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Anchoring and Scaffold Proteins for Kinases and Phosphatases

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ABSTRACT

Many hormones mediate their intracellular actions by triggering signal transduction pathways that alter the phosphorylation state of key regulatory proteins. Protein phosphorylation is a reversible process involving two classes of signaling enzymes: protein kinases, which catalyze the transfer of phosphate from ATP onto substrate proteins, and phosphoprotein phosphatases, which perform the dephosphorylation step. To insure tight control of hormonally initiated phosphorylation events, the activity of multifunctional kinases and phosphatases is precisely regulated and responds to fluctuations in diffusible second messengers such as Ca^{2+}, phospholipid, and cAMP. Another mechanism that contributes to their regulation is to restrict the location of these enzymes to certain subcellular compartments. Subcellular targeting enhances the selectivity of serine/threonine phosphatases and kinases by favoring their accessibility to certain substrate proteins. Compartmentalization is achieved through a "targeting moiety," which is defined as that part of a phosphatase or kinase that directs the catalytic subunit to a certain subcellular environment. The targeting moiety restricts the location of a phosphatase or kinase through association with a "targeting locus." These are often structural membrane proteins, cytoskeletal components, or cellular organelles. Targeting subunits for the type I phosphatase and protein kinase C have been identified; however, the focus of this chapter centers around a family of anchoring proteins, called AKAPs, that localize the type II cAMP-dependent protein kinase (PKA). Structure-function analysis suggest that each anchoring protein binds to the RII dimer through a conserved amphipathic helix region and is tethered to specific subcellular sites via association of a targeting domain with structural proteins or cellular organelles. Peptides patterned after the amphipathic region have been used to probe the functional significance of PKA anchoring inside cells and have begun to be established by that disruption RII/AKAP interaction *in vivo* has concomitant effects on certain PKA-mediated phosphorylation events. In addition, multivalent binding proteins such as AKAP79 and AKAP250 have been characterized and appear to serve as platforms for the assembly of kinase/phosphatase signaling complexes. Collectively, these studies suggest that the AKAPs represent a growing family of regulatory proteins that provide a molecular architecture that organizes the intracellular location of a single or multiple multifunctional kinase.

I. Introduction

Hormone action is a complicated and dynamic process that requires the efficient transmission of information from the extracellular environment to precise intracellular sites (Sutherland, 1972). The speed and precision of these events is a fascinating topic that has engaged researchers for more than three decades. The

work of numerous investigators has shown that many hormone-stimulated signaling cascades emanate from transmembrane receptors at the plasma membrane and proceed through intermediary G-proteins to promote the stimulation of adenylyl cyclase or the activation of phospholipases (Divecha and Irvine, 1995; Taussig and Gilman, 1995). The net effect of these signaling events is the generation of the soluble second messengers cAMP and diacylglycerol (DAG). This leads to the activation of the cAMP and Ca^{2+}/phospholipid-dependent protein kinases, called PKA and PKC, respectively (Krebs *et al.,* 1959,1985; Nishizuka, 1992). Site-selective activation and termination of the phosphorylation events that emanate from these kinases are critical events that regulate the specificity of hormone action. Although this process is ultimately controlled by the action of phosphoprotein phosphatases that catalyze the dephosphorylation of substrate proteins, it has become clear that other levels of enzyme regulation contribute to this process. One emerging mechanism is that the subcellular distribution of several kinases and phosphatases is restricted by association with targeting proteins or subunits (reviewed by Faux and Scott, 1996a; Hubbard and Cohen, 1993; Mochly-Rosen, 1995). Targeting can occur either by binding of a separate targeting subunit with the catalytic subunit of the enzyme (Fig. 1A) or by a specialized targeting domain of the enzyme itself (Fig. 1B). Either way, targeting restricts the location of a phosphatase or kinase through associations with specific targeting loci, usually structural membrane proteins, cytoskeletal components, or cellular organelles. Recently, several targeting proteins have been identified that anchor individual kinases or phosphatases close to their specific substrates. For

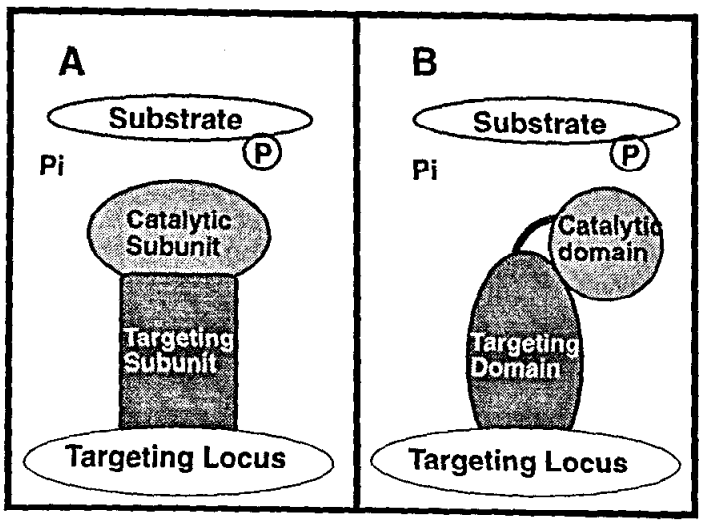

FIG. 1. Schematic diagram depicting the subcellular targeting of enzymes through targeting moieties. (A) Localization of the catalytic subunit to a targeting locus through association with a targeting subunit. (B) Localization of enzymes through a distinct targeting domain. [Adapted from Faux and Scott, 1996a.]

example, the type I phosphatase is localized at glycogen particles, endoplasmic reticulum, and nucleus through association with targeting subunits (Hubbard and Cohen, 1993) and the heterotrimeric phosphatase 2A is compartmentalized through interaction of its b subunit with microtubule and nuclear proteins (Sontag *et al.*, 1995). PKC is tethered to the cytoskeleton or at submembrane sites through association with a family of substrate/binding sometimes called Racks for *R*eceptors for *A*ctivated *C K*inase (Chapline *et al.*, 1993; Mochly-Rosen *et al.*, 1991). Likewise, the type II PKA is targeted by association of its regulatory subunit (RII) with *A-K*inase *A*nchoring *P*roteins, called AKAPs (Rubin, 1994; Scott and McCartney, 1994). This chapter will highlight the properties of targeting proteins that localize protein phosphatases, protein kinase C (PKC), but will also focus on anchoring proteins that compartmentalize the cAMP-dependent protein kinase (PKA). Latter sections will describe a new class of multivalent adapter proteins that coordinate the location of more than one kinase or phosphatase (Faux and Scott, 1996b).

