* Characterization of the AKAP-*IS* peptide.**

Dissociation constants (KD) for the optimized AKAP-*IS* peptide were measured by fluorescence anisotropy using both R subunit subtypes. The KD for RIα was 227 ± 55 nM (n=3) whereas RIIα bound more tightly with a KD of 0.45 ± 0.07 nM (n=3) (Figure 2.3A & 2.3B). A control peptide of identical amino acid composition but with a scrambled sequence did not interact with either R subunit type (Figure 2.3B). Parallel experiments performed with the Ht31 peptide, which is derived from AKAP-Lbc, generated KD values of 1277 ± 56 nM (n=3) for RIα and 2.2 ± 0.03 nM (n=3) for RIIα (Figure 2.3A & 2.3B).

Although 8 out of 17 residues are identical in the AKAP-*IS* and Ht31 sequences (Figure 2.3C), modeling studies show that the majority of the changes are located on the non-contacting polar face of the amphipathic helix (Figure 2.3C & 2.3D). Two factors predict that AKAP-*IS* is a more helical peptide than Ht31. Alanine residues at positions

Figure 2.2



C

Optimized AKAP-*IS* Sequence:

QIEYLAKQIVDNAIQQA

Figure 2.2: Optimization of PDSM consensus sequence

A) A two dimensional array of 320 AKAP-*IS* peptide derivatives was generated where each residue between positions 2 and 17 in the PDSM sequence (**above the array**) was replaced with every possible side-chain (**left of the array**). Amino acids are indicated using the one letter code. Binding of ^{32}P-labelled RII was detected by autoradiography. Peptide derivatives substituted at positions 6, 9, 10, 13 and 14 (**yellow columns**), proline substituted PSDM consensus sequences with reduced RII binding (**black rectangle**), and internal control peptides of native sequence (**white circles**) are indicated. AKAP-*IS* derivatives with higher apparent RII binding affinity (**green and purple squares**) are indicated. **B)** (**Top panel**) Solid phase RII binding of the original PDSM-consensus and the five peptides with higher affinity (indicated above each lane) were quantified by densitometry of autoradiographs. Representative data from three individual experiments is presented. (**Bottom Panel**) The relative binding affinities (arbitrary units) of each peptide (**indicated below each column**) are presented in graphic form. **C)** The optimized AKAP-*IS* sequence is indicated by the single letter amino acid code.

Figure 2.3

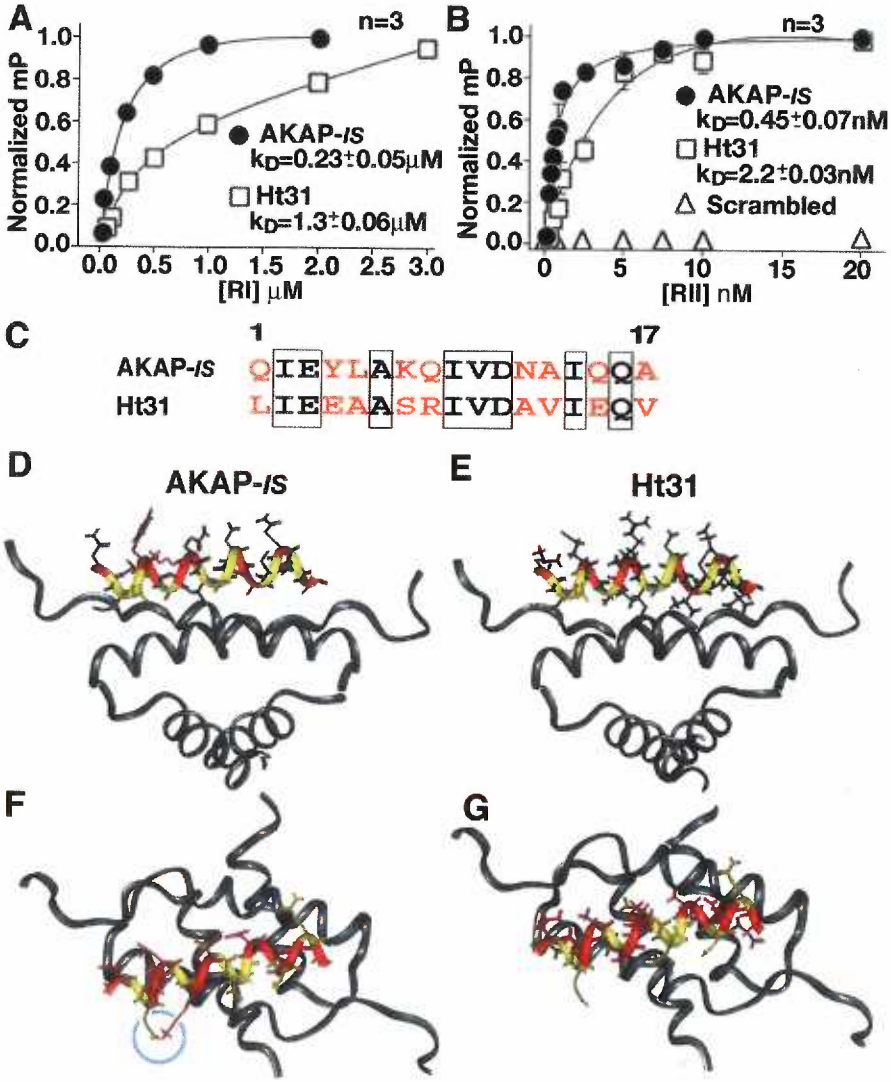

A
n=3
Normalized mP
AKAP-IS
kD=0.23±0.05μM
Ht31
kD=1.3±0.06μM
[RI] μM
B
n=3
Normalized mP
AKAP-IS
kD=0.45±0.07nM
Ht31
kD=2.2±0.03nM
Scrambled
[RII] nM
C
1
17
AKAP-IS QIEYLAKQIVDNAIQQA
Ht31 LIEEAASRIVDAVIEQV
D
AKAP-IS
E
Ht31
F
G

Figure 2.3: Biochemical and structural analysis of AKAP-*IS*. Dissociation constants (KD) of fluorescein-labeled AKAP-*IS* (**circles**) and Fluorescein-labeled Ht31 (**squares**) peptides were determined by fluorescence polarization. Saturation binding curves were generated with increasing concentration of RIα (**A**) or RIIα (**B**). Polarization values (mP) were determined upon reaching equilibrium and normalized to the highest value of saturation. Non-linear regression analysis was used to derive KD values (**inset**). The compiled data from three experiments are presented. Interaction of RII was not detected using a Fluorescein-labeled scrambled peptide (**open triangles**) of identical amino acid composition as the AKAP-*IS* sequence. **C)** Sequence alignment of the AKAP-*IS* and Ht31 sequences. Identical residues (**boxed**) and dissimilar residues (**red**) are indicated. **(D-G)** Molecular modeling of the AKAP-*IS*/RIIα complex used coordinates from the NMR structure of the Ht31/RIIα complex. The core peptide (**yellow**) and sites of divergence between AKAP-*IS* and Ht31 (**red**) are indicated. A side view reveals a change in RII contact side chains at positions 14 and 17 in AKAP-*IS* **(D)** when compared to the Ht31 **(E)**. Top view of AKAP-*IS* **(F)** reveals the formation of a salt bridge formed by residues E3 and K7 (**blue circle**) that is not found in Ht31 **(G)**.

13 and 17 in AKAP-*IS* are predicted to increase the helicity of the peptide (Figure 2.3D & 2.3E) and a salt bridge formed between Glu3 and Lys7 stabilizes the overall conformation of the peptide (Figure 2.3F & 2.3G). These data suggest why AKAP-*IS* binds to either R subunit more tightly than Ht31.

Cell based analysis of AKAP-*IS*

Expression constructs encoding AKAP-*IS* or the scrambled sequence fused to a V5/His epitope tagged Green Fluorescent Protein (GFP) were transfected into HEK293 cells (Figure 2.4A). Three cell-based approaches were used to characterize the AKAP-*IS* interaction with the PKA holoenzyme. First of all, immune complexes were isolated with a monoclonal antibody against the V5 epitope and the co-precipitation of PKA subunits was assessed by immunoblot using specific antibodies (Figure 2.4B). Both type II R subunits (RIIα and RIIß) and the catalytic (C) subunit were detected in the AKAP-*IS*-GFP immunoprecipitates (Figure 2.4B, top three panels, lane 4) but not found in immune complexes with the scrambled-GFP fusion protein (Figure 2.4B, lanes 3). Neither type I R subunit (RIα or RIß) co-purified with AKAP-*IS* (Figure 2.4B, fourth panel, lane 4), which probably reflects their lower binding affinities. Equivalent amounts of AKAP-*IS*-GFP or the scrambled peptide-GFP fusion proteins were present in the immune complexes as assessed by immunoblot using V5 monoclonal antibodies (Figure 2.4B, bottom panel).

Secondly, PKA activity co-precipitating with AKAP-*IS*-GFP or an Ht31-GFP fragment was measured by *in vitro* kinase assay using Kemptide as a substrate (Figure 2.4C). PKA activity was 2.6 fold higher in the AKAP-*IS*-GFP fraction than the Ht31-GFP fraction (Figure 2.4C, right panel) although equivalent levels of the fusion proteins were used (Figure 2.4C, left panel). Kinase activity was not co-purified with the scrambled peptide or an Ht31P-GFP control, which is unable to anchor PKA (Figure 2.4C, right panel). Control experiments confirmed that all PKA activity was blocked by the PKI 5-24 inhibitor peptide (Figure 2.4C, right panel).

Thirdly, immunofluorescence techniques demonstrated that overexpression of AKAP-*IS*-GFP (Figure 2.4D) displaced endogenous RII from anchoring sites at the perinuclear regions and Golgi of HEK293 cells (Figure 2.4E &F). In contrast, expression of the scrambled-GFP construct (Figure 2.4H) had no effect on the subcellular distribution of RII (Figure 2.4H & I). Similar results were obtained when these experiments were performed in COS7 cells (data not shown). These approaches demonstrate that AKAP-*IS* is a high affinity ligand for the type II PKA holoenzyme inside cells.

Figure 2.4

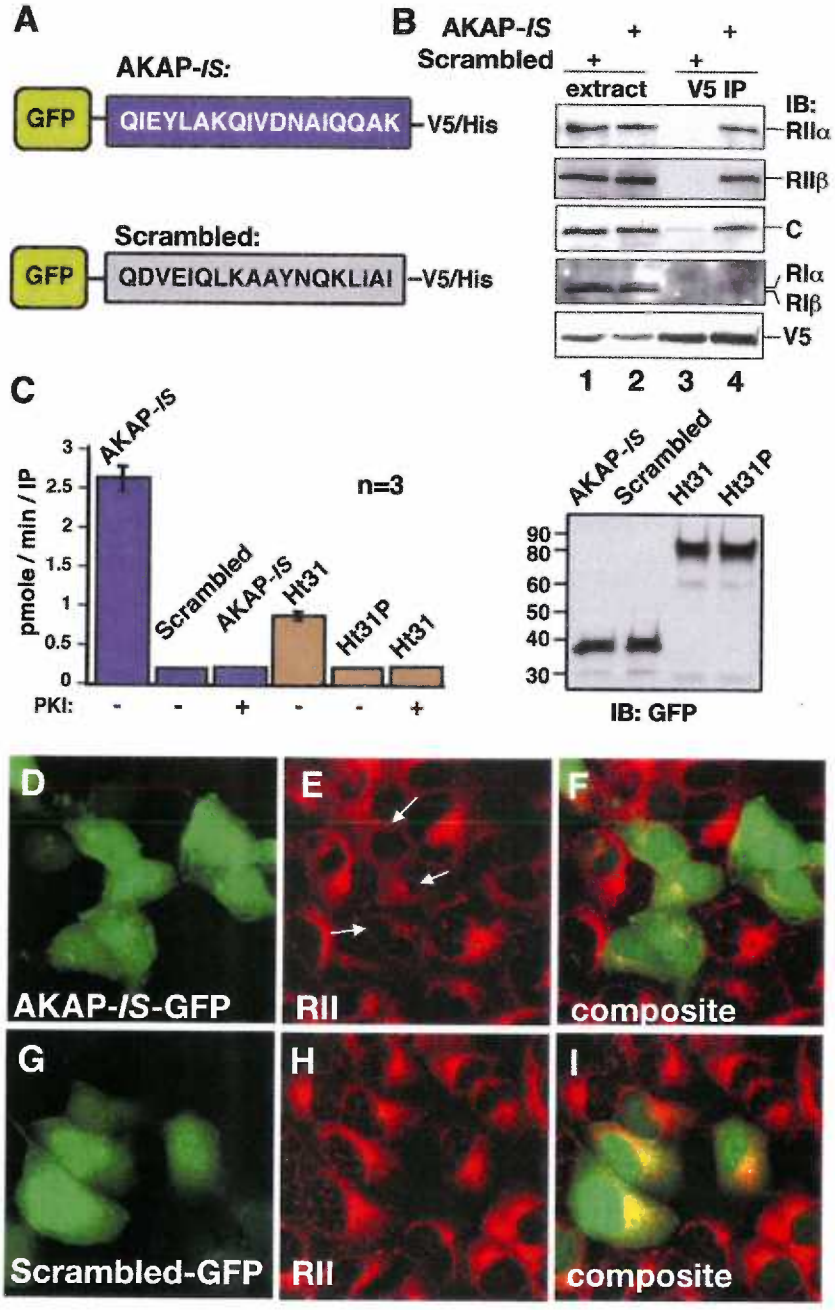

A
AKAP-IS:
GFP
QIEYLAKQIVDNAIQQAK
V5/His
Scrambled:
GFP
QDVEIQLKAAYNQKLIAI
V5/His
B
AKAP-IS
Scrambled
extract
V5 IP
IB:
RIIα
RIIβ
C
RIα
RIβ
V5
1 2 3 4
C
pmole / min / IP
n=3
AKAP-IS
Scrambled
AKAP-IS
Ht31
Ht31P
Ht31
PKI:
AKAP-IS
Scrambled
Ht31
Ht31P
IB: GFP
D
AKAP-IS-GFP
E
RII
F
composite
G
Scrambled-GFP
H
RII
I
composite

Figure 2.4: AKAP-*IS* interacts with PKA inside cells. A) Schematic representation of GFP fusion proteins encoding the AKAP-*IS* **(top)** and scrambled **(bottom)** peptides used in the cell based studies. Sequences are given by the one letter amino acid code. **B)** HEK293 cells were transfected with either construct and immune complexes were isolated from triton-X soluble extracts **(extract)** with a V5 antibody **(V5 IP)**. Co-purification of PKA holoenzyme subunits was detected by immunoblot using antibodies against the RIIα **(top panel)**, RIIß **(second panel)**, C subunit **(third panel)**, or RI subunits **(fourth panel)**. The GFP fusion proteins were detected by immunoblot using the V5 antibody **(bottom panel). C) (left panel)** The specific activity (pmol/min./IP) of PKA C subunit co-precipitating with chimeric AKAP fusion proteins (indicated above each column) was measured by filter paper binding assay using the Kemptide as a substrate. PKA activity was blocked when PKI (5-24) peptide (10uM) was added to the reaction mixture. The accumulated data from three independent experiments is shown. **(right panel)**. Immunoblots blots showing that that equal amounts of the GFP fusion proteins **(indicated above each lane)** were used in these experiments. **(D-I)** Cells transiently transfected with plasmids expressing AKAP-*IS* or the scrambled peptide for 24 hours were fixed and Immuncytochemical techniques were used to detect intrinsic GFP fluorescence **(green; D & G)**. The subcellular location of RII **(red; E & H)** was detected with a monoclonal anti-RII antibody and Texas-red conjugated secondary antibody on a Biorad 1024 UV/Vis confocal microscope. Arrows indicate the mislocalization of RII in AKAP-*IS* expressing cells. Composite images **(F and I)** are presented.

Functional Analysis of AKAP-*IS*.

A physiologically relevant model of PKA anchoring is the time-dependent down-regulation (rundown) of AMPA responsive currents in hippocampal neurons upon intracellular dialysis of the Ht31 peptide (Rosenmund et al., 1994). This system was reconstituted in HEK293 cells by co-expressing the neuronal anchoring protein AKAP79 and the AMPA channel subunit GluR1. Whole-cell patch-clamp techniques provided a sensitive means to record GluR1 currents upon the delivery of bioactive peptides through the patch pipette (Figure 2.5A). Control recordings demonstrated that GluR1 currents were stable over a 10-minute time course (Figure 2.5A, open circles & 2.5B). Perfusion of AKAP-*IS* (1μM) evoked a pronounced and rapid reduction (41.4 ± 3.6 %; n= 14) in GluR1 currents that was almost complete within 5 minutes (Figure 2.5A, closed circles & 2.5B). In contrast, perfusion of the Ht31 peptide (1μM) evoked a less robust (20.8 ± 4.6%; n= 11) and slower response (Figure 2.5A, triangles & 2.5B). Interestingly, the magnitude and time course of the AKAP-*IS* response was similar to the effect of PKI 5-24 peptide (10μM), a potent and specific inhibitor of the kinase (Figure 2.5A, open squares, & 2.5B). Collectively this functional data suggests that AKAP-*IS* is a potent cell-based antagonist of AKAP79/PKA interaction. Additional support for this notion was provided by *in vitro* binding assays showing that AKAP-*IS* [IC_{50}= 8 nM (n=5)] was more effective at displacing RII from interaction with AKAP79 than the Ht31 peptide [IC_{50}= 55 nM (n=5); Figure 2.5C].