II. Phosphatase Targeting

Serine/threonine protein phosphatases counterbalance a variety of hormonal events such as insulin action and β-adrenergic stimulation. However, phosphatase control of these complex cellular processes proceeds through only a limited number of catalytic subunits. Three prominent types of phosphatase catalytic (C) subunit have been identified, termed PP-1, PP-2A and PP-2B, which have been categorized on the basis of their substrate preferences, activation pathways, and sensitivity to inhibitor proteins or naturally occurring toxins. These findings led Philip and Patricia Cohen to propose that the regulation of so many phosphorylation events by such few phosphatases must require multiple levels of enzyme regulation (Cohen and Cohen, 1989). These included modification of catalytic activity and the selective compartmentalization of certain phosphatase C subunits. The idea of targeting subunits was instigated by studies on hormonal regulation of glycogen metabolism, where it was recognized that a substantial proportion of PP-1 is associated with glycogen particles (Cohen and Cohen, 1989; Hubbard and Cohen, 1991). It is now likely that each phosphatase type is regulated by targeting subunits (Fig. 2A). The PP-1 targeting subunits perform two distinct functions: localization of the C subunit and adaptation of phosphatase activity to favor the dephosphorylation of certain substrates. For example, the glycogen-associated type I phosphatase, PP-1G, is a heterodimer composed of a 37 kDa C subunit and a targeting subunit (G), sometimes called RG (Hubbard *et al.*, 1990; Tang *et al.*, 1991). The muscle type I phosphatase form, GM, is a 124 kDa protein, whereas the liver form, GL, is a distinct but related protein of 33 kDa (Doherty *et al.*, 1995; Moorhead *et al.*, 1995). In yeast, the G subunit is encoded by an 85 kDa protein, GAC-1, that coordinates glycogen levels (Francois, 1992). Both

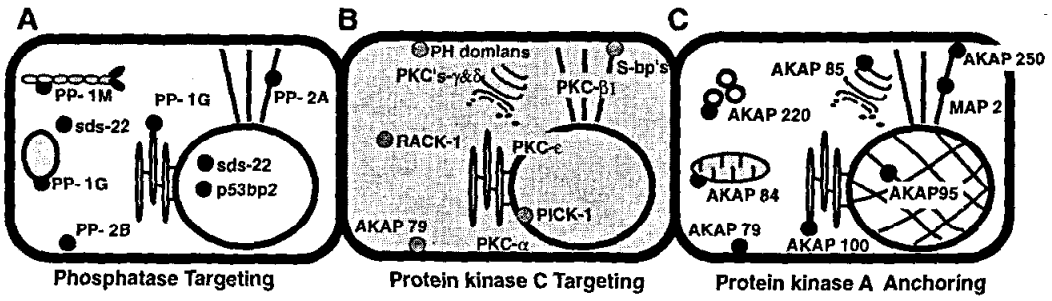

FIG. 2. The cellular location of targeting subunits. Targeting of (A) protein phosphatases, (B) protein kinase C, and (C) protein kinase A through targeting subunits. The subcellular locations of known targeting subunits that attach their respective enzymes to specific organelles are indicated. [Adapted from Faux and Scott, 1996a.]

mammalian G subunits target PP-1 to either glycogen particles or to the membranes of the sarcoplasmic reticulum (Hubbard *et al.*, 1990). The glycogen binding and PP-1 binding sites are in the N-terminal regions of the protein, whereas the membrane binding domain includes a stretch of hydrophobic residues at the C-terminus (Doherty *et al.*, 1995). Mammalian GM function is hormonally regulated. Insulin action stimulates the phosphorylation of GM. This, in turn, favors glycogen synthesis by modifying PP-1 activity to enhance the dephosphorylation of sites on glycogen synthase and phosphorylase kinase. In contrast, β-adrenergic stimulation triggers glycogenolysis through phosphorylation of GM by the cAMP-dependent protein kinase, which permits the concomitant release of PP-1 (Hubbard and Cohen, 1991,1993). PP-1 compartmentalization is not restricted to association with glycogen particles and sarcoplasmic reticulum membranes but is found in other cellular organelles. This raises the question of how PP-1 is targeted to many different locations. In muscle, PP-1 is complexed with myofibrils through regulatory M subunit(s), which also modifies the substrate specificity of the enzyme (Hubbard and Cohen, 1993). The delicate specificity achieved with distinct targeting subunits is evident when the properties of the skeletal and smooth muscle type 1 phosphatases are compared. Smooth muscle PP-1M enhances the dephosphorylation of smooth muscle myosin but not skeletal muscle myosin, whereas the skeletal muscle PP-1M enhances the dephosphorylation of skeletal muscle myosin to a much greater extent than smooth muscle myosin. The smooth muscle enzyme is a heterotrimeric complex consisting of the 37 kDa C subunit and regulatory subunits of 130 kDa and 20 kDa, called M-130 and M-20 (Chen *et al.*, 1994; Shimizu *et al.*, 1994; Shirazi *et al.*, 1994). The PP-1 binding region includes a series of seven ankyrin repeat sequences in the N-terminus of M-130, whereas a leucine zipper motif in the C-terminus of M-20 may participate in association to myosin or other proteins (Chen *et al.*, 1994; Shimizu *et al.*, 1994;

Shirazi *et al.,* 1994). Interestingly, leucine-rich repeats may form the PP-1 binding site on another targeting subunit, SDS-22, that facilitates the metaphase/anaphase transition during mitosis in yeast (Renouf *et al.,* 1995). Nuclear targeting of PP-1 also proceeds through association with a nuclear inhibitor of PP-1, called NIPP-1 (Van Eynde *et al.,* 1995) and the tumor suppressor accessory protein, p53 binding protein, p53bp2 (Helps *et al.,* 1995). p53bp2 is a potent inhibitor of phosphatase activity and, like M-130, it contains ankyrin repeat sequences that have been implicated in PP-1 binding and a src homology SH3 domain that may be involved in other protein-protein interactions (Helps *et al.,* 1995).

Recent studies suggest protein phosphatase 2A (PP-2A) may be targeted in a manner that is similar to PP-1 (Fig. 2A). The PP-2A holoenzyme is a heterotrimer consisting of a 36 kDa catalytic (C) subunit, a 65 kDa structural subunit (A), and a third protein, termed B or phosphatase regulatory (PR) subunit. The A and C subunits form a core enzyme, whereas multiple B subunit families exist that increase the complexity of PP2A tissue-specific expression and subcellular targeting (Csortos *et al.,* 1996; Mayer-Jaekel *et al.,* 1993; McCright and Virshup, 1995). The Bα subunit is localized to the centrosome and microtubule network in CV-1 cells and the ABαC form of PP-2A interacts with microtubules and the SV40 small tumor antigen (Sontag *et al.,* 1993). Furthermore, immunofluorescence experiments suggest that distinct pools of PP-2A appear to be localized at the endoplasmic reticulum, golgi, and nucleus (Sontag *et al.,* 1995). A clear objective for future studies will no doubt be to establish if the subcellular location of PP-2A is directed specifically by the B subunit or whether additional targeting interactions are maintained through the core enzyme.

A proportion of protein phosphatase 2B (PP-2B) or calcineurin is targeted to the membrane (Fig. 2A). In bovine brain, particulate PP-2B form is inactive (Klee *et al.,* 1988). This may be explained by evidence that the phosphatase is inhibited and targeted to submembrane sites through association with AKAP75, an anchoring protein that also binds PKA (Coghlan *et al.,* 1995a,b). Other studies suggest that cytosolic PP-2B associates with one of its physiological substrates, the transcription factor NFATp (Wesselborg *et al.,* 1996). However, PP-2B binding does not depend upon the phosphorylation state of NFATp, suggesting a role in targeting. In fact, more recent studies suggest that calcium signaling induces that association of the NF-AT4 isoform with PP-2B and that the entire complex is transported into the nucleus (Shibasaki *et al.,* 1996). Furthermore, it has been proposed that a phosphatase activity is maintained in the NF-AT4/ PP2B nuclear complex that serves to counterbalance the activity of nuclear NA-AT4 kinases. This type of mechanism may explain the role of PP-2B in T cell activation that requires several signaling events, including the dephosphorylation and nuclear translocation of NA-AT, before transcription of cytokine genes can proceed (Shibasaki *et al.,* 1996).

III. PKC Targeting Proteins

Protein kinase C (PKC) now represents a family of at least 11 enzymes that are activated by various combinations of Ca^{2+} and/or diacylglycerol (reviewed by Newton, 1995). While there is a considerable literature on the structure of each PKC isoform, the role of each PKC subtype in hormone-mediated events is not so clear. The problem is compounded by evidence that most cells express multiple isoforms with nearly identical ligand binding and substrate specificity (Newton, 1995; Nishizuka, 1992). Not surprisingly, compartmentalization of individual PKC isoforms has been offered as an explanation for how individual enzyme subtypes attain some measure of selectivity toward their physiological substrates (Fig. 2B). Accordingly, studies with isoform-specific antisera demonstrate that PKC subtypes do have distinct subcellular locations, albeit in specified cell types. It has been shown that phorbol ester treatment causes a rapid redistribution of several PKC isoforms overexpressed in 3T3 fibroblasts. PKC-α predominates in the endoplasmic reticulum and cell margins, PKC-βII is attached to the actin cytoskeleton, PKC-γ is concentrated in the golgi, and PKC-ε is found at the nuclear membranes (Goodnight *et al.,* 1995). Although PKC targeting most certainly requires protein-lipid interactions, it is now apparent that protein-protein interactions also participate in the compartmentalization of the enzyme (Newton and Keranen, 1994; Newton and Koshland, 1990). Wolf and Sahyoun (1986) were the first to demonstrate that PKC bound to a variety of cytoskeletal and postsynaptic density (PSD) proteins in a phosphatidylserene (PS)-dependent manner. Since then, several laboratories have identified PKC targeting proteins (reviewed by Mochly-Rosen, 1995). Using a variety of techniques, at least three classes of PKC binding proteins have been identified (Fig. 2B).

PKC substrate/binding proteins bind PKC in the presence of PS and have been detected by a gel-overlay procedure. A distinguishing feature of this class is that phosphorylation regulates their association with PKC (Liao *et al.,* 1994). It has been proposed that PS supports a ternary complex of PKC and the substrate/binding protein. Phosphorylation simultaneously abolishes PKC-substrate/binding protein interaction and PS binding (Liao *et al.,* 1994). So far, cDNAs for 11 substrate/binding proteins have been isolated that encode proteins involved in membrane-skeleton linker functions such as vinculin and talin or structural components that link to the actin cytoskeleton such as MARCKS-related protein, adducins, annexins, and desmoyokin (Chapline *et al.,* 1993,1996). Although the substrate/binding proteins exhibit a binding affinity for PKC, as assessed by the overlay assay in the absence of ATP, it remains to be seen whether this family of proteins associate with the enzyme *in vitro.* It is possible that these proteins merely represent PKC substrates that release the enzyme slowly once the phosphotransfer reaction is complete.

*R*eceptors for *A*ctivated *C-k*inase (RACKs) also bind PKC but are not sub-

strates for the kinase. RACKs bind at a site that is distinct from the substrate binding pocket of the kinase and in a PS-independent manner (Mochly-Rosen, 1995). Peptides to a prospective PKC binding site derived from one of the RACKs, annexin I, inhibit PKC as assessed by the overlay assay. These peptides have also been reported to block insulin-induced translocation of PKC in *Xenopus* oocytes (Ron and Mochly-Rosen, 1995). So far only one, called RACK 1, has been cloned. This protein shows some sequence similarity to the β subunits of G-proteins, as it contains seven WD40 motifs. A second suggested function of RACK-1 is to enhance the phosphorylation of histones when complexed with PKC (Ron *et al.,* 1994); however, it is hard to reconcile this function with its proposed subcellular location at the plasma membrane. Nevertheless, RACK-1 and PP-1 targeting subunits may be functionally related as they regulate the activity of their bound enzymes through subcellular targeting and through adaptation of the substrate specificity.

A third class of PKC targeting proteins, designated *P*roteins that *I*nteract with *C-k*inase (PICKs), have been identified by two-hybrid screening. So far, only one of these proteins, called PICK-1, has been characterized and has been shown to be a PKC substrate. Immunofluorescence studies show that PICK-1 is localized at the perinuclear regions and two-hybrid analysis suggests that it exclusively binds the catalytic core of PKC-α (Staudinger *et al.,* 1995). However, the failure of PICK-1 to bind native PKC or the regulatory domain has led to the suggestion that this targeting protein recognizes determinants that are masked in the dormant holoenzyme (Staudinger *et al.,* 1995). Unlike the substrate/binding proteins, both phospho- and dephosphorylated forms of PICK-1 bind equally well to the kinase. Furthermore, PICK-1 does not contain any regions with homology to the RACKs. Potentially, a fourth class of targeting protein may exist, as recent evidence suggests that plextrin homology (PH) domains on the Burton tyrosine kinase and RAC kinase bind PKC. PH domains might modulate the substrate specificity of PKC, since binding to a 14-3-3-like protein, stratifin, enhances the phosphorylation of histones (Dellambra *et al.,* 1995). Despite the growing number of PKC interacting proteins that have been identified, it should be recognized that there are as yet very few data to support the concept that these proteins function as targeting subunits *in vivo.*

IV. PKA Anchoring

The work of numerous investigators have shown that many hormone-stimulated signaling cascades emanate from transmembrane receptors at the plasma membrane and proceed through intermediary G-proteins to promote the stimulation of adenylyl cyclase (Wedegaertner *et al.,* 1995; Taussig and Gilman, 1995). The net effect of this transduction unit is the generation of the diffusible second messenger cAMP, which binds and activates the cAMP-dependent protein

kinase (PKA) (Walsh *et al.,* 1968). The kinase is activated by the release of two catalytic subunits (C) from the regulatory (R) subunit-cAMP complex. An array of PKA isozymes are expressed in mammalian cells and genes encoding three C subunits (Cα, Cβ, and Cγ) and four R subunits (RIα, RIβ, RIIα, and RIIβ) have been identified (reviewed in Scott, 1991). Two holoenzyme subtypes, called type I and type II, are formed by the combination of RI or RII with the C subunits (Brostrom *et al.,* 1971; Corbin *et al.,* 1973).