Figure 2.5

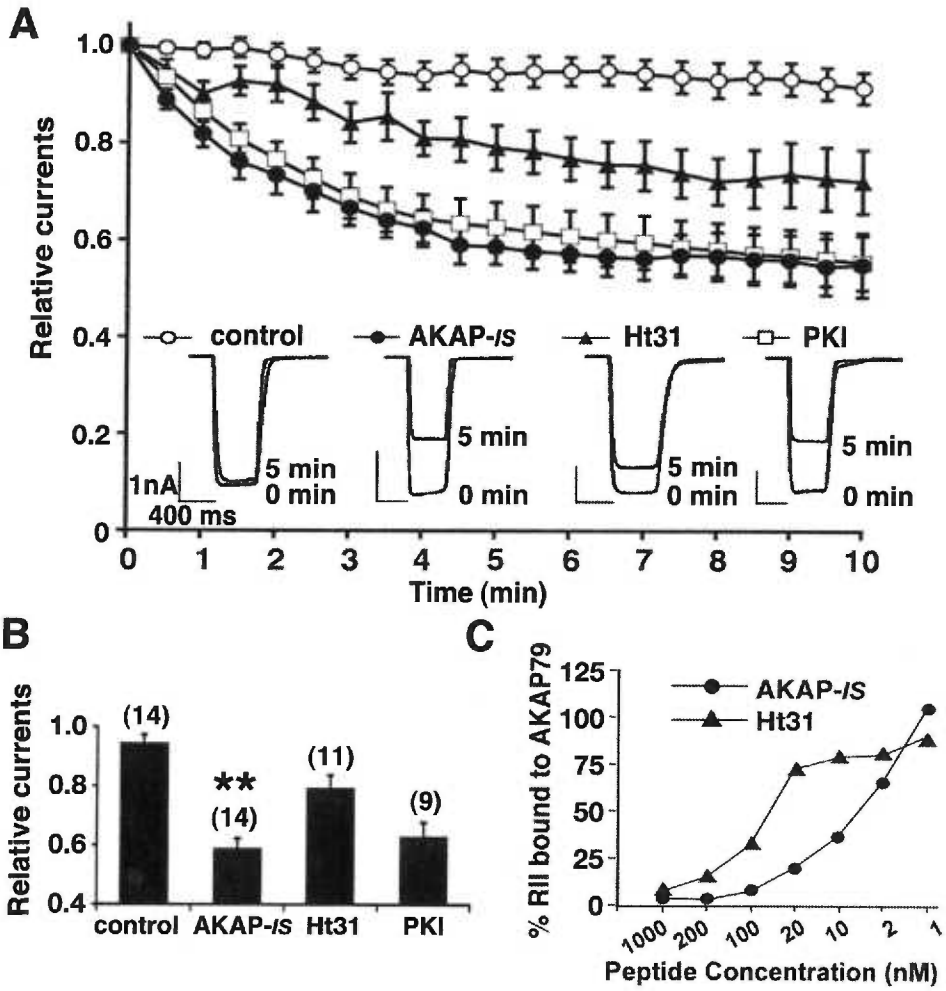

A
Relative currents
1.0
0.8
0.6
0.4
0.2
0
control
AKAP-IS
Ht31
PKI
1nA
400 ms
5 min
0 min
0 1 2 3 4 5 6 7 8 9 10
Time (min)
B
Relative currents
(14)
**
(14)
(11)
(9)
control
AKAP-IS
Ht31
PKI
C
% RII bound to AKAP79
125
100
75
50
25
0
AKAP-IS
Ht31
1000 200 100 20 10 2 1
Peptide Concentration (nM)

Figure 2.5: AKAP-*IS* is a potent antagonist of PKA anchoring. A) Whole cell patch clamp recording techniques were used to measure the effects of AKAP-*IS* (1 υM; **closed circles**), Ht31 (1 υM; **closed triangles**), or PKI (10 υM; **open squares**) peptides on the time dependant rundown of GluR1 receptor currents expressed in HEK293 cells. The accumulated data from 9-14 experiments including control currents (**open circles**) are presented. Inset shows representative current trace from 0 and 5 min. Amplitude and time scale bars are presented **B)** Graphical representation of the peak current amplitudes upon glutamate stimulation five minutes after delivery of the peptides (indicated below each column). Each bar is normalized to the peak amplitude found at time 0. Amalgamated data from a number experiments (indicated above each column). Star indicates significant difference between AKAP-*IS* and Ht31 (**$p<0.01$). **C)** *In vitro* competition assays. Recombinant AKAP79 (100 ng per sample) immobilized to glutathione sepharose bound was loaded with recombinant ^{32}P-labelled RII. Decreasing concentrations of AKAP-*IS* (**closed circles**) or Ht31 (closed triangles) peptide were added. After washing, the remaining RII was detected by autoradiography. RII binding (% bound) and peptide concentrations (nM) are indicated.

Discussion.

Bioinformatics is a powerful methodology in biomedical research. Computational biology approaches are routinely used to search genomes for conserved structural motifs and to chart of the evolutionary history of gene super-families (Eisenberg et al., 2000). The MEME software used in this study was originally designed to identify distantly related protein sequences from a user defined substitution-scoring matrix. This is an ideal system to analyze PKA anchoring sites on AKAPs, which represent structurally related regions of low sequence identity on otherwise unrelated proteins. Consequently, we have combined this innovative bioinformatic approach with high-throughput screening of peptide arrays and a solid-phase RII binding assay to design a potent antagonist of PKA anchoring. Although, the latter two techniques allowed us to quickly pair down our initial pool of sixty candidate peptides to five "high affinity" AKAP sequences, the most critical element in the design strategy was analysis by the MEME program. Not only did the consensus sequence it derived bind RII more tightly, but the subsequent analysis of 320 derivatives during the optimization procedure only prompted a single substitution in the final AKAP-*IS* peptide. Thus our product is a novel reagent that binds RII with significantly higher affinity than naturally occurring AKAP peptides.

Ht31 is the prototypic AKAP sequence that has been to used to study PKA anchoring for the past decade (Carr et al., 1991). This twenty-four residue peptide is derived from human anchoring protein AKAP-Lbc and binds RII with a dissociation constant (K_D) of 2-4 nM (Carr et al., 1992a; Diviani et al., 2001). Our biochemical studies show that AKAP-*IS* binds RII with a 5-fold higher affinity (K_D = 0.4 nM) and our

cell-based experiments confirm that it is a more effective inhibitor of PKA anchoring *in vivo*. Two observations emphasize this latter point: 1) an AKAP-*IS*-GFP fusion protein more effective isolated PKA activity from cells than an Ht31 derivative, and 2) the intracellular dialysis of the AKAP-*IS* peptide promoted a more rapid and complete attenuation of GluR1 currents than Ht31. In fact, our electrophysiological data indicate that the rundown in GluR1 channels upon AKAP-*IS* mediated disruption of PKA anchoring occurs with a similar rate and magnitude as complete inhibition of the kinase by PKI 5-24. Thus, AKAP-*IS* has clear advantages over the currently available anchoring inhibitor peptides and should prove to be a superior reagent to for disrupting anchored PKA pools in cells, tissues and possibly whole animals.

The tertiary structure of AKAP-*IS* may explain its higher PKA anchoring affinity. Modeling studies using coordinates from the Ht31 /RIIα structure suggest that most aliphatic side-chains which contact the RII protomer are retained in both peptides (Newlon et al., 2001). Interesting exceptions to this observation are the alteration of the RII contact residues alanine 13 and alanine 17 (Figure 2.3C). Surprisingly, these changes remove hydrophobic contacts to the RII protomer from the peptide surface and, *a priori*, would have been predicted to destabilize the complex. However, alanine is the most favored residue for helix formation, and when combined with the introduction of a lysine at position 7 on the solvent exposed surface of the peptide, serves to increase the predicted helical content of the unbound peptide. This likely involves the formation of an intramolecular salt bridge between Glu3–Lys7 in AKAP-*IS* (Figure 2.3F). The kinetic structural mechanism for AKAP binding is presently unknown but the following two scenarios exist: 1) the peptide binds to RII then folds into a helical structure (binding-

induced folding) or 2) the peptide folds into an α-helix and then binds to RII (conformational regulation of binding). The former hypothesis is less likely as the loss of intermolecular hydrophobic interactions would serve to destabilize the complex (Figure 2.3D). Alternatively, if the recognition surface of the AKAP is already assembled, then increasing the helical content of the peptide could shift the equilibrium towards a binding competent form. In this case, the observed on-rate would be maximized under conditions where the helical form predominates. Indirect support for this notion is provided by our electrophysiology data showing that AKAP-*IS* is a more potent and rapid antagonist of PKA anchoring inside cells.

Although AKAPs were initially thought to interact only with the type II PKA holoenzyme, there is now ample evidence showing that many anchoring proteins also target the type I kinase (Huang et al., 1997b; Li et al., 2001; Miki and Eddy, 1998). In fact, substitution of aliphatic side-chains in the hydrophobic face of the Ht 31 helix increases affinity for RI (Miki and Eddy, 1999). However, the native RI binding affinities of AKAP79 and AKAP-Lbc are 500-fold less for RI than for RII. This suggests that these AKAPs will preferentially associate with the type II PKA inside cells when both kinase sub-types are available (Burton et al., 1997; Herberg et al., 2000). Likewise, AKAP-*IS* exhibits a 500-fold preference for RII over RI in vitro, and its interaction with RI cannot be detected inside cells. This isoform selectivity is not surprising given that the five AKAP sequences used to generate the progenitor AKAP-*IS* consensus are recognized as high affinity RII binding proteins (Colledge and Scott, 1999). In contrast, anchoring proteins such as D-AKAP-1/sAKAP84/149 and D-AKAP-2 exhibit less

selectivity for either R subunit and have been designated as dual-function AKAPs (Huang et al., 1997a; Huang et al., 1997b). Recent evidence suggests that a single nucleotide polymorphisim (SNP) that is identified in the aging population causes a Valine to Isoleucine mutation the anchoring helix of D-AKAP-2. This mutation increases RI binding affinity 3 fold but has no effect on the RII/ D-AKAP-2 interaction. In an accompanying paper the same authors used peptide array technologies to generate a high affinity binding peptide with a 100-fold preference for RIα. Thus peptide antagonists are now available to test the hypothesis that isoform selective kinase anchoring occurs in a cellular context.

CHAPTER THREE

Rab32 is an A-kinase Anchoring Protein and Participates in Mitochondrial Dynamics

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Abstract

A-Kinase Anchoring Proteins (AKAP) tether the cAMP dependent protein kinase (PKA) and other signaling enzymes to distinct subcellular organelles. Using the yeast two-hybrid approach we demonstrate that Rab32, a member of the Ras superfamily of small molecular weight G-proteins, interacts directly with the type II regulatory subunit of PKA. Cellular and biochemical studies confirm that Rab32 functions as an AKAP inside cells. Anchoring determinants for PKA have been mapped to sites within the conserved α5 helix that is common to all Rab family members. Subcellular fractionation and immunofluorescent approaches indicate that Rab32 and a proportion of the cellular PKA pool are associated with mitochondria. Transient transfection of a GTP-binding deficient mutant of Rab32 promotes aberrant accumulation of mitochondria at the microtubule organizing center. Further analyses of this mutant indicate that disruption of the microtubule cytoskeleton results in aberrantly elongated mitochondria. This implicates Rab32 as a participant in synchronization of mitochondrial fission. Thus Rab32 is a dual function protein that participates in both mitochondrial anchoring of PKA and mitochondrial dynamics.

Introduction

The transmission of environmental cues to precise sites within the cell frequently involves the assembly of multiprotein signaling complexes (Hunter, 2000; Jordan et al., 2000; Pawson, 1995). These transduction units, which often include effector proteins, signal transduction enzymes and their substrate proteins are maintained by scaffolding or anchoring proteins (Smith and Scott, 2002). These "signal organizing" molecules ensure that their complement of anchored enzymes are optimally positioned to receive activation signals and are placed in close proximity to their substrates. One example is the A-Kinase Anchoring Proteins (AKAPs) that sequester the cAMP dependent protein kinase (PKA) and other signaling enzymes at a variety of intracellular sites (Colledge and Scott, 1999; Feliciello et al., 2001).

Although a principle function of many kinase anchoring proteins is to orchestrate protein phosphorylation events, it is now evident that they participate in a wider range of signaling events. For example several anchoring proteins have been implicated in the recruitment and localization of Ras family small molecular weight GTPases. AKAP-Lbc binds PKA and functions as a Rho selective guanine nucleotide exchange factor to induce actin stress fiber formation in fibroblasts (Diviani et al., 2001). Scar/WAVE-1 assembles a signaling complex that includes PKA, the Abelson tyrosine kinase (Abl), Rac-1 and the Arp2/3 complex to, coordinate lamellapodial extension at the leading edge of motile cells (Westphal et al., 2000). Anchoring proteins that bind to other second messenger regulated protein kinases apparently perform analogous functions. The mammalian ortholog of the *C. elegans* cell-polarity gene, mPAR-6, interacts with either Cdc42 or Rac1, and an atypical Protein Kinase C (aPKC) to establish cell shape and polarity in the

developing embryo (Etemad-Moghadam et al., 1995; Joberty et al., 2000; Kuchinke et al., 1998; Lin et al., 2000; Qiu et al., 2000). Collectively, these findings highlight a secondary role for kinase anchoring proteins in the coordination of small G-protein location and signaling to down stream effectors.

Here we report that Rab32, a member of the Rab subfamily of Ras small molecular weight G-proteins, functions as an AKAP *in vivo*. Most Rab proteins have been implicated in membrane dynamics, where they coordinate the assembly of protein networks that regulate membrane fusion/fission, exocytosis, and cytoskeletal trafficking (Takai et al., 2001). These events require the Rab proteins to cycle from a cytoplasmic localized GDP-bound "off" state to a membrane-localized GTP-bound "on" state (Novick and Zerial, 1997). Accordingly, Rab proteins have been found at various intracellular sites including the endoplasmic reticulum, golgi apparatus, endosomes, intracellular vesicles, and the plasma membrane (Takai et al., 2001). Our cell-based studies demonstrate that Rab32 is associated with the mitochondria through potential lipid modification of two C-terminal cysteines. Furthermore, ectopic expression of a GTP-binding defective mutant of Rab32 perturbs mitochondrial distribution, suggesting a specific role for this Rab protein at mitochondria.

Experimenal Procedures

Yeast two-hybrid: The cDNA from the RIIα dimerization/AKAP-binding domain was PCR amplified and subcloned into the *Eco*RI-*Bam*HI sites in the pLexA yeast expression vector. This gene encodes an N-terminal LexA DNA binding domain fused to the first 45 amino acids of RIIα. 500μg of a human-brain MATCHMAKER cDNA library (Clontech) was screened for AKAPs using the yeast two-hybrid assay as described in (Hollenberg et al., 1995).

Cloning full length Rab32: An Expressed Sequence Tag (EST) clone for Rab32 was obtained from Genome Systems (Genbank AI281920, Image 1882928). This clone contained the cDNA sequence representing amino acids 19-225 of Rab32 including the termination codon. A synthetic oligonucleotide was designed to contain the 90 base pairs corresponding to the 5' region of Rab32 cDNA. The partial Rab32 EST was used as a template for PCR amplification with the 90-mer 5' primer and a reverse primer that corresponds to the 3' end of the Rab32 coding sequence. The resulting PCR product was subcloned into the bacterial expression vector pGex4T-1. Full length Rab32 was confirmed by sequencing.

Construction of expression vectors: The full length Rab32 cDNA was PCR amplified from the pGEX4T template with synthetic oligonucleotide primers and directly subcloned into mammalian and yeast expression vectors. For the yeast expression vector (pLexA), and the mammalian expression vector (Flag-pcDNA3.1), PCR fragments were subcloned into the 5' *Eco*RI sites and 3' *Bam*HI sites. All Rab32 mutants were generated using

Quick-Change™ mutagenesis (Stratagene) with specific primers. All PCR reactions were carried out using Pfu Turbo polymerase (Stratagene). Each expression construct was sequenced to determine correct reading frame and the existence of appropriate mutations.