Although many hormones use parallel pathways to activate PKA, some measure of specificity must be cryptically built into each signaling cascade to ensure that the correct pool of kinase becomes active in the right place and at the right time (Harper *et al.,* 1985). Compartmentalization of the kinase seems to represent a regulatory mechanism that may increase the selectivity and intensity of a cAMP-mediated hormonal response. In order to facilitate this process, up to 75% of type II PKA is targeted through association of the regulatory subunit (RII) with *A-Kinase Anchoring Proteins*, called AKAPs (reviewed by Rubin, 1994, and Scott and Carr, 1992). Additionally, there is now some indication that the type I PKA is also compartmentalized (Rubin *et al.,* 1972; Skalhegg *et al.,* 1994). In recent years, numerous AKAPs have been identified that target PKA to a variety of subcellular locations (Scott and McCartney, 1994). These proteins represent a growing family of signaling molecules that function to localize PKA, thereby favoring access to certain substrates (Fig. 2C).

V. AKAPs

Initially, anchoring proteins were identified by contaminating proteins that co-purified with the RII after affinity chromatography on cAMP-sepharose (Sarkar *et al.,* 1984). However, detailed study of AKAPs was made possible by the original observation of Lohmann *et al.* (1984) that many, if not all, of these associated proteins retain their ability to bind RII after they have been immobilized to nitrocellulose or similar solid-phase supports. As a result, the standard technique for detecting AKAPs is an overlay method that is essentially a modification of the Western blot (reviewed by Carr and Scott, 1992). Using the overlay technique, we have surveyed various mouse, bovine, and human tissues and have detected AKAPs ranging in size from 21–300 kDa (Carr *et al.,* 1992). From this type of study, it would appear that a typical cell expresses 5–10 distinct AKAPs that presumably adapt the type II PKA for specific functions. Also, the expression of some AKAPs may be hormonally regulated, as treatment of granulosa cells with FSH induces the expression of an 80 kDa anchoring protein (Carr *et al.,* 1993). The RII overlay method has also been developed into an efficient interaction cloning strategy wherein cDNA expression libraries have been screened using recombinant RII as a probe.

A. RII/AKAP INTERACTION

Recent work in our laboratory and others has allowed us to refine our model for the topology of the anchored PKA holoenzyme. This model, presented in Figure 3, illustrates the essential features of AKAPs. Each anchoring protein participates in at least two distinct types of binding: protein-protein interactions with the R subunit of PKA and subcellular targeting of the PKA-AKAP complex through association with structural proteins, membranes, or cellular organelles. In order to define the minimum region of RII required to bind AKAPs, a family of RII deletions and chimeric proteins were screened for their ability to bind AKAPs by the overlay method (Luo *et al.,* 1990; Scott *et al.,* 1990). From these studies, we concluded that RII dimerization was an absolute prerequisite for anchoring and that the AKAP binding site resided in the amino-terminal 79 residues of RII (Scott *et al.,* 1990). This model was later refined by Erlichman, Rubin, and colleagues, who concluded that sites within the first 50 residues of RIIβ were sufficient for anchoring (Li and Rubin, 1995; Luo *et al.,* 1990). A more precise analysis demonstrated that the localization and dimerization determinants were contained in the first 30 residues of each RII protomer (Hausken *et al.,* 1994). In fact, deletion of residues 1 to 5 abolished the anchoring function but had no qualitative effect upon dimerization (Hausken *et al.,* 1994). Subsequent mutagenesis studies identified isoleucines at positions 3 and 5 as determinants for association with Ht 31 and several other AKAPs. Based on these studies, it is our working hypothesis that RII dimerization and two isoleucines on each protomer are principle determinants for association with AKAPs (Fig. 3).

Complimentary studies have focused on mapping the corresponding site on AKAPs that binds to RII (Carr *et al.,* 1991, 1992). In fact, a goal of early cloning studies was to compare several AKAP sequences in order to identify a conserved

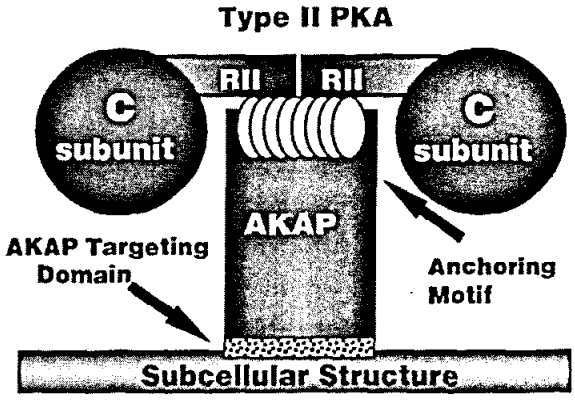

FIG. 3. Topology of the anchored PKA holoenzyme complex; topology of anchored type II PKA. This model illustrates the essential features of AKAPs: (A) a conserved RII binding site and (B) a unique targeting domain for localization to intracellular sites.

motif responsible for RII binding. Although each of the RII binding sequences was related (Fig. 4A), each region exhibited a propensity for helix formation and exhibited a striking segregation of hydrophobic and hydrophilic side-chains when analyzed on a helical-wheel projection (Fig. 4B). This observation led to the proposal that the RII binding motif on AKAPs involved an amphipathic helix (Carr *et al.*, 1991). Subsequent studies performed on Ht 31 demonstrated a requirement for this region in RII binding: substitution of selected side-chains for proline, which perturbs helix formation, abolished RII binding (Carr *et al.*, 1991) and peptides encompassing the predicted helical region of Ht 31 bound RII or the type II PKA holoenzyme with nanomolar affinity (Carr *et al.*, 1992). Subsequent studies by ourselves and others have confirmed that corresponding regions on other AKAPs are components of their RII binding sites (Coghlan *et al.*, 1994; Glantz *et al.*, 1993; Lester, 1996; McCartney *et al.*, 1995). Therefore, we have proposed that amphipathic helices provide principle sites of contact with the RII dimer.

B. AKAP TARGETING

The PKA anchoring model (Fig. 3) implies that AKAPs must contain at least two classes of functional domains: a conserved amphipathic helix that binds RII and a unique targeting site that is responsible for association with subcellular structures. So far, immunochemical and subcellular fractionation techniques have

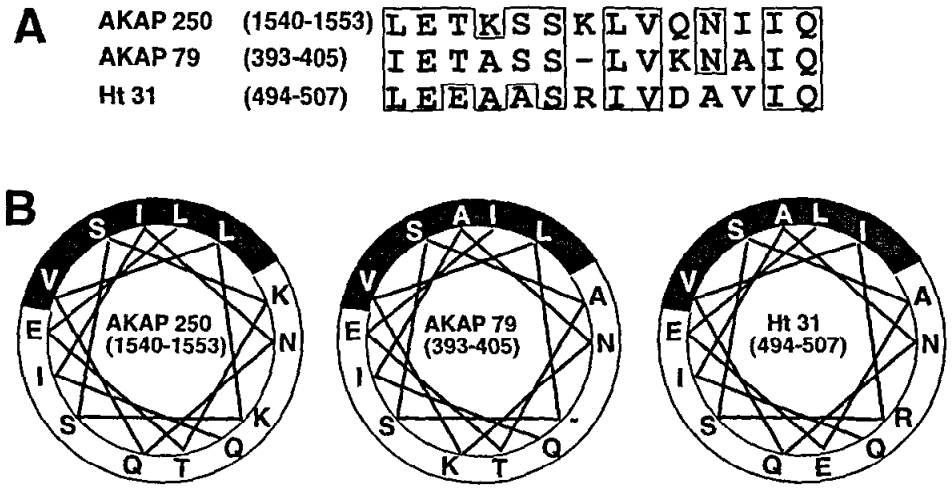

FIG. 4. The conserved RII binding motif on AKAPs. Association with the type II regulatory subunit of PKA occurs through a region of conserved secondary structure on the AKAPs. (A) Sequence alignment of the RII binding regions on three AKAPs. Boxed areas represent regions of homology. (B) Each sequence is portrayed in a helical-wheel projection (3.6 side-chains per turn), which is used to orient amino-acid side chains in an alpha-helical conformation. Shaded areas represent the clustering on hydrophobic residues.

identified AKAPs at the centrosomes, endoplasmic reticulum, golgi, microtubules, mitochondria, membranes, nuclear matrix, and secretory granules (Coghlan *et al.,* 1994; Keryer *et al.,* 1993; Lin *et al.,* 1995; McCartney *et al.,* 1995; Ndubuka *et al.,* 1993). More biochemical and molecular approaches have mapped a few AKAP targeting sequences. A C-terminal octadecapeptide repeat sequence serves as an intracellular targeting domain for the microtubule-associated protein MAP 2 (Joly *et al.,* 1989), whereas two noncontiguous N-terminal basic regions, called T1 and T2, have been proposed to facilitate submembrane attachment of AKAP75 (Glantz *et al.,* 1993).

C. AKAP 220

Recently, we have cloned and characterized a novel AKAP, called AKAP220, that seems to be targeted to the peroxisomes (Lester *et al.,* 1996). Peroxisomes are small organelles present in all cell types that function prominently in cellular lipid metabolism, such as the β-oxidation of fatty acids. These organelles have been associated with cAMP-responsive events such as androgen biosynthesis (Kragler *et al.,* 1993). Peroxisomal targeting may be conferred by the last three residues of the protein, Cys-Arg-Leu, which conforms to a peroxisomal targeting signal 1 (PTS-1) motif (McNew and Goodman, 1996; Swinkels *et al.,* 1992). Work originally performed on the C-terminus of the firefly luciferase has demonstrated that a similar tripeptide, Ser-Lys-Leu, is necessary and sufficient for peroxisomal targeting (Distel *et al.,* 1992). Analysis of mammalian peroxisomal targeting motifs have determined that Leu is the only invariant residue in the triplet, whereas Ser can be replaced by Cys or Ala at the first position and Arg can be substituted for Lys at position two. Therefore, the Cys-Arg-Leu triplet in AKAP220 is likely to facilitate peroxisomal localization of AKAP220 and may represent its targeting domain (McNew and Goodman, 1996; Swinkels *et al.,* 1992; Wiemer *et al.,* 1995). Our immunocytochemical analysis (Fig. 5) supports this association of AKAP220 with peroxisomes in RINm5F cells. Moreover, double-labeling immunofluorescence experiments show that AKAP220 and RII exhibit distinct but overlapping subcellular distributions (Fig. 5A&B). When these findings are considered with the biochemical evidence that RII and AKAP220 associate, it provides convincing evidence to suggest that a pool of type II PKA is anchored to the peroxisomes. Although the role of PKA anchoring to the peroxisomes is not clear, functional studies are in progress to determine whether disruption of PKA/AKAP220 interactions alters any peroxisomal events.

D. PKA ANCHORING AND cAMP-RESPONSIVE EVENTS

One of the developments during the past few years has been the production of peptide inhibitors of PKA anchoring (Carr *et al.,* 1992). Peptides encompassing the amphipathic helix region of Ht 31 effectively compete for RII-AKAP inter-

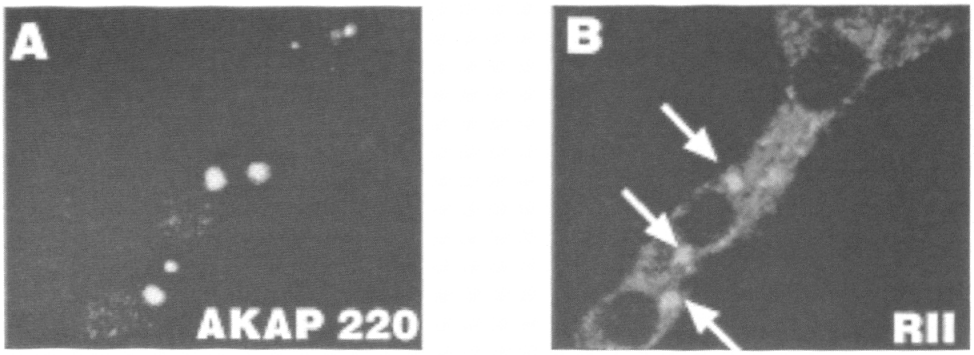

FIG. 5. Peroxisomal targeting of RII and AKAP 220 in RINm5F cells. Immunohistochemical analysis of RINm5F cells was performed using affinity-purified antibodies against (A) RII (1:200) and (B) AKAP 220 (1:100). Immunofluorescent detection was performed in a Lietz confocal microscope by the methods described in Lester *et al.* (1996).