Bacterial expression and purification of recombinant Rab32: Recombinant Rab32 and mutants were expressed from the bacterial expression vector pGex4T in the BL-21/DE3 strain of *Escherichia coli* and purified as N-terminal GST fusions using glutathione sepharose (Pharmacia).

RII interaction assays: RII overlays were carried out using murine [^{32}P] RIIα as previously described (Carr and Scott, 1992). For Yeast two hybrid analysis, the cDNA encoding RIIα 1-45 was subcloned into the Gal4 activation domain containing, yeast expression vector, pACT2. This gene encodes an N-terminal Gal4 activation domain fused to RIIα 1-45. Each pLexA-Rab32, -Rab32L188P, -Rab5, -Rab6, or -Rab7 was co-transformed with the pACT2- RIIα 1-45 into the yeast strain L40 as described above.

Autospot peptide array: Peptide arrays were synthesized on cellulose paper using an Auto-Spot Robot ASP222 (ABiMED) as previously described (Frank, 1992). After synthesis, the N-termini were acetylated with 2% acetic acid anhydride in DMF. The peptides were then deprotected by 1 hour treatment with DCM/TFA, 1:1, containing 3% triisopropylsilane and 2% water.

Immunoprecipitations and PKA activity assay: Cells 50-80% confluent were transfected using lipofectamine plus reagents (Invitrogen) following manufacturers instruction. 5_g of plasmid DNA (Flag-Rab32 or Flag-Rab32L188P) were used to transfect HEK-293 cells in 10cm dishes. Transfections were carried out for 24 hours followed by lysis in IP buffer (20mM HEPES pH7.5, 150mM NaCl, 1mM EDTA, 1% Triton-X 100). Recombinant proteins were immunoprecipitated using sepharose conjugated anti-flag monoclonal antibody (Sigma). PKA kinase assays were performed as previously described (Corbin and Reimann, 1974).

Northern blot: A Northern blot containing immobilized samples of mRNA from several human tissues (Clontech) was assayed using a [^{32}P] radiolabeled 113-bp probe corresponding to the C-terminal hypervariable domain of Rab32. Human RNA dot-blot analysis was performed following manufacturers instructions (Clontech).

Western blot analysis and subcellular fractionation of WI-38 cells: Antibodies to Rab32 were raised in rabbits (Covance) against the unique peptide CEENDVDKIKLDQETLRAEN (Princeton Biomolecules). Anti-Rab32 antibodies were affinity purified against recombinant Rab32 coupled to Affigel-10/15 following manufacturers instructions (Bio-Rad). WI-38 cell extracts were prepared by lysing confluent cells in buffer: 20mM HEPES, 150mM NaCl, 1mM EDTA, 1% Triton-X, and protease inhibitor cocktail (Roche). Lyastes were incubated on ice for 10 minutes and centrifuged for 20 min at 20,000xg. 20 μg of supernatants were subjected to Western blot analysis. Subcellular fractionation studies were carried out as described (Lutsenko

and Cooper, 1998). Briefly, a 10cm dish of confluent WI38 cells was harvested by trypsin digestion, washed, and resuspended in 1ml of HB buffer (10 mM HEPES-NaOH, 250mM sucrose, and protease cocktail, pH7.5). Cells were dounce homogenized (10-15 strokes) and subjected to differential centrifugation as described. 20μg of proteins from whole cell extract, P1 (nuclear pellet), and P2 (mitochondria enriched) fractions were subjected to western blot analysis. Control experiments were performed using anti-COX subunit I antibody (Molecular Probes).

Confocal microscopy: Cells were seeded on glass coverslips and incubated overnight at 37°C under 5% CO_2. To detect mitochondria, coverslips were incubated with MitoTracker Red™ (Molecular Probes) for 30 minutes at 37°C under 5% CO_2. In recombinant Rab32 localization studies, mitochondria were detected by co-expression of the plasmid pEYFP-Mito (Clontech). RII monoclonal antibodies (Transduction Labs) were used in co-localization studies. Coverslips were washed twice with PBS and fixed for 10 min. in PBS/3.7% formaldehyde. For immunocytochemistry was performed as is in (Westphal, et al., 2000). Fitc-, Texas Red-, or Cy5-conjugated secondary antibodies were used for detection of the primary antibody (Jackson Labs). Cells were washed and mounted using the Prolong system (Molecular Probes). Immunofluorescent staining, MitoTracker Red™, or intrinsic YFP fluorescence was visualized on a BioRad MRC1024 UV laser-scanning confocal microscope.

GDP and GTP binding assays: GDP off- and GTP on-rates were measured as previously described (Self and Hall, 1995). Briefly, purified recombinant GST-Rab32

was resuspended in assay buffer (50mM Tris-HCl, 50mM NaCl, and 5mM $MgCl_2$, 5mM DTT pH 7.5) supplemented with 10mM EDTA. GDP off-rate was determined in high magnesium conditions. GTP on-rates were determined in low magnesium conditions. Samples were dissolved in scintillation fluid followed by counting the radioactive emissions. Data is expressed as the percent nucleotide bound normalized to time 0 (100%). The Nucleotide dependent-RII binding studies were conducted as follows: 1mg of GST-Rab32 bound to Glutathione sepharose was nucleotide depleted in (50mM Tris-HCl, 50mM NaCl, 10mM EDTA, 5mM $MgCl_2$, 5mM DTT, pH 7.5) followed by loading with 1mM GDP or 1mM GTPγS for 30 min. at room temperature. The nucleotides were stabilized on Rab32 by extensive washing in high magnesium buffer (50mM Tris-HCl, 50mM NaCl, 5mM $MgCl_2$, 5mM DTT, pH 7.5 plus either 1mMGDP or 1mM GTPγS). 1μg of recombinant RIIα was incubated with 1μg nucleotide loaded Rab32 for 1 hour at room temperature followed by extensive washing in high magnesium buffer. The extent of RII binding was determined by western blot analysis using a monoclonal anti-RIIα antibody (Transduction Labs).

Rab32 functional experiments: Cos7 cells at 50-80% confluence were transfected with 1ug of the mammalian expression vectors Flag-Rab32, -Rab32T39N, -Rab32ΔCC, -Rab32T39NΔCC, or Rab32L188P using Lipofectamine Plus (Invitrogen). Transfected cells were incubated at 37°C, 5% CO_2, for 8 hours. Cells were labeled with MitoTracker Red™ (Molecular Probes) for 30 min, and processed for immunocytochemistry. Recombinant Rab32 and mutants were detected using a Fitc-conjugated anti-Flag antibody. Fifty Rab32 and mutant expressing cells were scored for a mitochondrial-

collapse phenotype in which the mitochondrial stain was predominantly found at the microtubule organizing center or around the nucleus. Three independent experiments were carried out and the results are shown as the percentage of transfected cells with the observed phenotype for each Rab32 construct. To detect cellular markers, primary antibodies against, EEA-1 (Transduction Labs), LAMP-1 (Transduction Labs), Calnexin (StressGen), and GM130 (Transduction Labs), were used in immunocytochemistry assays, and detected by Cy5-conjugated secondary antibodies. Immunofluorescent staining and MitoTracker Red was visualized on a BioRad MRC1024 UV laser-scanning confocal microscope. For microtubule depolimerization studies, transfected Cos7 cells were treated with 5μM Nocodazole (sigma) for 1 hour prior to fixation.

Results

Identification and Characterization of Rab32 as an RII binding protein:

Biochemical and structural analyses have indicated that the first 45 amino acids of the type II regulatory subunit (RII) comprise the primary determinants for interacting with AKAPs (Hausken et al., 1994; Newlon et al., 1999). To identify new AKAPs, the first 45 amino acids of the murine RIIα were used as "bait" to screen a human brain cDNA library using the yeast two-hybrid assay. Fifty positive colonies were identified from $2x10^6$ co-transformants as assessed by activation of both the HIS3 and LacZ genes. Forty of these positive colonies encoded previously identified AKAPs including Yotiao, AKAP220, and S-AKAP84 (Figure 3.1A). One positive colony encoded residues 106-225 of Rab32 that, based on primary amino acid similarity and domain architecture, is believed to be a small molecular weight G-protein of the Rab family (Accession # NP_006825)(Bao et al., 2002). Independent confirmation of this result was provided when the bacterially expressed Rab32 106-225 fragment bound recombinant [^{32}P] RIIα as assessed by a solid-phase binding assay (Figure 3.1B).

The PKA anchoring site on AKAPs includes a region of 14-18 residues that forms an amphipathic helix (Carr et al., 1991; Newlon et al., 2001). To define this region within Rab32, an array of overlapping 20-mer peptides (offset by three amino acids) covering residues 151 to 225 were synthesized on a membrane support using an AutoSpot Robot (Figure 3.2A). A single peptide corresponding to amino acids 178-197 (NINIEEAARFLVEKILVNHQ) strongly bound RIIα as assessed by the solid-phase

Figure 3.1

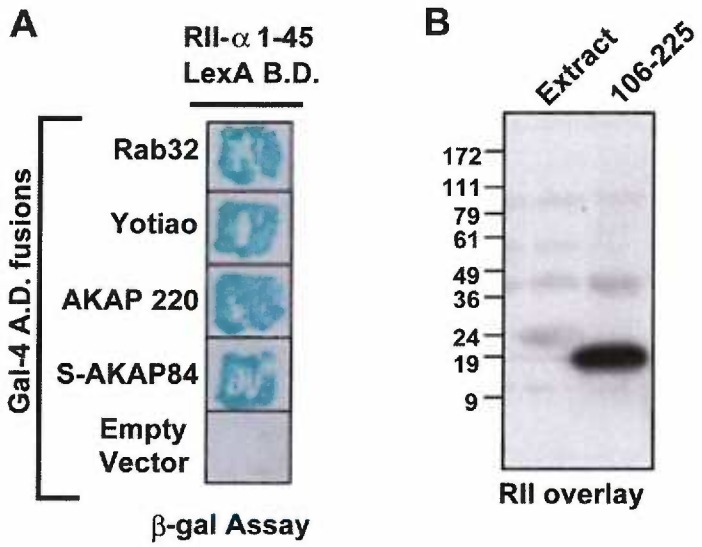

Figure 3.1: Identification of Rab32 as a putative AKAP.

Murine RIIα 1-45/LexA fusion was used as "bait" to screen a human brain cDNA library using the yeast two-hybrid assay. **A)** Detection of positive colonies expressing Rab32 and other AKAP fragments were detected by β-galactosidase. The name of each AKAP is indicated. **B)** Control and bacterial extracts expressing the Rab32 106-225 fragment (indicated above each lane) were separated on SDS-PAGE (4-15%) and electrotransferred to nitrocellulose. RII binding was as sessed by ove rlay using [^{32}P] labeled RIIα and detected by autoradiography. Molecular weight markers are indicated.

Figure 3.2

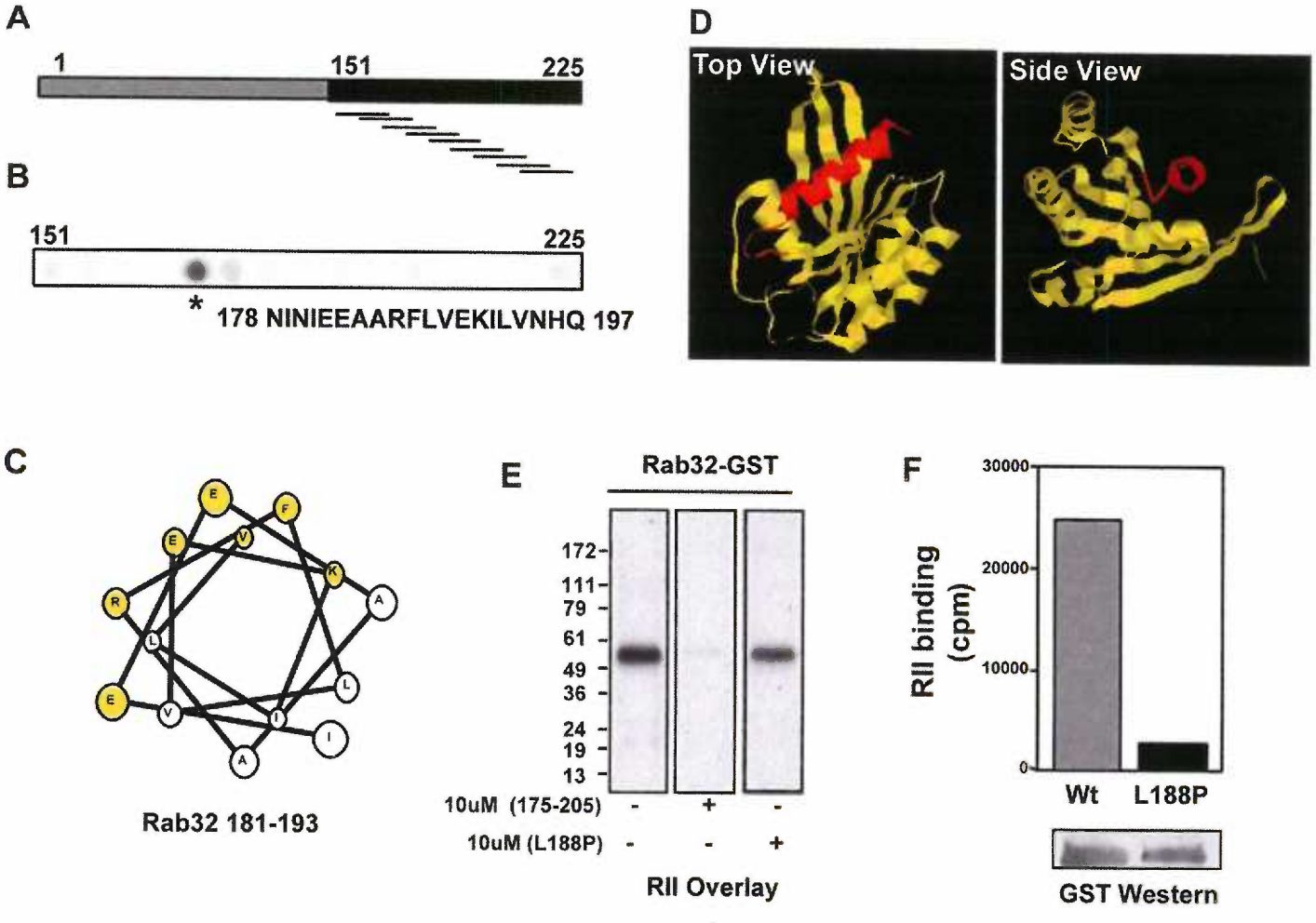
A
1
151
225
B
151
225
* 178 NINIEEAARFLVEKILVNHQ 197
D
Top View
Side View
C
Rab32 181-193
E
Rab32-GST
172
111
79
61
49
36
24
19
13
10uM (175-205) - + -
10uM (L188P) - - +
RII Overlay
F
RII binding (cpm)
30000
20000
10000
0
Wt
L188P
GST Western

Figure 3.2: Mapping the RII binding domain of Rab32.

A) Schematic representation of the mapping strategy to identify the RII binding domain of Rab32. A family of 20-mer peptides (each offset by 3 residues) spanning the region 153-224 of Rab32 were synthesized and immobilized to a membrane support. **B)** Solid-phase binding of RII was assessed by the RII overlay procedure. The sequence of the RII binding peptide is indicated using the one letter amino acid code (*). **C)** Helical wheel alignment of residues 181-193 of Rab32. Hydrophobic residues (white circles) and hydrophilic residues (yellow circles) are indicated. **D)** Ribbon diagram of the Rab3a backbone based upon coordinates provided in (Ostermeier and Brunger, 1999). Top **(Left panel)** and side views **(Right panel)** are presented. The α5 helix is marked (red). **E)** Purified recombinant GST-Rab32 fusion protein (2μg) was separated by SDS-PAGE and electrotransferred to nitrocellulose. RII overlays were performed in the absence of competitor peptide **(Left panel)**, in the presence of 10μM Rab32 175-205 peptide **(Middle panel)**, or in the presence of 10μM Rab32 175-205 L188P peptide **(Right panel)**. Molecular weight markers are indicated. **F)** Solution binding of recombinant RII and Rab32. [^{32}P] RIIα was incubated with either GST-Rab32 or GST-Rab32L188P mutant followed by glutathione sepharose purification of the complex. RII binding was assessed by measuring the counts per minute (cpm) corresponding to [^{32}P] RIIα bound to Rab32 **(Top panel)**. Equal amounts of GST fusion proteins were used in these experiments **(Lower panel)**.

binding assay (Figure 3.2B). Computer predictions of its secondary structure suggest that residues 178-197 form an amphipathic helix (Figure 3.2C). All Ras family members share a similar structure with an arrangement of six central strands of β-sheet and five α-helical regions. Structural analysis of Rab3a, a protein with 71% identity to Rab32, suggests that the RII binding region falls within the conserved α5 helix (Ostermeier and Brunger, 1999). Modeling studies using the coordinates from the Rab3a structure suggest that the α5 helix of these proteins are located on an exposed surface of the molecule and would be available for docking to RII (Figure 3.2D, both panels). Experimental confirmation of these observations was provided when the Rab32 175-205 peptide blocked RIIα interaction with a full length Rab32 GST fusion protein (Figure 3.2E, middle panel). A control peptide, where leucine 188 was replaced with proline to disrupt secondary structure, was unable to block RII interaction (Figure 3.2E, right panel). These data imply that RII interacts with Rab32 through an amphipathic helix formed by residues 178-197 of the protein. Solution binding assays confirmed that recombinant Rab32 bound [^{32}P] RIIα whereas the Rab32L188P mutant did not (Figure 3.2F). Together, these results show that Rab32 can interact with full length RII *in vitro* and that this interaction occurs through a predicted amphipathic helix in a manner similar to other AKAPs.