action at nanomolar concentrations (Fig. 6) and have been used to disrupt the subcellular location of PKA inside cells (Rosenmund *et al.,* 1994). Accordingly, these "anchoring inhibitor peptides" are proving to be valuable reagents for functional studies that probe the role of PKA anchoring in certain cAMP-responsive cellular events. Our first study, published in 1994, focused on the modulation of the AMPA/kainate-responsive glutamate receptor ion-channels. Electrophysiological studies had demonstrated that agents with elevated intracellular cAMP increase the open probability of the channel (Greengard *et al.,* 1991; Wang *et al.,* 1991,1993) and it is presumed that channel opening is maintained through PKA phosphorylation events. We were able to demonstrate that perfusion of cultured hippocampal neurons with "anchoring inhibitor peptides" caused a time-dependent rundown in AMPA-kainate responsive currents, whereas perfusion of control peptides, which were unable to compete for RII binding, had no effect on channel activity. These findings suggested that the anchoring inhibitor peptides were able to displace PKA from anchored sites close to the channel. Additional support for this concept was provided by evidence that perfusion of excess purified bovine catalytic subunit could overcome the anchoring inhibitor effect, presumably by artificially increasing the intracellular concentration of the kinase close to the channel. These studies were the first to demonstrate that PKA anchoring is a determinant for certain cAMP-responsive events. Since then, these peptides have been used to demonstrate that PKA anchoring is required for the modulation of L-type Ca^{2+} channels (Johnson *et al.,* 1994).

One limitation of the original peptides was that they were not able to cross the cell membranes. This reduced the versatility of these reagents, as their delivery into cells required microinjection techniques. As a result, we could only examine the role of PKA anchoring in a few isolated instances where it was possible to measure effects on a single-cell basis. However, we have recently developed a

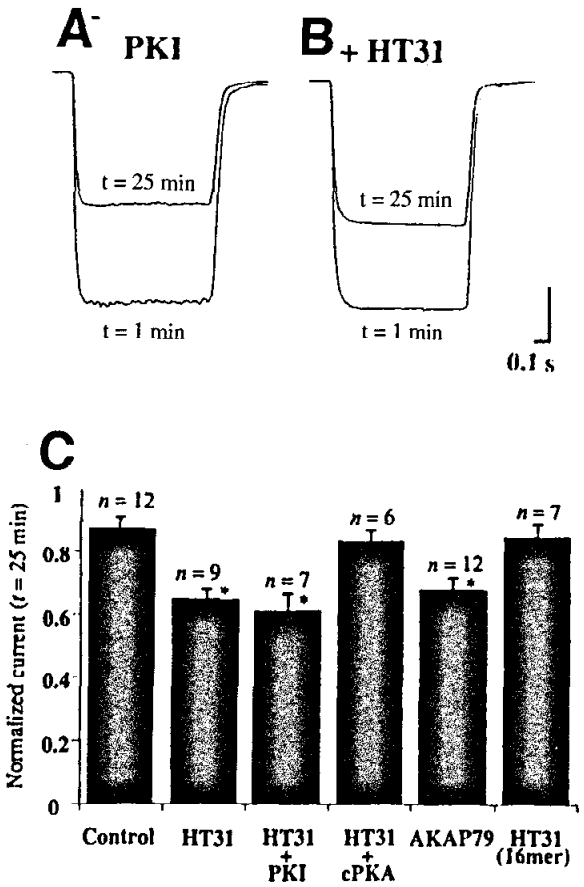

FIG. 6. Displacement of PKA by anchoring inhibitor peptides blocks regulation of AMPA/kainate channels by PKA. (A) Inward currents evoked by kainate (20 μM) at 1 and 25 min after the start of whole-cell recordings are superimposed. (Left) The current decreased in the presence of the PKI(5-24) peptide (1 μM) that blocks kinase activity. (B) The current decreased to a similar extent in the presence of the Ht 31 peptide that is a competitive inhibitor of PKA anchoring. (C) Controls showing the amplitude of AMPA/kainate currents in the presence of ATP (5 mM) 25 min after application of various bioactive peptides and C subunit of PKA (0.3 μM). Peptide concs were Ht 31 (1 μM), PKI 5-24 (1 μM), and AKAP79 388-409 (1 μM). [Adapted from Rosenmund *et al.*, 1994.]

second generation of cell-soluble "anchoring inhibitor peptides." This was inspired by the studies of Eichholtz *et al.* (1993), who demonstrated that myristylated forms of the PKC pseudosubstrate inhibitor entered cells and were functional. As a result, myristylated versions of the Ht 31 "anchoring inhibitor peptide" and the PKI 5–24 peptide were produced and have been successfully used as cell-soluble reagents in neurons and lymphocytes. In a recently completed study, we have demonstrated that application of the myristylated anchoring inhibitor peptide relieves cAMP-dependent inhibition of interleukin-2 (IL-2) transcription. Following on from these peptide studies, we have embarked upon the screening and identification of small molecule inhibitors of PKA anchoring. So

far, one small molecule has been characterized that is cell soluble and appears to uncouple RII/AKAP interaction and mimic the effects of the anchoring inhibitor peptides in cAMP-dependent IL-2 transcription. Future studies are planned to establish the selectivity and toxicity of these compounds inside cells.

VI. Multienzyme Signaling Complexes

Although many of the studies described in this chapter highlight the role of targeting proteins that localize individual signaling enzymes, work published in the last year has identified a new class of multivalent adapter proteins that coordinate the location of multienzyme signaling complexes (Faux and Scott, 1996b). The molecular "glue" for these complexes is provided by two related but distinct classes of adapter protein. Scaffold proteins such as the yeast protein sterile 5, which simultaneously associates with several kinases of a signaling pathway, forming an ordered module that permits sequential activation of each enzyme (Fig. 7A) and anchoring proteins such as AKAP79 and AKAP250, which are tethered to subcellular structures and localize their complement of enzymes close to their site of action (Fig. 7B&C).

A. THE STERILE 5 COMPLEX

Ligand-activated signal transduction pathways link cell surface receptors or activation of tyrosine kinases to changes in gene expression. In many cases, this occurs through a series of protein-protein interactions that trigger the mitogen-activated protein (MAP) kinase cascade (reviewed by Marshall, 1995). This pathway proceeds from the membrane-bound guanine nucleotide binding protein Ras, through the sequential activation of the cytoplasmic serine/threonine kinases Raf, MAP-kinase kinase (MEK), and MAP kinase (MAPK), and leads to specific gene expression in the nucleus. In the budding yeast *S. cerevisae,* there are at least five MAP kinase cascades. Each pathway is initiated by a distinct upstream regulator

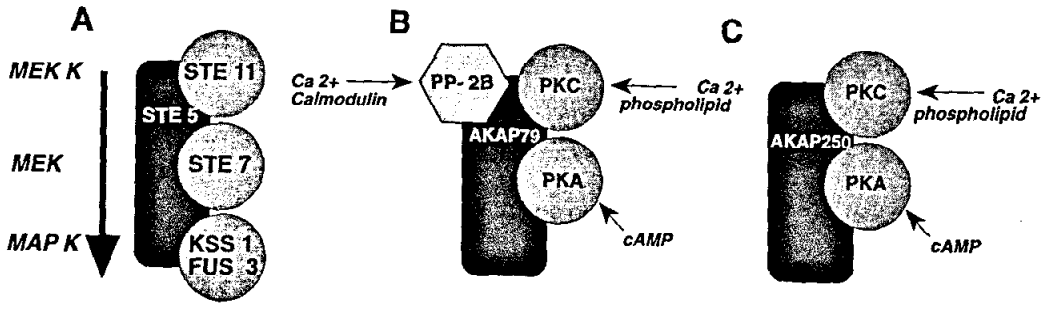

FIG. 7. Multienzyme signaling complexes. (A) Coordination of yeast MAP kinase cascade through STE 5. (B) The AKAP79 signaling complex targets PP-2B, PKC, and PKA to subsynaptic sites in neurons. (C) The AKAP250 complex targets PKA and PKC to the cytoskeleton.

and individual MEKK-MEK-MAPK modules control mating, cell-wall integrity, pseudohyphal development and invasive growth, sporulation, and osmoregulation (Herskowitz, 1995; Yashar *et al.,* 1995). The kinases in these pathways may be segregated through association with scaffold proteins. The pheromone mating response is initiated through ligand-induced changes in G-protein-linked receptors to activate a kinase, STE 20. This leads to the stimulation of STE 11, a MEKK homolog, which phosphorylates and activates STE 7, a MEK homolog, which, in turn, phosphorylates and activates the MAPK homologs, FUS 3 or KSS 1 (Choi *et al.,* 1994; Marcus *et al.,* 1994; Printen and Sprague, 1994). This signaling pathway is tightly controlled because each enzyme associates with a scaffold protein called STE 5 (Fig. 7A). Sterile 5 was initially identified as a recessive mutation in the yeast mating pathway 20 years ago and has recently been shown to encode a 917 amino acid protein. Initial clues to its function came from yeast two-hybrid experiments reported by three groups that demonstrated that STE 11 and STE 7 bind to distinct sites on STE 5, while FUS 3 and KSS 1 compete for another site on the protein (Fig. 7A). These findings were confirmed through deletion analysis demonstrating that a STE 5 1–336 fragment binds FUS 3 or KSS 1, whereas the STE 5 336–917 fragment only binds STE 7 and STE 11. Recent studies suggest that there may be additional components of the complex, as the G-protein β subunit, STE 4, and possibly STE 20 interact with STE 5 (Leeuw *et al.,* 1995; Whiteway *et al.,* 1995) (Fig. 7A). On the basis of these and other biochemical studies with bacterially expressed proteins, it is clear that STE 5 possesses all the qualities of a "classic" scaffold protein, as each enzyme binds to a distinct region of the protein (Fig. 7A). However, additional protein-protein interactions exist between each of the kinases in the absence of the scaffold protein, as two-hybrid studies indicate that STE 7 and STE 11 interact with either FUS 3 or KSS 1 in yeast strains lacking STE 5.

Although components of the STE 5 complex have been identified, the regulatory role of this signaling scaffold remains to be determined. Obviously, the clustering of successive members in the MAP kinase cascade is optimal for the tight regulation of the pathway. Regulation could occur through assembly and disassembly of the complex or by dephosphorylation by a phosphatase such as MSG5 (Doi *et al.,* 1994). It has also been proposed that scaffold proteins such as STE 5 may provide some selectivity by preventing cross-talk between functionally unrelated MAP kinase modules in the same cell. Indeed, a recent study using MEK gain-of-function mutations in *S. cerevisae* has demonstrated that STE 5 limits interactions of STE 7 with other MAPK activation pathways, contributing to STE 7 specificity (Yashar *et al.,* 1995). STE 5 is also able to maintain two different combinations of kinases, STE 11/STE 7/FUS 3 or STE 11/STE 7/KSS 1. An additional level of regulation could be obtained if the STE 5 complexes are differentially compartmentalized or additional enzymes associated with the scaffold. A likely candidate would be the phosphatase MSG5, which is known to

dephosphorylate FUS 3 (Doi *et al.,* 1994). Clearly, the demonstration that members of a yeast MAP kinase cascade are physically associated through a scaffold protein begs the question of whether a similar signaling scaffold exists in mammalian cells.

B. AKAP SIGNALING COMPLEXES

We have previously proposed that anchoring proteins contain at least two functional domains: an enzyme binding site and a targeting site responsible for anchoring to subcellular structures (Scott and McCartney, 1994). Until recently, all of the evidence supported the notion that each kinase or phosphatase was targeted through association with its own anchoring protein. However, it now appears that at least two anchoring proteins bind more than one enzyme at a time. In neurons, PKA is localized at postsynaptic densities by association with an anchoring protein called AKAP79. Biochemical studies show that AKAP79 also binds the Ca^{2+}/calmodulin-dependent protein phosphatase 2B, calcineurin (Coghlan *et al.,* 1995a,b) as well as several isoforms of PKC (Klauck *et al.,* 1996) (Fig. 7B). The structure of AKAP79 is modular and seems to resemble STE 5 in that deletion analysis, peptide studies, and co-precipitation techniques have demonstrated that each enzyme binds to a distinct region of the anchoring protein. Targeting of the AKAP79 signaling complex to the postsynaptic densities suggests a model for reversible phosphorylation in which the opposing effects of kinase and phosphatase action are co-localized in a signal transduction complex by association with a common anchor protein (Fig. 7B). Potential substrates for the AKAP79 transduction complex are likely to be synaptic receptor/channels and may include AMPA/kainate receptors and Ca^{2+} channels, which have recently been shown to be modulated by AKAP-targeted PKA, and NMDA receptors, which are activated by PKC and attenuated by calcineurin (reviewed by Klauck and Scott, 1995).

The apparent coordination of synaptic signaling by a modular anchoring protein raises the intriguing possibility that AKAP79 displays a similar function to the STE 5 scaffold. Both STE 5 and AKAP79 provide an additional level of regulation for protein phosphorylation events by restricting the action of kinases and phosphatases through protein-protein interactions. However, there are important differences. While AKAP79 dictates the subcellular location of two multifunctional kinases and a broad-specificity phosphatase, STE 5 orchestrates the location and activation of three interrelated kinases. To insure efficient transduction of this signal, both intermediary kinases STE 11 and STE 7 have limited substrate specificity and bind to precise sites on the scaffold protein. AKAP79, on the other hand, restricts the location of PKA, PKC, and calcineurin, enzymes with broad specificity. Another distinction is that the AKAP79 complex is able to respond to three distinct activation signals, whereas a single upstream event,

the activation of STE 20, is sufficient to transduce a signal from one kinase to the next in the STE 5 signaling scaffold. Despite these differences, the similarities between AKAP79 and STE 5 provide an opportunity to speculate on other proteins involved in signaling that may also serve as a scaffold. This latter view is supported by some recent data suggesting that a newly identified anchoring protein, AKAP250, contains binding sites for RII and PKC. AKAP250 was originally identified as an RII binding protein by an interaction cloning strategy that used radiolabeled RII as a probe. However, once the complete sequence of the AKAP250 cDNA was deduced, it became apparent that the amino terminal two thirds of the protein showed extensive homology to a clone 72, a protein that was identified as a PKC binding protein (Chapline *et al.,* 1996). In light of these findings, we have recently been able to map RII and PKC binding sites on AKAP250. These studies provide evidence that AKAP250 may represent a second kinase scaffold protein that serves to localize PKA and PKC at a defined subcellular site (Fig. 7C). Studies are currently in progress to characterize enzyme binding to AKAP79 and to establish whether additional signaling enzymes associate with the anchoring protein.