We next sought to determine if Rab32 is an AKAP *in vivo*. Flag-tagged versions of wild-type Rab32 and Rab32L188P mutant were expressed in HEK-293 cells. Immune complexes were isolated, and co-precipitation of PKA activity was measured by the filter paper assay (Corbin and Reimann, 1974). PKA activity was enriched 7.1 fold ± 2 (n =3) in Rab32 immune-complexes whereas there was no enhancement of kinase activity in the

Rab32L188P mutant immunoprecipitates (Figure 3.3A). Control experiments confirmed that this was specific PKA activity because the PKI (5-24) peptide, a specific inhibitor of the kinase, blocked all activity (Figure 3.3A). In parallel studies, only wild type Rab32 bound RII in the Flag immunoprecipitates as assessed by the RII overlay (Figure 3.3B) suggesting that Rab32 is the only AKAP responsible for the PKA activity. Thus transfected Rab32 associates with endogenous PKA holoenzyme inside mammalian cells.

Rab32 and closely related orthologs are the only Rab family members that function as AKAPs:

Each of the 60 genes that encode Rab proteins in the human genome is highly conserved in the α5 helix (Bock et al., 2001). Therefore, peptides corresponding to the α5 helix region from 9 other Rab proteins (sequences are presented in Figure 3.4A as a clustal alignment) were synthesized by the AutoSpot Robot to determine if other Rab family members could interact with RII. Solid-phase binding to RII was only detected for the Rab32 peptide (Figure 3.4B & 3.4C). Likewise only full-length Rab32 bound RII as assessed by the two-hybrid assay (Figure 3.4D). Structural studies on several AKAPs have established that alanines at position 6 of the PKA binding helix are critical anchoring determinants (Newlon et al., 2001). The methyl group of the alanine side-chain inserts into a hydrophobic pocket formed by the RII dimer. Rab32 has an alanine at this position, whereas most Rab family members have a phenylalanine at this site in the α5 helix (Figure 3.4A, star). To test the role of Ala 185 as an anchoring determinant, we introduced a phenylalanine by site directed mutagenesis. The Rab32A185F mutant protein was unable to bind RII in the overlay procedure (Figure 3.4E), and an A185F

Figure 3.3

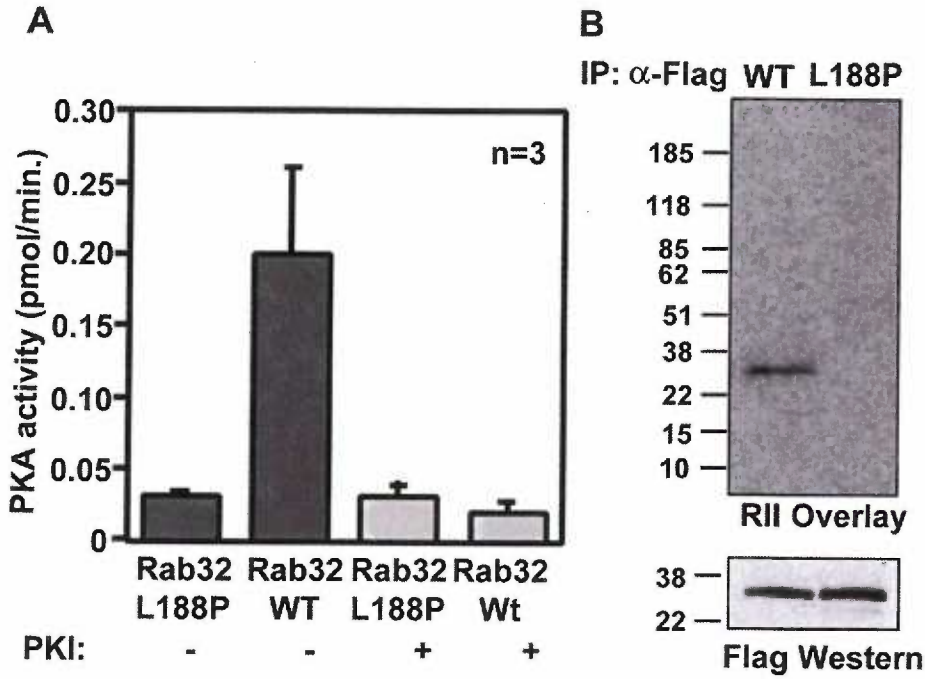

Figure 3.3: Rab32 can interact with the holoenzyme of PKA in mammalian cells. HEK-293 cells were transiently transfected with Flag-Rab32 or Flag-Rab32L188P cDNA. Triton-X soluble extracts were immunoprecipitated with sepharose conjugated anti-Flag antibody. **A)** Co-purification of the PKA holoenzyme was measured by assaying for PKA catalytic-subunit activity stimulated by exogenous cAMP. PKA activity was measured as pmol/min./mg of [^{32}P] incorporated into the PKA substrate kemptide using a filter paper binding assay (Corbin and Reimann, 1974). Specific PKA activity was blocked by 10μM of the inhibitor PKI (5-24) peptide. **B)** anti-Flag immunoprecipitates were separated on SDS-PAGE after the catalytic subunit had been eluted from the complex with exogenous cAMP, and electrotransferred to nitrocellulose. RII overlay assays **(Top panel)** were performed to determine if Rab32 was the only AKAP present in these fractions. Control experiments confirmed that equal levels of protein were immunoprecipitated in these experiments **(Lower panel)**. A representative example of three independent experiments is shown.

Figure 3.4

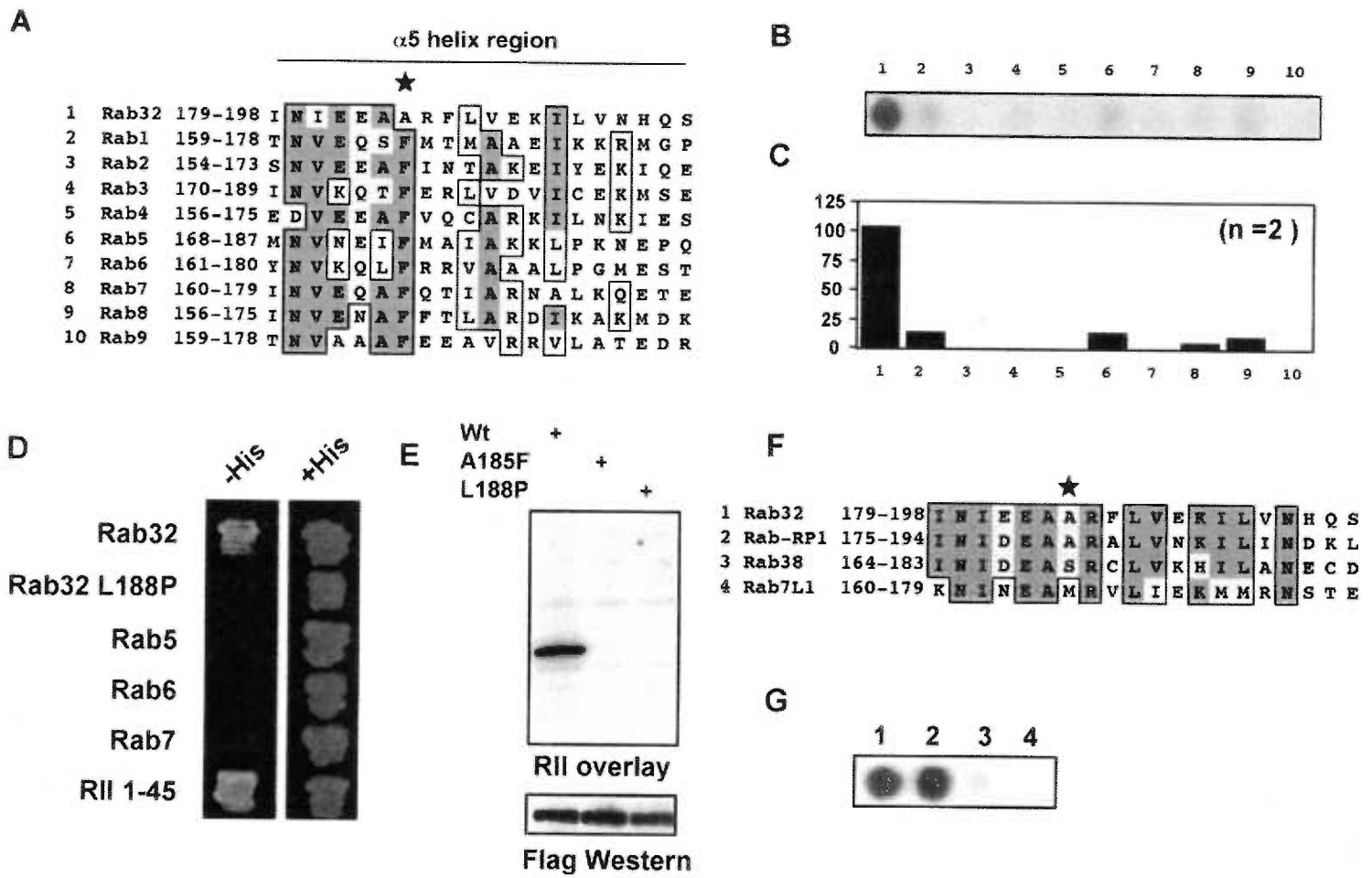
A
α5 helix region
1 Rab32 179-198 I N I E E A A R F L V E K I L V N H Q S
2 Rab1 159-178 T N V E Q S F M T M A A E I K K R M G P
3 Rab2 154-173 S N V E E A F I N T A K E I Y E K I Q E
4 Rab3 170-189 I N V K Q T F E R L V D V I C E K M S E
5 Rab4 156-175 E D V E E A F V Q C A R K I L N K I E S
6 Rab5 168-187 M N V N E I F M A I A K K L P K N E P Q
7 Rab6 161-180 Y N V K Q L F R R V A A A L P G M E S T
8 Rab7 160-179 I N V E Q A F Q T I A R N A L K Q E T E
9 Rab8 156-175 I N V E N A F F T L A R D I K A K M D K
10 Rab9 159-178 T N V A A A F E E A V R R V L A T E D R
B
1 2 3 4 5 6 7 8 9 10
C
125
100
75
50
25
0
(n =2)
1 2 3 4 5 6 7 8 9 10
D
-His
+His
Rab32
Rab32 L188P
Rab5
Rab6
Rab7
RII 1-45
E
Wt +
A185F +
L188P +
RII overlay
Flag Western
F
1 Rab32 179-198 I N I E E A A R F L V E K I L V N H Q S
2 Rab-RP1 175-194 I N I D E A A R A L V N K I L I N D K L
3 Rab38 164-183 I N I D E A S R C L V K H I L A N E C D
4 Rab7L1 160-179 K N I N E A M R V L I E K M M R N S T E
G
1 2 3 4

Figure 3.4: Only Rab32 and RabRP-1 contain determinants for PKA anchoring.
A) The sequences of Rab32 and nine other Rab proteins are aligned within the α5 helix region. The first and last residues of each sequence and the name of each Rab protein are indicated. Boxed and shaded regions depict sequence identity and similarity. Star indicates the conserved phenylalanine in most Rab proteins. **B)** A solid-phase peptide array of these Rab sequences was screened for RII binding by the overlay assay. Binding of [^{32}P] RIIα was detected by autoradiography. **C)** Quantitation of RII binding was assessed by densitometry. Arbitrary units were normalized to 1, indicating the highest level of RII binding. The data presented is an amalgamation of two independent experiments. **D)** Yeast two-hybrid analysis was used to determine if RII interacts with selected, full-length, Rab family members. Two-hybrid crosses of RIIα 1-45 fragment with full-length cDNAs for Rab32, Rab32L188P, Rab5, Rab6, or Rab7 fused to the LexA DNA binding domain. Interactions were assayed by growth on minimal media plates in the absence of histidine **(Left panel)**. As a toxicity control, all co-transformed yeast strains were able to grow in the presence of histidine **(Right panel)**. **E)** HEK-293 cell extracts expressing Flag-tagged Rab32 and mutants (indicated above each lane), were separated by SDS PAGE on 4-15% gels and electrotransferred to nitrocellulose. Binding of [^{32}P] RIIα was assessed by the overlay assay. **(Top panel)** RII binding was detected by autoradiography. Equal loading of recombinant Rab32 proteins was confirmed by western blot using anti-Flag antibodies. **F)** Sequence alignment of Rab32 and its most closely related family members. The first and last residues of each sequence and the name of each Rab protein are indicated. Boxed and shaded regions depict sequence similarity and identity. Star indicates the position of alanine 185 in the Rab32 sequence. **G)** A solid-phase peptide array of these closely related Rab sequences was screened for RII binding by the overlay assay. Binding of [^{32}P] RIIα was detected by autoradiography.

substituted peptide was also unable to interact with RIIα (data not shown). Database searches identified a Drosophila ortholog, Rab-RP1 that contained an alanine in a position analogous to residue 185 of Rab32. Peptide array analysis confirmed that Rab-RP1 bound RII in the overlay assay whereas two closely related Rab proteins lacking the conserved phenylalanine (Rab38 and Rab7L1) did not bind RII (Figure 3.4F & 3.4G). Collectively, these data suggest that Rab32 and orthologs are AKAPs.

Rab32 is associated with mitochondria:

The tissue distribution of Rab32 messenger RNA was determined by Northern blot using a specific 113bp probe. An mRNA species of 1.4kb was detected prominently in the liver and to a lesser extent in other tissues (Figure 3.5A). RNA dot blot analysis detected high levels of Rab32 mRNA in bone marrow, testis, colon, and fetal lung (data not shown). This latter observation prompted us to examine the subcellular distribution of the Rab32 protein in the human fetal lung fibroblast cell-line WI-38. As a prelude to these studies, anti-peptide antibodies were raised against residues 202 to 220 of Rab32 and affinity purified against the recombinant protein. A single protein band of 27 kDa was detected in cell extracts as assessed by Immuno-blot (Figure 3.5B, left panel). Pre-incubation with the antigenic peptide blocked antibody recognition of this 27kDa protein (data not shown). Control experiments using the pre-immune serum were negative (Figure 3.5B, right panel).

Immunofluorescent analysis of Rab32 in WI-38 fibroblasts revealed a staining pattern (Figure 3.5C) that overlapped with the mitochondrial marker MitoTracker Red™ (Figure 3.5D) but was distinct from tubulin (Figure 3.5E). This is most clearly demonstrated in a

Figure 3.5

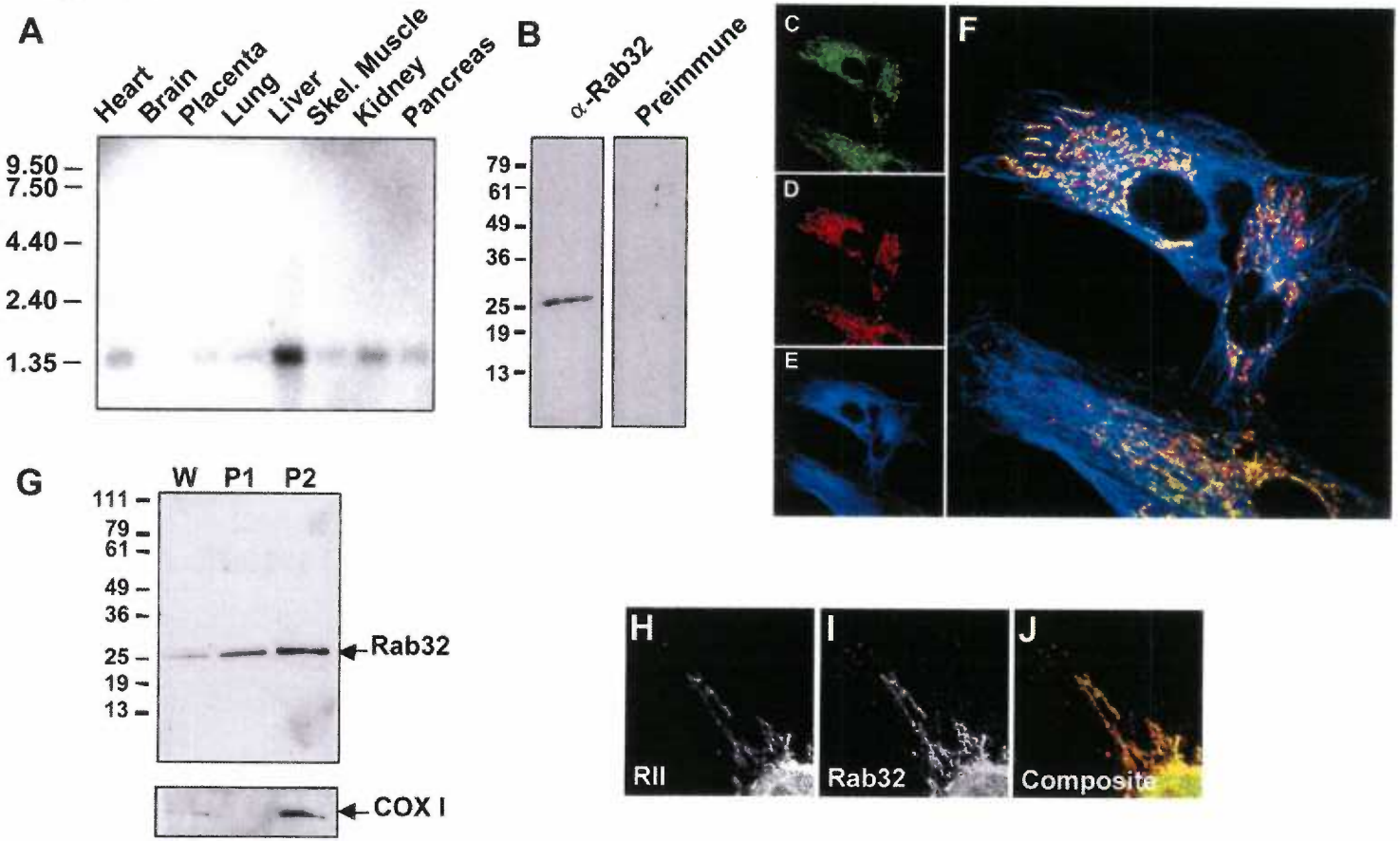
A
Heart
Brain
Placenta
Lung
Liver
Skel. Muscle
Kidney
Pancreas
9.50
7.50
4.40
2.40
1.35
B
α-Rab32
Preimmune
79
61
49
36
25
19
13
C
D
E
F
G
W
P1
P2
111
79
61
49
36
25
19
13
Rab32
COX I
H
RII
I
Rab32
J
Composite

Figure 3.5: The cellular and subcellular distribution of Rab32.
The tissue distribution of Rab32 mRNA was assessed by Northern blot analysis. **A)** A human multi-tissue (tissue sources indicated above each lane) was screened using a 113 base pair cDNA probe that corresponds to the C-terminal hypervariable region of Rab32. Hybridization was detected by autoradiography. The sizes of DNA markers are indicated. **B)** Triton-X soluble extracts from the WI-38 human fetal-lung fibroblasts were immunoblotted with affinity purified anti-Rab32 antibody **(Left panel)** or pre-immune sera **(Right panel)**. Signals were detected by chemiluminescence. Molecular weight standards are indicated. Confocal Immunofluorescence microscopy of WI-38 fibroblasts triple labeled with polyclonal antibodies against Rab32 **(C, green)**, cell permeable dye MitoTracker Red™ **(D, red)** and a monoclonal antibody against α-tubulin **(E, blue)**. A merged image **(F)** indicates the cellular distribution of all three signals. **G)** Subcellular fractionation of WI-38 cells was performed according to the materials and methods. Protein (20μg each) from whole cell (W), nuclear (P1), and mitochondria enriched (P2) fractions were subjected to SDS-Page and electrotransferred to nitrocellulose. Membranes were immunobloted using affinity purified polyclonal anti-Rab32 antibody **(Top panel)**, and a monoclonal anti-cytochrome oxidase subunit I **(Bottom panel)** as a marker for mitochondria. Immunocytochemistry and confocal analysis were used to demonstrate the subcellular location of RII **(H)** and Rab32 **(I)**. A merged image shows a significant overlap of the signals **(J)**.

composite image (Figure 3.5F). In support of this observation Rab32 was enriched in the P2 fraction (Figure 3.5G, top panel) when WI-38 cell extracts were subjected to subcellular fractionation. The partitioning of Rab32 correlated with the detection of the mitochondrial marker, Cytochrome Oxidase Subunit 1 (Figure 3.5G, bottom panel). Immunofluorescence data also suggested that a proportion of cellular RII signal is present at mitochondria (Figure 3.5H) and overlaps with the Rab32 signal (Figure 3.5I & 3.5J). Collectively, these results suggest that Rab32 and RII associate with mitochondria.

Mitochondria are arranged around the microtubule organizing center and utilize microtubules for their movement. However, Rab32 association with mitochondria is not dependent on the integrity of the microtubule cytoskeleton (Figure 3.6A-H). Cos7 cells were treated with the microtubule depolimerizing agent nocodazole and the location of Rab32, mitochondria, and microtubules were visualized by Immunofluorescence detection (Figure 3.6E-H). As expected nocodazole treatment disrupted the microtubule network (Figure 3.6G) and promoted a redistribution of mitochondria (Figure 3.6F & 3.6H) when compared to control cells (Figure 3.6B-D). Importantly, Rab32 was retained on the mitochondria after drug treatment (Figure 3.6E & 3.6H) indicating that it specifically associates with this organelle.

Mitochondrial targeting sequences have been identified in a number of proteins including other AKAPs (Huang et al., 1999; Lin et al., 1995), yet no such sequences were evident in Rab32. Previous studies have proposed that lipid modification of two C-terminal cysteines may participate in the membrane targeting of Rab proteins (Takai et al., 2001). We generated a Flag-tagged form of Rab32 that lacked the pair of C-terminal cysteine residues (Figure 3.6J). The Rab32ΔCC mutant did not associate with

Figure 3.6

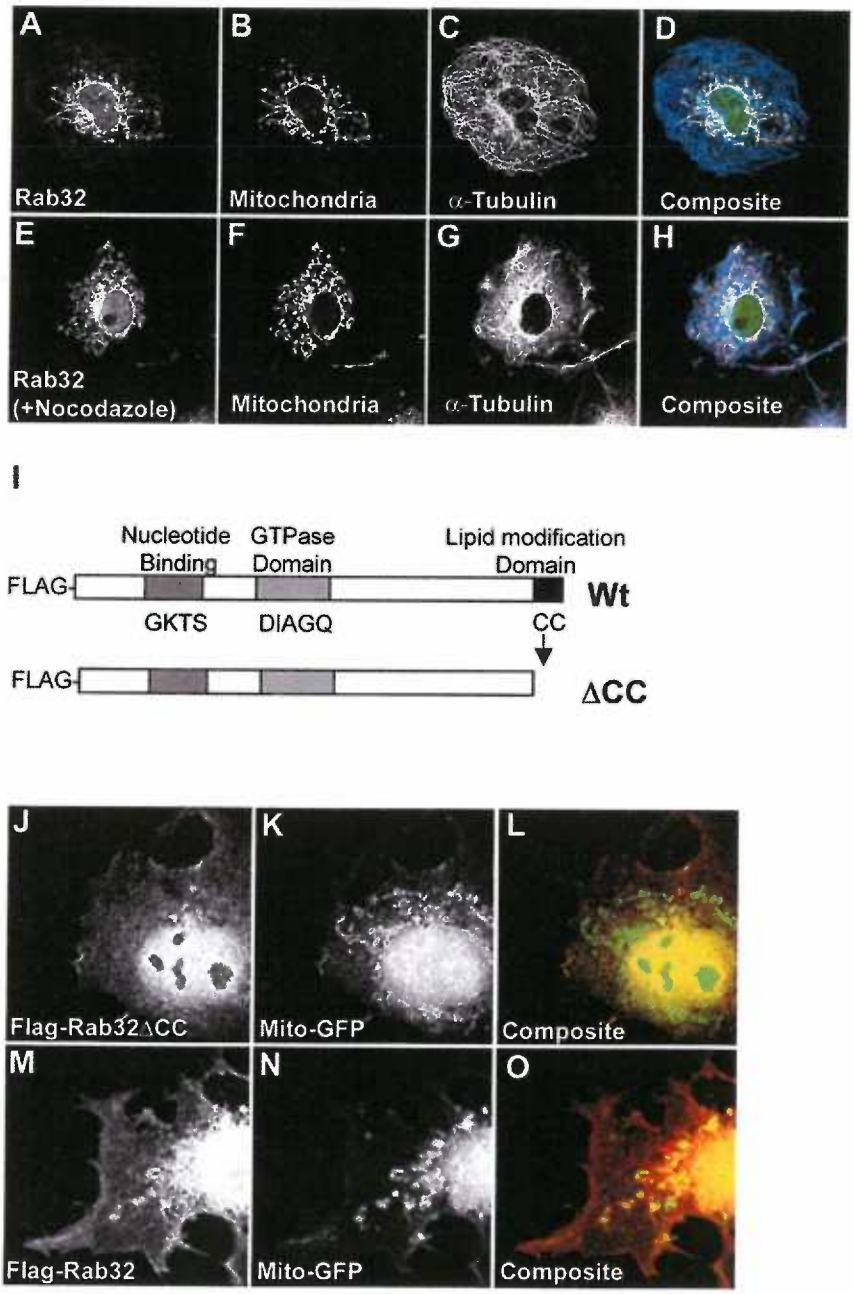
A
B
C
D
Rab32
Mitochondria
α-Tubulin
Composite
E
F
G
H
Rab32
(+Nocodazole)
Mitochondria
α-Tubulin
Composite
I
Nucleotide Binding
GTPase Domain
Lipid modification Domain
FLAG
Wt
GKTS
DIAGQ
CC
FLAG
ΔCC
J
K
L
Flag-Rab32ΔCC
Mito-GFP
Composite
M
N
O
Flag-Rab32
Mito-GFP
Composite

Figure 3.6: Mitochondrial targeting of Rab32.

Immunofluorescence analysis of Rab 32 location upon depolymerization of microtubules inCos7 cells. Control (**A-D**) or Cos7 cells treated with 5μM nocodazole for 30 minutes (**E-H**) were fixed and stained. Immunocytochemistry was performed using anti-Rab32 (**A & E, green**), MitoTracker Red™ (**B & F, red**), and a monoclonal antibody against α-tubulin (**C & G, blue**). Composite images are shown for each sample (**D & H**). **I)** Schematic representation of a nucleotide binding site, GTPase domain, and sites of putative lipid modification are indicated. Arrows indicate an amino acid substitution. **J)** Flag-tagged wild-type or Rab32ΔCC mutant were expressed in Cos7 cells. The subcellular distribution of recombinant Rab32ΔCC (**J**) or Rab32 (**M**) were analyzed using confocal fluorescence microscopy with an anti-Flag monoclonal antibody and Texas Red conjugated secondary (**J & M**). Mitochondria were visualized by using Mito-GFP targeting construct (**K & N**). Merged images are presented (**L & O**).

mitochondria when expressed in Cos7 cells (Figure 3.6K & 3.6L). Mitochondria were detected by co-expression of a GFP fusion protein that contained a mitochondrial targeting signal (Figure 3.6K & 3.6N). Control experiments demonstrated that wild-type Rab32 co-distributed with the Mito-GFP tag when both constructs were transfected into Cos7 cells (Figure 3.6M-O). Additional controls demonstrated that the Rab32L188P mutant, which is unable to anchor PKA, was correctly targeted to mitochondria (data not shown). These findings suggest that C-terminal lipid modification of Rab32 contributes to mitochondrial targeting, most likely to the outer mitochondrial membrane.

Biochemical and cellular analyses of Rab32:

Rab32 has four conserved guanine nucleotide binding regions including a possible GTPase domain (Figure 3.7A). Using an *in vitro* binding assay we found that [^{3}H] GDP dissociated from Rab32 with a T1/2 of 7.5 min. (n=3) (Figure 3.7B). Using a similar assay, GTP binding was measured with a T1/2 of 12 min. (n=3, Figure 3.7C). These studies show, as predicted, that Rab32 is a guanine nucleotide binding protein. On the basis of homology to other Ras family members we predicted that mutation of threonine 39, which lies within the GTP binding domain, would abolish interaction with this nucleotide (Figure 3.7A). As expected the Rab32T39N mutant bound GTP to a 12-fold lesser extent when compared to the wild-type protein (Figure 3.7D). Equal amounts of both Rab32 forms were used in these assays (Figure 3.7D insert). We next sought to determine if RII preferentially interacts with Rab32 in a nucleotide dependent manner. Rab32 was preloaded with either GDP or the non-hydrolyzable GTP analog, GTPγS. Rab32 could associate with RII whether it was loaded with GDP or GTPγS (Figure 3.7E).

Figure 3.7

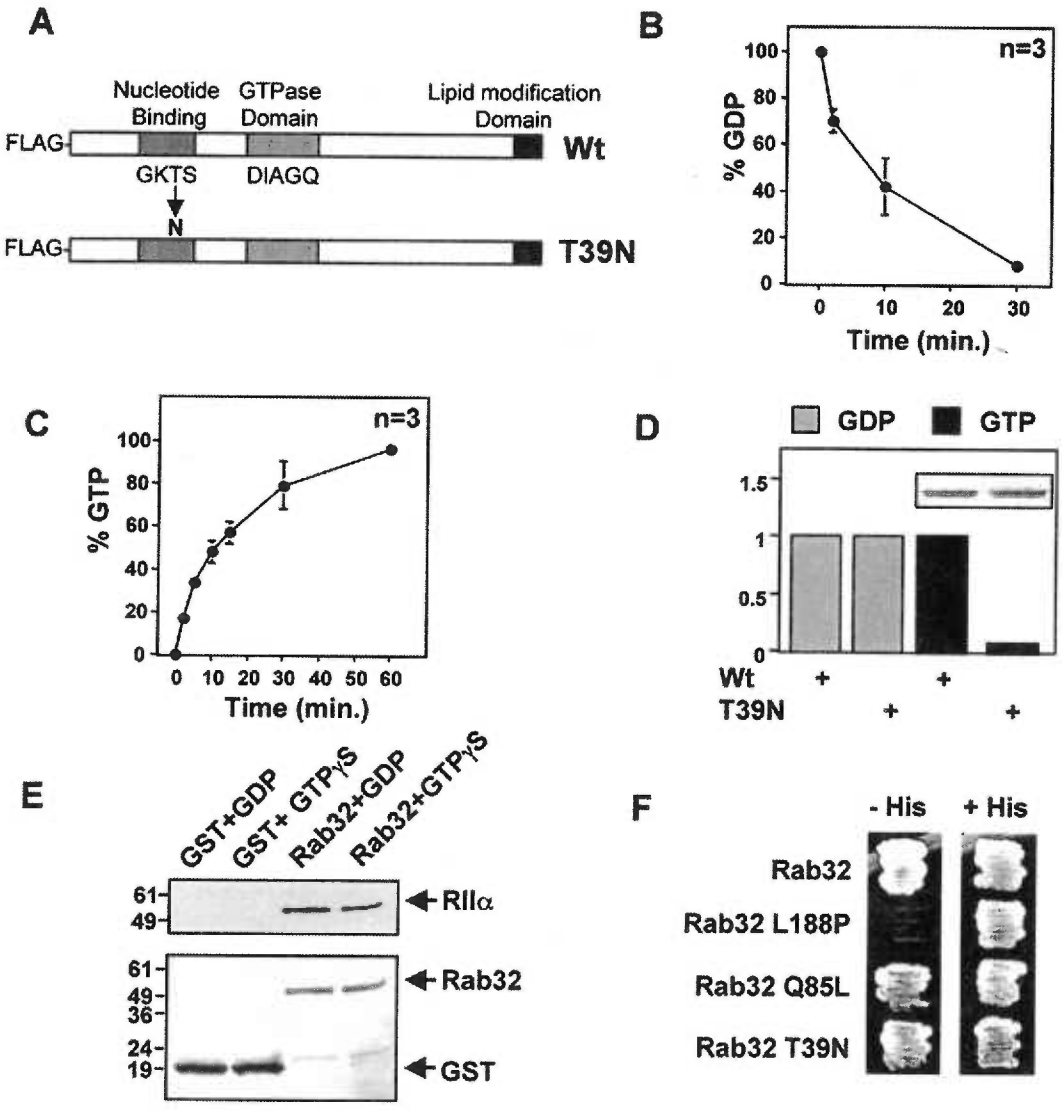
A
Nucleotide Binding
GTPase Domain
Lipid modification Domain
FLAG
Wt
GKTS
DIAGQ
N
FLAG
T39N
B
n=3
% GDP
Time (min.)
C
n=3
% GTP
Time (min.)
D
GDP
GTP
Wt
T39N
E
GST+GDP
GST+ GTPγS
Rab32+GDP
Rab32+GTPγS
RIIα
Rab32
GST
F
- His
+ His
Rab32
Rab32 L188P
Rab32 Q85L
Rab32 T39N

Figure 3.7: Nucleotide binding properties and GTPase activity of Rab32.