VII. Conclusions

Targeting subunits have emerged as a new class of regulatory molecule that contributes to the organization and specificity of signal transduction pathways. The broad specificity kinases and phosphatases seem to use these proteins not only to direct their catalytic subunits to particular parts of the cell but also to enhance specificity towards preferred substrates. This may represent a mechanism to prevent indiscriminate phosphorylation or dephosphorylation of phosphoproteins by these broad-specificity enzymes. The diversity of targeting subunits may reflect the diversity of cellular locations where these kinase and phosphatase enzymes act. One feature that seems to distinguish the phosphatase targeting subunits from kinase targeting subunits is that proteins such as the G or M subunits are components of the holoenzyme. In contrast, the kinase targeting subunits seem to represent separate entities that function independently of the holoenzyme. For example, myosin light chain kinase is attached to myosin, Raf activation occurs at the membranes, and many kinases containing PH domains are targeted to membranes. Undoubtedly, additional kinase targeting proteins will be identified. Now there is evidence that another multifunctional kinase, the Ca^{2+}/calmodulin kinase II, is targeted through association with anchoring proteins (McNeill and Colbran, 1995).

A variation on this theme seems to be the packaging of enzymes into signaling complexes. This may prove to be an efficient means for controlling the phosphorylation state of a given protein in response to multiple intracellular signals. Although conceptually similar, there are distinct differences between the

signaling complexes described in this chapter. While STE 5 and AKAP79 both coordinate the access of their associated enzymes to substrates, AKAP79 interacts with three multifunctional enzymes that are activated independently by second messengers, whereas STE 5 coordinates the action of several kinases in response to a single activation event. Although it may seem at odds with a classical view of signal transduction, signals passing through the STE 5 complex are not amplified. In fact, STE 5 may provide a framework that segregates one signaling event from the next, as there appear to be at least five distinct MAP kinase cascades in yeast. A unique feature of AKAP79 is that it localizes PKA, PKC, and calcineurin to the same subcellular sites, which potentially provides a mechanism to modulate the phosphorylation state of a single substrate protein in response to distinct second messenger signals. It remains to be seen whether AKAP250 will function in a similar manner as AKAP79. Despite these subtle differences, a common denominator between these adapter proteins is that they restrict the movement and increase the intracellular organization of serine/threonine kinases and phosphatases. These properties facilitate the diversity of signaling pathways that can be generated from connecting a few enzymes together. Moreover, the integration of separate second messenger signals at precise intracellular sites may hold a key to understanding how individual hormones can influence such specific intracellular phosphorylation events.

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DISCUSSION

David Klein: How do 14–3–3 proteins fit into the anchor scaffold proteins story?

John Scott: There is some evidence from the crystal structure of 14–3–3 to suggest that the dimeric structure of protein may function to bring together pairs of signaling enzymes. This is based upon biochemical and structural evidence that each 14–3–3 promoter contains a ligand binding site that can accommodate many ligands, including signaling enzymes such as Raf and protein kinase C. Therefore, it is formally possible that 14–3–3 could serve as a signaling scaffold. However, it is clear from the work of numerous investigators that the 14–3–3 family performs a myriad of other functions that are distinct from kinase targeting.

David Klein: Do the myz-peptides enter the nucleus?

John Scott: Immunofluorescent analysis of various cell types suggests that the myristyllated Ht 31 peptides are excluded from the nucleus. At this time we have been unable to establish whether the myristyl moiety seeks a hydrophobic environment and inserts into membranes.

David Klein: Does the Pro substitution alter cellular permeability?

John Scott: Surprisingly, the proline-substituted version of the anchoring inhibitor peptide is more soluble than the native version.

Robert Moss: I am interested in the hippocampal cultured neurons experiments and the administration of HT31 and PKI. How were they administered? Are they membrane permeable, or were the cells dialyzed? What was the time course for this action? What was the subtype of glutamate channels tested?

John Scott: All of our electrophysiology experiments (conducted in collaboration with Christian Rosemund and Gary Westbrook at the Vollum Institute) have focused on the kainate-responsive

glutamate receptor ion channels. Whole-cell recording was conducted at 30-second intervals over a time-course of 25 minutes after application of the peptides via a microdialysis procedure.

Etienne Baulieu: What are the main regulatory events governing AKAP synthesis and/or activity? Do AKAP proteins have any "molecular chaperone" property?

John Scott: We have conducted a few studies on the developmental and hormonal-induced expression of AKAPs. In 1993 we published a paper [Carr *et. al, J. Biol. Chem.* **268,** 20729–20732] demonstrating that follicle stimulating hormone (FSH) induces the expression of a 80 kDa AKAP in granuloma cells. This particular anchoring protein selectively binds to the RIIα isoform and FSH action results in a selective translocation of the type IIα PKA holoenzyme to the particulate fraction. This work is being followed up by a former postdoctoral fellow, Dr. Dan Carr.

Anthony Means: Is the binding of calmodulin and PKC to AKAP79 mutually exclusive? Is calmodulin involved in targeting AKAP79 to the post-synaptic density?

John Scott: This is an important question that we have spent a considerable amount of time addressing. Ca^{2+}/calmodulin is an antagonist of PKC/AKAP79 interaction. Calmodulin binds to the PKC binding site (residues 31 to 52) on AKAP70 and releases PKC activity from inhibition by the anchoring protein. Currently, we are focusing on the role of Ca^{2+}/calmodulin as a physiological regulator of PKC anchoring at the postsynaptic densities. We are also testing the hypothesis that calmodulin may serve as a targeting subunit for the AKAP79 signaling complex.

Phyllis Wise: Is there a relationship between the scaffolding or anchoring proteins and caveolae?

John Scott: None known as yet.

Michael Thorner: Do you have any evidence for a spontaneous mutation of an AKAP interfering with signal generation?

John Scott: No, although AKAP250 was originally isolated with sera from myasthenia gravis patients.