Bacterially purified GST-fusion proteins were assayed for guanine nucleotide binding. **A)** Schematic diagram depicting a mutation in the nucleotide binding domain of Rab32. A nucleotide binding site, GTPase domain, and sites of putative lipid modifications are indicated. **B)** GST-Rab32 (2μg) was preloaded with 2μCi of [^{3}H] GDP. GDP off-rate is presented as %GDP bound to Rab32 over time. **C)** Nucleotide depleted Rab32 was incubated with [γ^{32}P] GTP. The GTP on-rate for GST-Rab32 was measured and is presented as the % of GTP bound over time. Amalgamated data from three experiments are presented. **D)** Biochemical characterization of guanine nucleotide binding to recombinant wild-type GST-Rab32, and GST-Rab32T39N mutant. [^{3}H] GDP-binding (gray bars) and [γ^{32}P] GTP binding (black bars) to Rab32 and Rab32T39N are indicated. Results are presented as the % nucleotide bound normalized to 1. 2μg of GST recombinant proteins were used and shown in the inset. A representative experiment from three independent analyses is presented. **E)** GST-pulldowns of GDP loaded Rab32 or GTPγS loaded Rab32 in the presence of recombinant RII (1μg). Proteins were separated by SDS-PAGE and transferred to nitrocellulose. **(Top panel)** Co-precipitation of RII was detected by western blot using an anti-RII monoclonal antibody. **(Bottom Panel)** Control experiments showing equal loading of proteins. **F)** Yeast co-expressing pLexA Rab32 mutants and pACT2 RII 1-45 were assayed for growth on minimal media without hisitidine **(Left panel).** As a toxicity control, yeast harboring these plasmids can grow in media supplemented with histidine **(Right panel).**

Yeast two-hybrid assay confirmed this result as the Rab32T39N mutant and Rab32Q85L, a potential GTPase inactive form, interacts with RII (Figure 3.7F). These data show that Rab32 interacts with RII in a nucleotide independent state.

In cellular studies, overexpression of the GTP binding deficient mutant Rab32T39N caused the condensation of mitochondria at the microtubule organizing centers as assessed by immunofluorescence techniques (Figure 3.8A-C). In contrast, overexpression of the wild-type protein had little or no effect on mitochondrial distribution (Figure 3.8D-E). The frequency of mitochondrial collapse was measured in transfected cells (50 cells each) from three separate experiments (Figure 3.8G). Mitochondrial collapse was detected in 56 ± 4% of cells expressing the Rab32T39N mutant (Figure 3.8G) and only 8.6 ± 2% of the wild type cells exhibited this phenomena (Figure 3.8D-E & 3.8G). Mitochondrial collapse did not appear to involve PKA anchoring since expression of the PKA binding deficient mutant, Rab32L188P, did not alter the incidence of mitochondrial collapse (Figure 3.8G). In addition, expression of a Rab32T39N, L188P double mutant caused the same degree of mitochondrial collapse as the Rab32T39N mutant (Figure 3.8G). However, correct targeting to the mitochondria appears to contribute to this phenomena as expression of Rab32T39N, ΔCC, a GTP binding deficient mutant lacking the C-terminal cysteine pair, displayed a reduced incidence of mitochondrial collapse (32 ± 2%, Figure 8G). Since endogenous Rab32 was detected in Cos7 cells (Figure 3.6A & 3.6E), our cellular experiments suggest that Rab32T39N acts as a dominant negative mutant. In specificity-control experiments, we did not observe any abnormal clustering of endosomes (Figure 3.8H-J), lysosomes

Figure 3.8

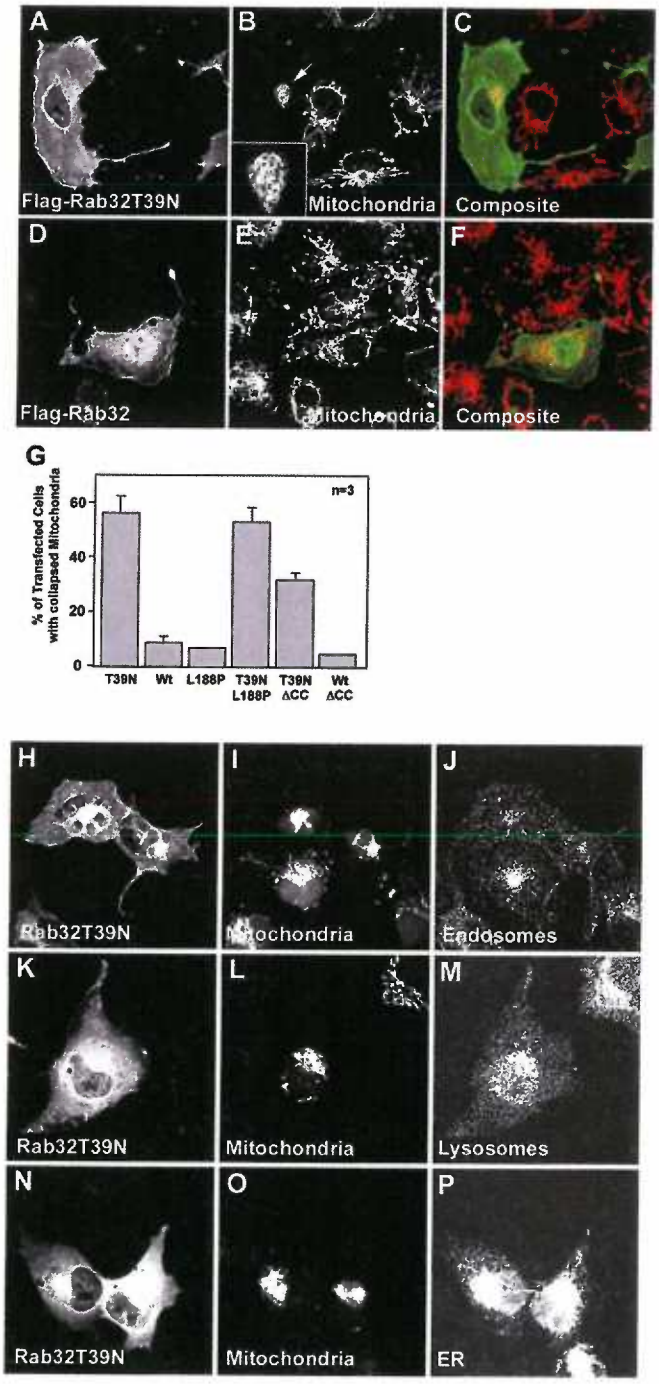
A
B
C
Flag-Rab32T39N
Mitochondria
Composite
D
E
F
Flag-Rab32
Mitochondria
Composite
G
% of Transfected Cells with collapsed Mitochondria
n=3
60
40
20
0
T39N
Wt
L188P
T39N L188P
T39N ΔCC
Wt ΔCC
H
I
J
Rab32T39N
Mitochondria
Endosomes
K
L
M
Rab32T39N
Mitochondria
Lysosomes
N
O
P
Rab32T39N
Mitochondria
ER

Figure 3.8: The GTP binding mutant Rab32T39N induces mitochondrial collapse around the microtubule organizing center.

Cos7 cells were transiently transfected with Flag-Rab32 or Flag-Rab32T39N. Cells were labeled with MitoTracker Red™ for 30 min, fixed and permeabalized before the Rab proteins detected with a Fitc-conjugated anti-Flag antibody. **A)** Rab32T39N mutant or **D)** Rab32, and **(B & E)** mitochondria are shown. **(C & F)** Composite images are presented. A mitochondrial collapse phenotype is observed in Rab32T39N expressing cells **(B, inset).**

(G) Mitochondrial collapse was scored in cells expressing various Rab32 mutants (indicated below each column). Data is presented as the number of cells exhibiting the phenotype per number of cells transfected. 50 Rab expressing cell were analyzed in three independent experiments for each construct.

Control experiments were performed to determine if expression of Rab32T39N **(H, K, & N)** alters the morphology or distribution of other organelles. **(I, L, O)** Mitochondria were visualized with MitoTracker Red™, **(J)** Endosomes were stained with antibodies against the marker protein EEA-1, **(M)** Lysosomes were stained with antibodies against the marker protein LAMP-1, and **(P)** Endoplamic Reticulumn were stained with antibodies against the marker protein Calnexin.

(Figure 8K-M), or endoplasmic reticulumn (Figure 3.8N-P) in cells exhibiting a Rab32T39N induced mitochondrial collapse.

Rab proteins can function in membrane fusion, membrane fission and regulate organelle motility. Our data thus far does not distinguish between these events. Expression of Rab32T39N at low levels promotes an intermediate phenotype in which the mitochondria are elongated and highly interconnected (Figure 3.9 A-D). To further explore this effect Rab32T39N transfected cells were subjected to nocodazole treatment for 1hour prior to fixation (Figure 3.9E). Depolymerization of the microtubules did not result in a dispersion of the mitochondria (Figure 3.9F & 3.9G) but there was a preponderance of highly interconnected and elongated mitochondria (Figure 3.9G). Often there were fewer mitochondria per cell suggesting that expression of Rab32T39N promoted abnormal fusion (data not shown). In contrast, Cos7 cells expressing wild-type Rab32 are individually dispersed throughout the cytoplasm upon nocodazole treatment (Figure 3.9 I-L).

Figure 3.9

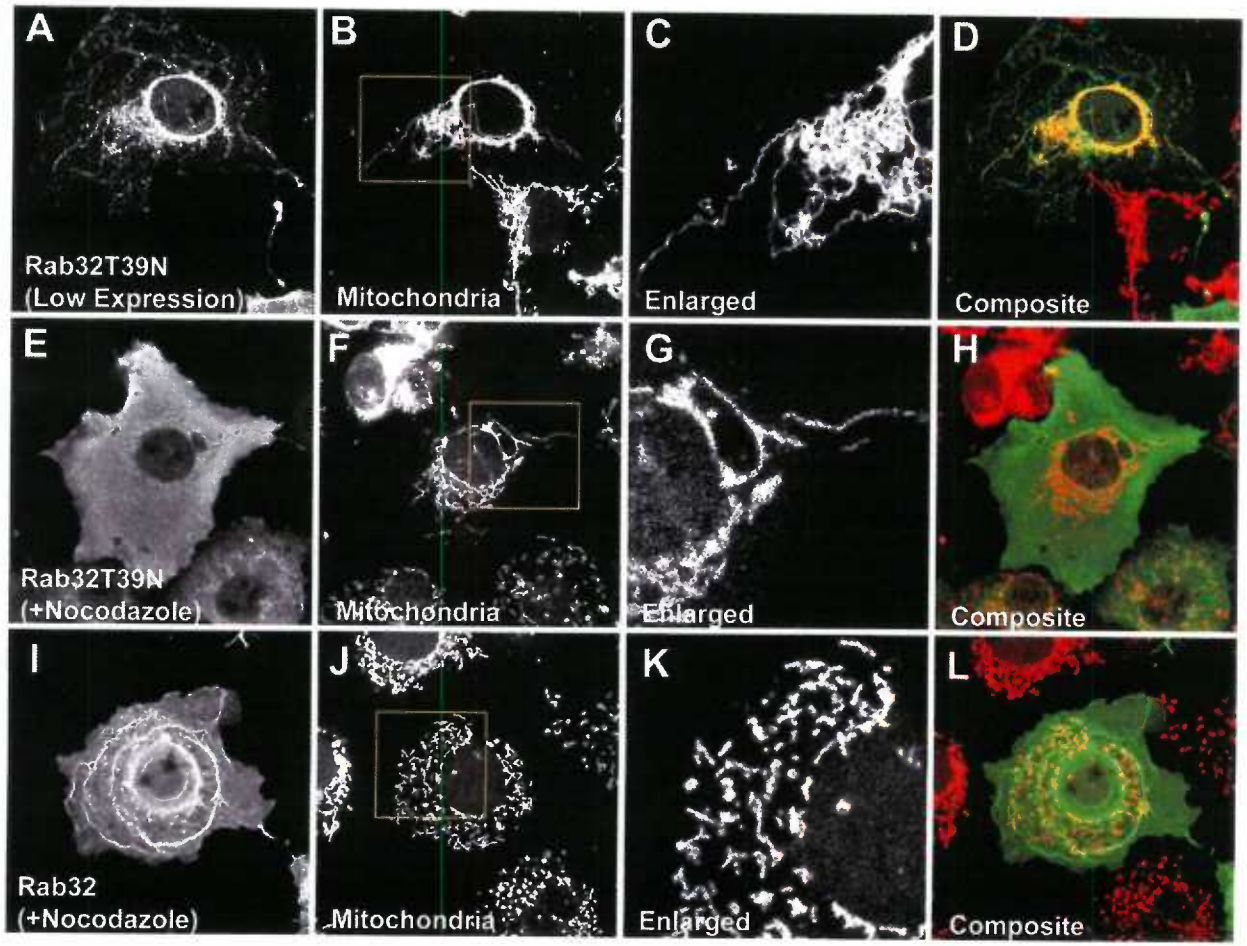
A
Rab32T39N
(Low Expression)
B
Mitochondria
C
Enlarged
D
Composite
E
Rab32T39N
(+Nocodazole)
F
Mitochondria
G
Enlarged
H
Composite
I
Rab32
(+Nocodazole)
J
Mitochondria
K
Enlarged
L
Composite

Figure 3.9: Expression of Rab32T39N leads to aberrant mitochondrial fusion. Analysis of mitochondrial morphology was performed on cells expressing various levels of Rab32T39N **(A-H)** and the wild-type protein **(I-L). A)** Analysis of collapsed mitochondrial morphology in Cos7 cells expressing low levels of Flag-Rab32T39N. **B)** Mitochondria were detected with MitoTracker Red™ stain and **C)** a 3x enlargement of the boxed region shown in B (yellow box). **D)** Composite image of **(A)** and **(B)** are presented. **E)** Analysis of collapsed mitochondrial morphology in Cos7 cells expressing Flag-Rab32T39N treated with 5μM nocodazole for 1 hour. **F)** Mitochondria were detected with MitoTracker Red™ stain and **G)** a 3x enlargement of the boxed region shown in F (yellow box). **H)** Composite image of **(E)** and **(F)** are presented. **I)** Analysis of dispersed mitochondrial morphology in Cos7 cells expressing Flag-Rab32 treated with 5μM nocodazole for 1 hour. **J)** Mitochondria were detected with MitoTracker Red™ stain and **K)** a 3x enlargement of the boxed region shown in J (yellow box). **L)** Composite image of **(I)** and **(J)** are presented.

Discussion

Rab32 is a member of the Ras superfamily of small molecular weight G-proteins and belongs to a subclass of these proteins that have a non-consensus catalytic domain (Bao et al., 2002). We now show that Rab32 functions as an A-kinase Anchoring Protein (AKAP) as it associates with the cAMP-dependent protein kinase (PKA) inside cells. Although we have previously shown that two Rho family GTPases interact with AKAPs (Diviani et al., 2001; Westphal et al., 2000), we provide the first evidence for a direct interaction between a small molecular weight G-protein and the PKA holoenzyme. Interestingly, two other Rab family members interact with protein kinases. Rab8 binds to a GC kinase in a GTP-dependent manner (Ren et al., 1996), and Rab3d co-purifies with a ser/thr kinase in the RBL-2H3 mast cell-line (Pombo et al., 2001). Our study further emphasizes two additional properties of Rab32: it is localized to mitochondria and it participates in the control of mitochondrial dynamics.

AKAPs are a growing family of functionally related proteins that are classified on the basis of their interaction with the R subunits of the PKA holoenzyme. Common properties include a conserved helical region of 14 to 18 residues that binds to the R subunit dimer (Carr et al., 1991; Newlon et al., 2001). Our mutagenesis and peptide-binding experiments have located PKA anchoring determinants between residues 178 to 197 of Rab32 which include the α5 helical region that is conserved in all Ras family members (Bourne et al., 1991). Our modeling studies used the coordinates from the crystal structure of Rab3a, to predict that the α5 helix of Rab32 is exposed and available to participate in PKA anchoring. Interestingly other members of the Ras super family

utilize this helix in a variety of protein-protein interactions (Maesaki et al., 1999; Morreale et al., 2000). However, only Rab32 and Rab RP-1, a closely related Drosophila ortholog, bind to RII experimentally. This indicates that determinants reside within the α5 helices of both isoforms that accommodate PKA anchoring. Surprisingly, the ability to bind PKA may be determined by a single amino acid side-chain. Rab32 and RabRP-1 contain an alanine at position 185 within the helix (numbering based on the Rab32 sequence), whereas most other Rab proteins have a phenylalanine at this position. In fact, substitution with phenylalanine at this site abolishes PKA binding to Rab32, suggesting that a bulky hydrophobic side-chain at this site perturbs PKA anchoring. Likewise, introduction of phenylalanine at the corresponding sites in AKAP79, mAKAP, AKAP-KL, AKAP18 or AKAP-Lbc abolishes interaction with RII as assessed by peptide array analysis (N.M. Alto and J.D. Scott, unpublished observations). These findings emphasize the high degree of specificity that is built into the PKA anchoring regions.

A proposed function for AKAPs is to direct PKA and other signaling enzymes close to their substrates (Colledge and Scott, 1999; Smith and Scott, 2002). This is generally achieved by targeting domains that direct the AKAP signaling complex to discrete cellular environments. Our subcelluar fractionation and immunolocalization data indicate that Rab32 is localized at the mitochondria. While this is the first report of a Rab protein associated with this organelle, other AKAPs such as sAKAP84/D-AKAP-1 and D-AKAP-2 are targeted to the outer mitochondrial membranes (Huang et al., 1997a; Huang et al., 1997b). Mitochondrial targeting of sAKAP84/D-AKAP-1 is mediated by a consensus mitochondrial targeting sequence (Huang et al., 1999; Lin et al., 1995). Rab32 does not contain this sequence, but rather has a pair of cysteines at the C-terminus that

are necessary for correct targeting. Both residues are conserved in most Rab proteins and are likely to be sites of lipid modification. Such modifications may be required for insertion and retention of Rab32 in the mitochondrial membrane (Glomset and Farnsworth, 1994). However, additional protein-protein interactions are likely to specify mitochondrial targeting since high-level expression of Rab32 leads to the detection of the protein throughout the cytoplasm (Figure 3.8D). Overexpression of Rab32 may saturate its "receptor protein" on the mitochondrial membrane leading to an excess accumulation in the cytoplasm. It is likely that the C-terminal hypervariable domain of Rab32 is responsible for such interactions (Chavrier et al., 1991), however the exact mechanism of targeting remains to be determined.

Most Rab proteins cycle between an on state where GTP is bound and an off state where GDP is bound. Accordingly, Rabs can elicit GTP dependent biological responses by activating down stream effector. We show that PKA can associate with Rab32 in presence of GTP or GDP. This is consistent with the notion that Rab32 functions as a conventional AKAP as there are several examples of anchoring proteins that bind PKA and contain functionally independent domains that possess other biological activities (Diviani et al., 2001; Westphal et al., 2000). Alternatively, there may be multiple pools of Rab32, some of which harbor anchored PKA. This is supported by our immunolocalization data indicating that not all of the mitochondrial targeted Rab32 is associated with PKA. Thus, the Rab32/PKA complex may participate in distinct cellular processes from the free Rab protein. Defining the molecular interactions regulated by Rab32 should give insight into the PKA function of this complex. For example, specific

Rab32 effector proteins, GTPase activating proteins, and Guanine nucleotide exchange factors may serve as PKA substrates.

Rab family members participate in membrane trafficking events that are often associated with organelles such as the endoplasmic reticulumn, the golgi network, endosomes and membrane vesicles. Functions for some of these proteins have been described by genetic analyses in yeast, flies, and mice or by the expression of interfering mutants in mammalian cells. Using the latter approach we have established that transient transfection of a GTP-binding deficient form, Rab32T39N, selectively induces the collapse of mitochondria at microtubule organizing centers, yet has no effect on the distribution of other organelles (Figure 3.8). We propose that the Rab32T39N mutant acts as a dominant negative inhibitor of the endogenous Rab32 protein. Consistent with this notion, endogenous Rab32 is found in Cos7 cells and localizes to the mitochondria (Figure 3.6A). Furthermore, expression of the non-mitochondrial targeted double mutant, Rab32T39N ΔCC, exhibited a significant reduction in the incidence of mitochondrial collapse. This data suggests that mitochondrial localization of the Rab32T39N mutant contributes to the phenotype. It is possible that the Rab32T39N interferes with an upstream activator such as a guanine nucleotide exchange factor (GEF) that is localized at mitochondria. This proposition is analogous to the mechanism by which GTP-binding deficient Ras mutants act as dominant inhibitors of specific Ras-GEF proteins (Farnsworth and Feig, 1991).

Our analysis of the Rab32T39N mutant points toward a role for Rab32 in the regulation of mitochondrial fission events as we detect perinuclear clusters of mitochondria upon overexpression of this interfering mutant. Similar effects are

observed in cells expressing an interfering mutant of the dynamin-related GTPase Drp1 which functions to sever the outer mitochondrial membrane in yeast, *C. elegans*, and mammalian cells (Labrousse et al., 1999; Sesaki and Jensen, 1999; Smirnova et al., 2001). Expression of Drp-1 interfering mutants results in a frequency of collapsed mitochondria and disruption of microtubule network with nocodazole reveals a fewer number of elongated organelles (Smirnova et al., 2001). These observations are remarkably similar to the changes in mitochondrial morphology that we have recorded upon drug treatment in Rab32T39N expressing cells. Furthermore, low expression of Rab32T39N reveals an intermediate phenotype in which the mitochondria are partially collapsed. We observed a dense network of seemingly fused mitochondria in these cells. A potential mechanism for the action of the Rab32T39N mutant is to shift the balance of mitochondrial fusion and fission events toward increased fusion. Therefore, the cellular process regulated by Rab32 is most likely mitochondrial division. Future studies will be aimed at elucidating the complement of effector proteins involved in the assembly of a Rab32 mediated mitochondrial fission complex, and the role of anchored PKA in the regulation of these events.

CHAPTER FOUR

Conclusions and Future Directions

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AKAPIS:

Conclusions

The identification of pharmacological inhibitors that potently and specifically disrupt protein-protein interactions is of experimental interest. Typically two different strategies are used for their design. First, a rational approach based on the detailed structural information of target molecules and computer aided protein analysis is used to make chemical modifications in existing drugs or for the creation of drugs *de novo* (Amzel, 1998). Rational design approaches are hampered by the difficulty in obtaining detailed structural information about proteins in a complex and the chemical modifications do not always perform as predicted (Kast and Hilvert, 1997; Shao and Arnold, 1996). Second, libraries of randomized sequences can be screen for a functional activity (Mayer et al., 1999). The majority of drugs developed using these methods are antagonists that fit into small, well-defined binding pockets of enzymes or receptors. However, the fact that small molecule inhibitors cannot antagonize large protein interfaces, and the challenges of making such inhibitors cell permeable, has made designing drugs to disrupt protein-protein interactions quite challenging (Nord et al., 1997). Alternative to these methods, it is possible to use naturally occurring proteins as templates to design high affinity inhibitory reagents (Nygren and Uhlen, 1997).

Work presented in this thesis describes the creation and testing of a highly potent, synthetic peptide inhibitor of PKA-AKAP interaction. Past studies have indicated that small molecules do not have the ability to antagonize this protein-protein interaction (J.D. Scott, personal communication). Thus an alternative approach was taken in the pursuit of

developing an antagonist of PKA anchoring *in vivo* (**Figure 4.1**). First we identifed the minimal RII binding domains of endogenous AKAPs and classified them based on affinity. Next, using the high affinity sequences as templates, we generated a consensus sequence with a probability-based computer algorithm. This synthetic peptide was further optimized by screening a library of single point substitutions for increased binding to RII. We have designated the final peptide sequence as AKAPIS for its design "*in silico*".

Our biochemical and cellular data suggest that AKAPIS can be used as a highly potent inhibitor of PKA anchoring and is better than the existing inhibitors used today. Several lines of evidence support this: First, AKAPIS can immunoprecipitate both isoforms of RII, suggesting that they interact *in vivo*. Second, overexpression of AKAPIS results in the displacement of RII from sites at which it is normally anchored. In an attempt to determine if AKAPIS is a more potent inhibitor of PKA anchoring than previously described inhibitors, we directly compared AKAPIS to Ht31 in different experimental systems. In biochemical experiments, AKAPIS has 4- to 5-fold higher affinity for RII than Ht-31. The dissociation constant for AKAPIS measured by fluorescence polarization is 0.45 nM versus to 2.2 nM for Ht31 under identical conditions. Furthermore, AKAPIS interacts with more type-II PKA then Ht31 inside cells, presumably through the displacement of PKA from endogenous AKAPs. These data are important as they show not only that AKAPIS is a more potent inhibitor of PKA anchoring than Ht31, but also suggest that Ht31 does not completely displace all of the RII from anchored sites within the cell. This notion is supported by our functional data. Using a reconstituted system, Dr. Naoto Hoshi found that AKAPIS could displace more

Figure 4.1

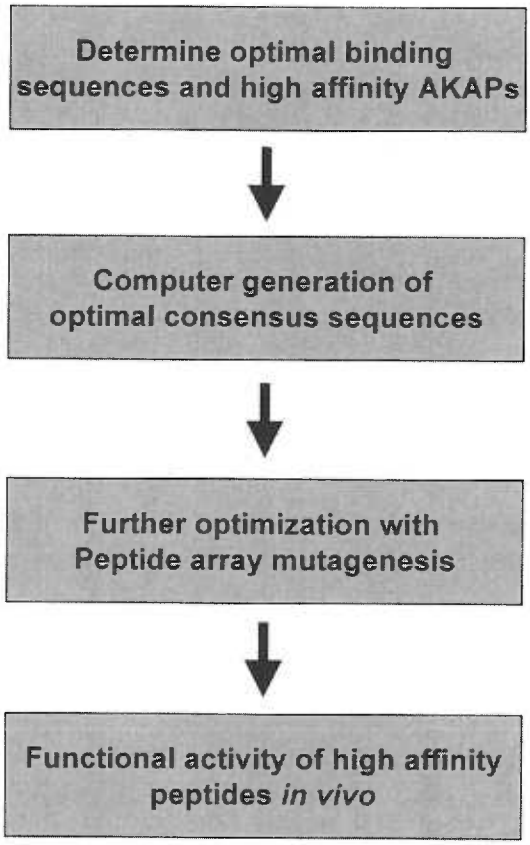

Figure 4.1: Flow Chart for the Design of AKAPIS

PKA from an AMPA channel complex then Ht31. AKAPIS not only displaces more PKA from endogenous AKAPs than Ht31, but also does so with kinetics that mimics complete kinase inhibition by PKI. Collectively, the data presented in this dissertation demonstrates that AKAPIS is a highly potent inhibitor of PKA anchoring and will be an important reagent for future studies.

AKAPIS:

Future Directions

Future directions will be aimed at using AKAPIS to identify novel physiological processes that require anchored PKA. The AKAPIS peptides we have synthesized can be delivered into cultured cells by either microinjection or through a patch-pipet in electrophysiological experiments. In addition, I have created a functional chimeric cDNA that can be transfected into mammalian cells. The expressed protein from this construct can be immunoprecipitated with antibodies that recognize an epitope-tag or directly visualized in cells by intrinsic YFP fluorescence. However, the ability to express AKAPIS, quickly, and in a non-invasive manner will greatly enhance future studies. For example, to test the effects of PKA anchoring in real-time (i.e. using live cell imaging), AKAPIS must be delivered quickly and efficiently during the time-course of an experiment.

One potential method for delivering AKAPIS into cells is to conjugate it to TAT, Ant, or poly-arg peptides (Lindgren et al., 2000)(Matsushita, 2001). Recently, it was demonstrated that highly basic peptides could cross biological membranes efficiently and

independently of transporters or specific receptors, and promote the deliver of large peptides or proteins into cells. For example, the TAT protein from HIV-1 has a highly basic domain and conjugation of this domain can facilitate the delivery of biologically active peptides *in vivo* (Nagahara et al., 1998). Likewise, the third α-helix of the Antennapedia (ANT) homeodomain protein performs in a similar manner (Nagahara et al., 1998). Because these peptides are highly basic, researchers have found that an 11x arginine (poly-arg) tag can work in an analogous manner to both the TAT and Ant peptides (Matsushita et al., 2001). Thus, future studies will be directed at designing, TAT, ANT, or poly-arg conjugated AKAPIS peptides for non-invasive delivery into cells **(Figure 4.2)**. Each peptide will be fluorescently conjugated to facilitate detection *in vivo* and tested for both efficient delivery and functional expression.

To examine the effects of PKA anchoring in a whole animal model it would be possible to create transgenic mice that express AKAPIS in a tissue specific manner. One approach is to use the Cre-lox system to selectively express AKAPIS in different tissues. This system is based on the ability of Cre-recombinase to catalyze the deletion of a cDNA fragment between two directionally oriented *loxP* sites (Nagy, 2000). This enzyme can be expressed in various mouse tissues at a precise developmental stage under the control of a tissue specific promoter. When loxP sites flank a cDNA, this cDNA can be excised by homologous recombination in a temporal and spatial manner through the tissue specific expression of Cre-recombinase. For our purposes, we could use a modification of the well-described Cre-reporter system to selectively express AKAPIS in a tissue specific manner **(Figure 4.3)**. With one transgene it would be possible to decipher the role of PKA anchoring in multiple tissues and cell types. Moreover, as the

Figure 4.2

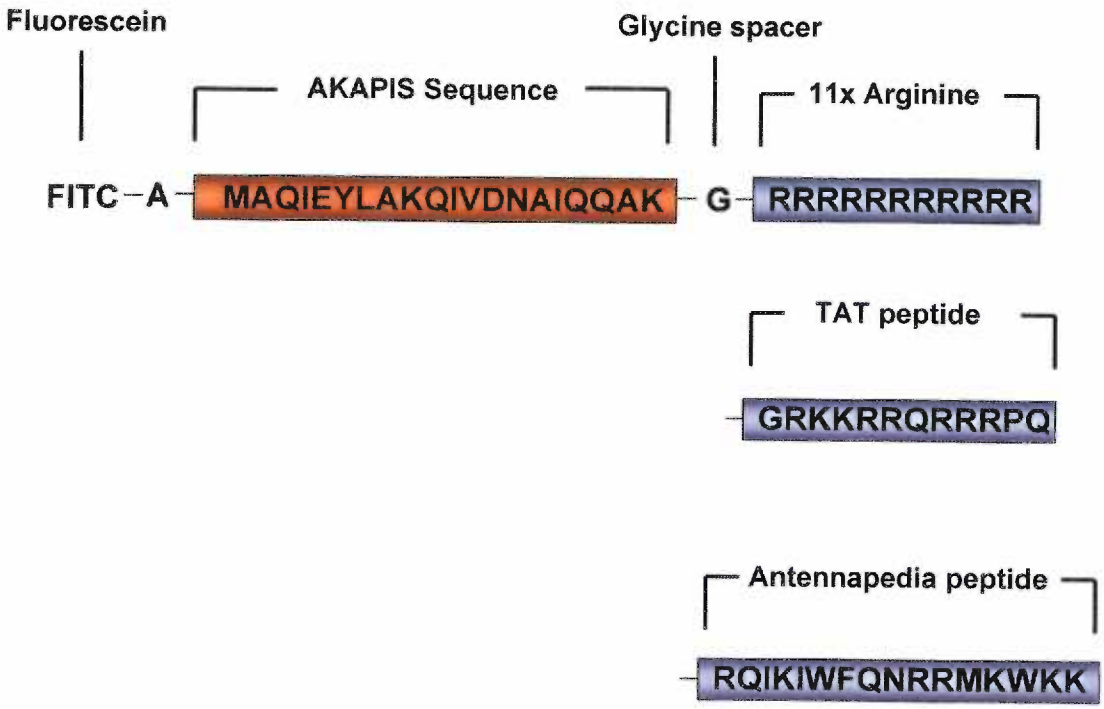

Figure 4.2: Design of a Cell Permeable AKAPIS peptide

AKAPIS peptide will be made cell permeable by C-terminal conjugation of 11x arginine, Tat, or Antennapedia peptides (right). A glycine spacer will be used to separate the AKAPIS peptide from the cell permeable peptides. N-terminal FITC conjugate will be used for visualization in cells. Both the AKAPIS and a control scramble peptide (not shown) will be designed.

Figure 4.3

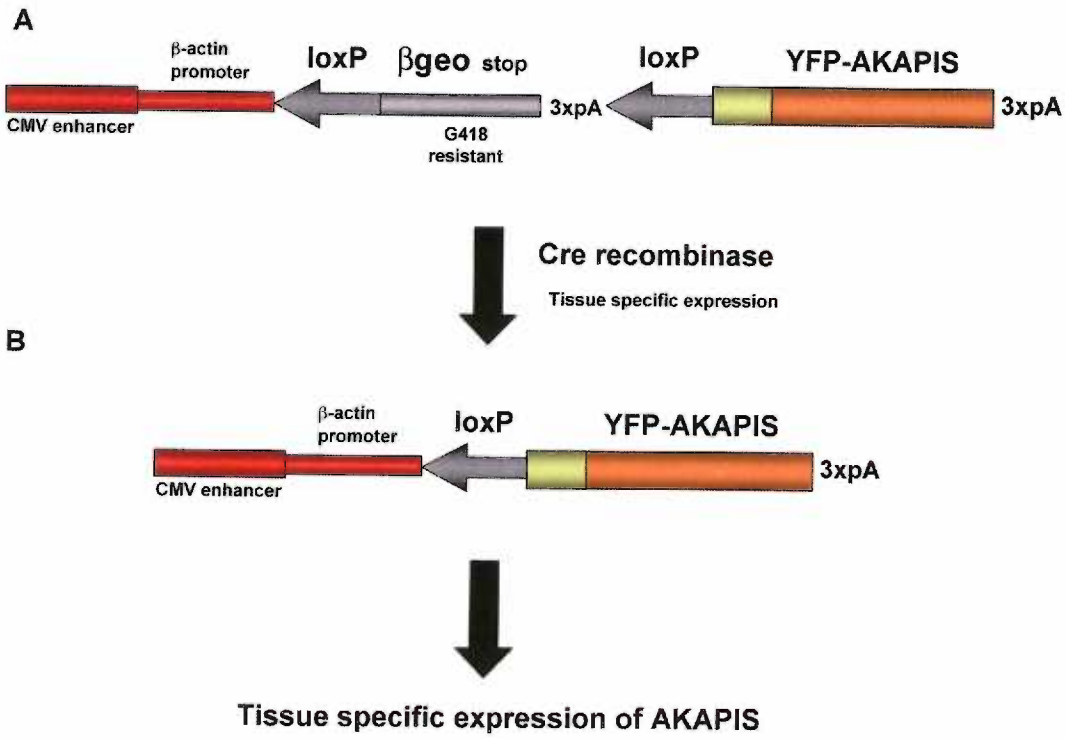

Figure 4.3: Strategy for Tissue Specific Expression of AKAPIS

A) Design of the AKAPIS expression vector. The model used to design the tissue specific expression of AKAPIS is a modification of the Z/AP double reporter for Cre excision (Lobe et al.) An AKAPIS transgene will be designed in the following manner. The βgeo cassette (*LacZ*/neoR) with a stop codon will be inserted between two tandem *loxP* sites. An upstream CMV enhancer/chicken β-actin promoter will drive the expression of βgeo. Therefore βgeo can be expressed but AKAPIS is not. The YFP-AKAPIS-V5His will be inserted down stream of the *loxP*-flanked βgeo/3xPa. Expression of the AKAPIS trangene will occur upon removal of the βgeo cassette by Cre-recombinase. Expression of AKAPIS will be monitored by both loss of βgeo and intrinsic YFP fluorescence from the expressed transgene.
B) For the tissues specific expression of AKAPIS. transgenic mice will be crossed to mice expressing Cre-recombinase under a tissue specific promoter (see www.mshri.on.ca/nagy/Cre-pub.html for available lines). Cre-recombinase will recombine the lox P sites resulting in excision of the βgeo cassette and stop codon in tissue or cell type specific manner. Recombination will result in the expression of AKAPIS chimeric protein. Expression of AKAPIS can be monitored by intrinsic YFP fluorescence.

Cre-reporter system becomes more advanced (ie. tetracycline inducible expression of Cre-recombinase) it may be possible to temporally, spatially, and quantitatively express AKAPIS. Unfortunately, the interpretation of these types of studies may be somewhat complicated because there are multiple AKAPs in a single cell type and AKAPIS expression does not discriminate between these molecules. However, these transgenic experiments, in combination with AKAP "knock ins" in which an RII binding allele of a specific AKAP is deleted, are the only methods that can determine the roles of PKA anchoring in whole animal physiology.

Here, I have described the generation and testing of a highly potent AKAP inhibitor peptide. Future studies will be aimed at using this peptide to explore new roles of PKA anchoring *in vivo*. Together, cellular delivery of peptide conjugates and tissue specific expression of AKAPIS in a whole animal model may elucidate physiological processes that require anchored PKA.

Rab32 functions as both an AKAP and Mediator of Mitochondrial Fission: Conclusions

One aim of researchers in the AKAP field is to identify all of the existing proteins that anchor PKA to subcellular locations. This pursuit has resulted in the characterization of at least 20 family members found in a variety of species (see Chapter 1). Unfortunately, the lack of a well-conserved RII binding-domain has made it challenging to use whole genome searches for this purpose. Therefore, experimental screening methods are still the best approach. Work presented in this thesis describes the identification and characterization of a novel AKAP, Rab32. Rab32 was found to interact with RII in a yeast two-hybrid screen. At the time, there were no published accounts of Rab32 and only its nucleotide sequence could be found in the Genbank database. Rab32 interacts with RII through an amphipathic helical domain found at the C-terminus. Interestingly, this domain is conserved among all Rabs, however experimental data suggests that Rab32 is the only family member that can function as an AKAP. Rab32 can interact with the functional PKA holoenzye *in vivo*, passing strict criteria for acceptance into the AKAP family.

As I discussed in Chapter 1, all AKAPs have a subcellular targeting domain. In the case of Rab32, both immunofluorescent and biochemical data show that it is associated with the mitochondria. We hypothesize that Rab32 is associated with mitochondria through a predicted C-terminal lipid modification. This notion is supported by experimental evidence that overexpression of Rab32 leads to a small but detectable level at the mitochondria and deletion of the last two cysteines abolishes this localization. Interestingly, mitochondrial targeting can be saturated by gross overexpression of

exogenous Rab32 suggesting that additional protein-protein interactions are necessary for targeting. To the best of our knowledge, Rab32 is the first example of a small G-protein that can interact with PKA and it is the third AKAP identified that targets to the mitochondria. At the time there was no information regarding the function of Rab32 at the mitochondria. For this reason, only speculation could be made as to the role of PKA and cAMP signaling in a Rab32 mediated reaction. Thus, much of the work presented in this thesis was aimed at determining the role of Rab32 at the mitochondria.

While it is clear that the pleiotropic nature of mitochondria is required for the normal function of this organelle, very few molecules have been implicated in the regulation of mitochodrial dynamics (Yaffe, 1999). To date, only three gene families have been show to regulate mitochondrial dynamics in multicellular organisms **(Table 4.1)**. These include three members of the kinesin family(Tanaka et al., 1998), the fuzzy onion (*fzo*) gene family (Hales and Fuller, 1997; Santel and Fuller, 2001), and a member of the dynamin-family of large GTPases, DRP-1 (Labrousse et al., 1999; Smirnova et al., 2001). Interestingly, expression of mutant forms of these genes, or direct gene deletion results in a redistribution of mitochondria from the cell periphery to the microtubule organizing center (MTOC). Therefore, this phenotype may be a marker for proteins involved in mitochondria dynamics. To probe the function of Rab32 at the mitochondria, a biochemicaly-characterized dominant-negative mutant form of Rab32 was overexpressed in Cos7 cells. Expression of mutant Rab32 leads to a collapse of mitochondria from the cell periphery to the microtubule-organizing center. Because this phenotype is often observed when perturbing the genes involved in mitochondrial dynamics, it is likely that

Table 4. 1: Proteins involved in mitochondrial dynamics

Proteins	Localization	Mutant Phenotype	Function	Rererences
Drp1, Dnm1P	Cytosol, Sites of division	Mitochondrial aggregation, Dominant negative cause abnormal mitocondrial fusion	Dynamin Related protein involved in mitochondrial division	Labrousse Smirnova et al.
KIF5B,Kif1B, KLP67A	Mitcrotubules	Redistribution of mitochondria to the microtubule organizing center	Microtubule motor protein involved in microtubule transport of mitochondria	Tanaka et al.
Fuzzy Onions, Fzo1p, Mitofusin,	Mitochondria, outer membrane	Redistribution of mitochondria to the microtubule organizing center,	Regulates fusion of mitochondria	Hales et al. Santel et al.
Rab32	Mitochondria, outer membrane	Mitochondrial aggregation, Dominant negative cause abnormal mitochondrial fusion	Mitochondrial fission, A-kinase Anchoring Protein	Alto et al.

Rab32 plays a specific role at this organelle (**Table 4.1**). To further probe the function of Rab32 at mitochondria, we explored the nature of this phenotype. The collapsed mitochondria were redistributed from the MTOC by depolimerizing microtubules with nocodazole. Under these conditions, the mitochondria appear to be elongated and abnormally fused compared to their wild-type counterparts. Highly fused mitochondria are also observed upon low expression of dominant-negative Rab32. Together these data supports the role of endogenous Rab32 in mitochondrial division (**Figure 4.4**). Thus Rab32 is now the fourth mammalian gene product identified that regulates mitochondrial dynamics.

In summary, the work presented here suggests that Rab32 is both an AKAP and a regulator of mitochondrial dynamics. Both features of this molecule are unique for the Rab family of small G-proteins. While there are now three AKAPs that interact with mitochondria, Rab32 is the first small G-proteins found at this organelle. Furthermore, Rab32 is the fourth mammalian gene product identified that function in mitochodrial dynamics. Although the dynamics of form and distribution are necessary for the correct function of this organelle, very little is known about the molecular nature of these events. Both findings provide the groundwork for future studies regarding cAMP signaling at the mitochondria and the mechanisms that govern mitochondrial division.

Figure 4.4

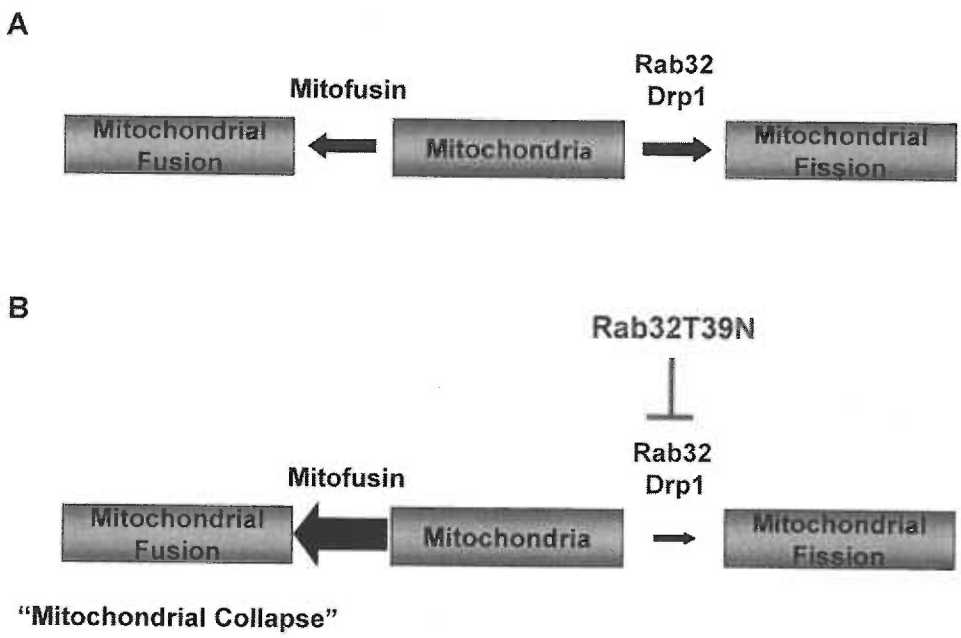

Figure 4.3: Rab32 Regulates Mitochondrial Division

A) Under normal conditions, the rate of mitochondria fusion (left) and fission (right) are in a state of dynamic equilibrium. Both Rab32 and the dynamin like GTPase, DRP-1, regulate Mitochondrial fission. Conversely, mitochondrial fusion is mediated by the protein mitofusin. Together, these molecules maintain a constant number of mitochondria in quiescent cells.

B) Hypothesis that endogenous Rab32 regulates mitochondrial fission events. Overexpression of Rab32T39N, a dominant-negative mutant form, may antagonize the function of endogenous Rab32 at the mitochondria through an unknown mechanism. If Rab32 is required for normal fission events, then decreases in fission instigated by inhibition of Rab32 will lead to abnormally fused mitochondria (see chapter 3, figures 3.8 and 3.9). When the balance is shifted towards fusion, mitochondria collapse around the microtubule organizing center.

Rab32 as an AKAP and mediator of Mitochondrial Dynamics: Future Directions

There is very little known about the role of cAMP signaling or PKA anchoring at the mitochondria. Because Rab32 is involved in mitochondrial dynamics, and specifically membrane fission, it will be interesting to test if cAMP signaling or anchored PKA plays a role in this process. Second messengers have been identified that regulate mitochondrial fusion and fission in some cell types. Addition of phorbol esters or injection of calcium ions can induce fragmentation of mitochondria (Chen, 1988). If cAMP leads to changes in mitochondria dymanics then delivery of the AKAPIS peptide that I described in Chapter Two could be used to determine if these events are PKA anchoring dependent. It will also be interesting to determine if any Rab interacting proteins, such as GAPs, GEFs, or effectors, are PKA substrates. This could add an increased level of regulation for Rab32 and the mitochondrial division apparatus, however further accessory proteins remain to be identified.

Many questions still remain about the role of Rab32 at the mitochondria, and in particular its role in mitochondrial division needs to be defined. One possibility is that Rab32 recruits effector proteins to the mitochondria that function in the division of mitochondria. A prime candidate for this type of regulation is the protein DRP-1. DRP-1 is a dynamin related protein involved in the fission of mitochondria. (Santel and Fuller, 2001; Smirnova et al., 2001). It is found at the constriction sites of dividing mitochondria and studies have indicated that DRP-1 catalyzes the division of outer, but not inner membranes (Labrousse et al., 1999). Moreover, DRP-1 seems to be recruited from the cytoplasm to the outer membrane through an unknown mechanism. Thus it plausible

that GTP-loaded Rab32 could directly recruit DRP-1 or DRP-1 interacting proteins to sites of division. This hypothesis can be tested by first determining if GTP loaded Rab32 can physically interact with cellular DRP-1 *in vitro*. If so, it would be interesting to see if Rab32 is localized to the restriction sites of mitochondria. Immuno-gold labeling and electron microscopy would be useful for these types of studies. Along with testing DRP-1, unbiased approaches can be used to identify specific effector proteins for Rab32. Two types of experiments would be useful for this purpose. First, GTPγS loaded Rab32, a non-hydrolyzable analog of GTP, could be used for in *vitro* pulldown assays from whole tissue or cellular extracts. Potential Rab effectors could be visualized following SDS-PAGE Gel Electrophoresis and in gel protein stain. Effectors protein sequences would be identified with Tandem Mass Spectroscopy. The second approach is to use the yeast two-hybrid assay. Dominant-active Rabs that preferentially associate with GTP inside of cell have been used to screen cDNA libraries using the yeast two-hybrid approach. Many true effector proteins have been identified in this way.

Future studies also include the identification of regulatory proteins such as GEFs and GAPs for Rab32. An interesting phenomenon that we observed is that expression of dominant-negative Rab32 (Rab32T39N) lacking membrane targeting domains results in a decreased incidence in mitochondrial collapse compared to the targeted dominant-negative mutant. One possible mechanism by which Rab32T39N acts in a dominant-negative manner is through the titration of an upstream activator of endogenous Rab32 (Powers et al., 1989). This upstream activator is most likely a Guanine Nucleotide Exchange Factor (GEF). Because nucleotide exchange is thought to be concomitant with membrane targeting of Rab proteins (see Chapter 1), and mitochondrial targeted

Rab32T39N is more active than the untargeted mutant, it is likely that a specific Rab32-GEF resides on the mitochondrial membrane. This could be studied by purifying mitochondrial fractions from cells and adding them to an *in vitro* Rab32 GEF assay. If specific GEF activity was found, biochemical purification of this factor could be performed. In summary, the future directions that I have presented here are aimed at determining the role of PKA in a Rab32 mediated division apparatus. It is likely that either regulatory or effector proteins may be PKA substrates. The identification of these accessory proteins is required to test this hypothesis. Moreover, defining upstream activators, downstream effectors, and termination factors for Rab32 will greatly increase our understanding of how this small G-protein functions at the mitochondria.

In conclusion, the primary focus of this thesis was to identify and characterize novel A-Kinase Anchoring Proteins. In this regard, work presented here describes the design and functional characterization of a synthetic AKAP mimic peptide that can inhibit PKA anchoring *in vivo*. These studies provide groundwork to test the kinase anchoring hypothesis in many cell types and tissues. In addition to this work, data presented also describes the small G-protein Rabe32 as both an AKAP and a regulator of mitochondrial dynamics. Based on these findings, studies can now be performed to examine the role of cAMP signaling at the mitochondria and to further describe the molecular events that govern mitochondrial division.

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