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Minireview

Protein Kinase A Anchoring*

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Specificity is perhaps the most enigmatic property of hormone-mediated signaling pathways, especially when one considers that more than 30 hormones employ the ubiquitous second messengers, Ca^{2+}, phospholipid, or cAMP, to relay messages from the cell membrane to intracellular effectors. In most cases the net signaling effect is to activate protein kinases or phosphoprotein phosphatases, which, in turn, alter the phosphorylation state of cellular target proteins. Although both enzyme classes have been intensely researched since the late 1950s, it is still unclear how individual hormones activate the correct pool of kinase or phosphatase to trigger specific intracellular events. One explanation that has recently attracted attention is a "targeting hypothesis" proposing that phosphorylation events are controlled in part by where kinases and phosphatases are located in the cell (1). In accordance with this proposal, it has become apparent that intracellular targeting of both enzyme classes is determined by association with "targeting subunits" or "anchoring proteins." While there have been several general reviews on this subject (1–3), this article will focus on subcellular targeting of the cAMP-dependent protein kinase (PKA).[1]

Regulation and Compartmentalization of the cAMP-dependent Protein Kinase

The primary action of cAMP in eukaryotic cells is to activate PKA (4). The PKA holoenzyme is a heterotetramer composed of a regulatory (R) subunit dimer that maintains two catalytic (C) subunits in a dormant state. Holoenzyme dissociation ensues upon binding of cAMP to tandem sites in each R subunit. This alleviates an autoinhibitory contact that releases the active C subunit. The active kinase is then free to phosphorylate substrates on serine or threonine residues, which are presented in a sequence context of Arg-Arg-Xaa-Ser/Thr or Lys-Arg-Xaa-Xaa-Ser/Thr. Given the frequent occurrence of these sequence motifs in many proteins, it is obvious that unrestricted access of the C subunit to its substrates would lead to indiscriminate phosphorylation. Consequently, several regulatory mechanisms are in place to ensure that cAMP levels and kinase activity are tightly controlled. Total cAMP levels are determined by a balance of cellular adenylyl cyclase and phosphodiesterase activities (5, 6), and signal-terminating mechanisms such as desensitization of adenylyl cyclase or compartmentalized activation of phosphodiesterases ensure further localized reduction of the second messenger (7, 8). Although access to cAMP is the primary requirement for PKA activation, additional factors are responsible for returning the kinase to the inactive state. The R subunits are expressed in excess over C subunits favoring rapid reformation of the holoenzyme when cAMP levels return to the basal state (9). In addition, the ubiquitous heat-stable inhibitor, PKI, may well serve as a fail-safe device, which sequesters free C subunit and mediates export of the kinase from the nucleus, which is devoid of R subunits (10).

For some time now it has been thought that the cellular specificity of PKA signaling is related to the existence of multiple C and R subunit isoforms. In mammals, three C subunit isoforms (α, β, and γ) exist, and although there are subtle differences in the kinetic characteristics and cAMP sensitivities of Cα- and Cβ-containing holoenzymes, these two predominant C subunit isoforms are virtually indistinguishable with respect to substrate specificity and interaction with R subunits (4, 11). In contrast, the dimeric R subunits exhibit both distinct cAMP binding affinities and differential localization within cells (4, 12). The type I PKA holoenzyme (containing either RIα or RIβ) is predominantly cytoplasmic, whereas >75% of the type II PKA holoenzyme is targeted to certain intracellular sites through association of the RII subunits (RIIα or RIIβ) with cellular binding proteins known as anchoring proteins (previously reviewed by Rubin (13) and Scott and McCartney (14)). Thus, it has recently been proposed that differences in subcellular targeting of type I and type II PKA are additional factors contributing to specificity in cellular responses.

AKAPs

The first RII-binding proteins were identified as contaminating proteins, which co-purified with RII after affinity chromatography on cAMP-Sepharose. However, a detailed study of these proteins was made possible by the observation that many, if not all, of these associated proteins retain their ability to bind RII after they have been immobilized on nitrocellulose (15). As a result, the standard technique for detecting RII-binding proteins is an overlay method that is essentially a modification of the Western blot (reviewed by Carr and Scott (16)). Using this technique, RII-binding bands ranging in size from 15 to 300 kDa have been detected in a variety of tissues, and it would appear that a typical cell contains 5–10 distinct binding proteins (17). The RII overlay method has also been refined into an efficient interaction cloning strategy wherein cDNA expression libraries are screened using RII as a probe. This has led to the cloning of numerous RII-binding proteins (17–26). More recently, the RII-binding proteins were renamed A-kinase anchoring proteins or AKAPs to account for their proposed PKA-targeting function (26). A model is presented in Fig. 1, which illustrates the essential features of AKAPs. Each anchoring protein contains two classes of binding sites: a conserved "anchoring motif," which binds the R subunit of PKA and a "targeting domain," which directs the subcellular localization of the PKA-AKAP complex through association with structural proteins, membranes, or cellular organelles.

Anchoring Motifs

Early work in the field focused on mapping the sites on RII required for interaction with AKAPs. Initially, a family of deletion mutants and chimeric proteins was screened by the overlay to define the minimum region of RII required to bind MAP2 and AKAP75 (27). It was concluded that RII dimerization was a prerequisite for anchoring, and AKAP binding required the first 30 residues of RII (27–29). However, the localization and dimerization determinants were later shown to be distinct as deletion of residues 1–5 abolished the anchoring but had no qualitative effect upon dimerization (29). This led to the identification of isoleucines at positions 3 and 5 as essential determinants for association with AKAPs (29, 30). Since leucines and isoleucines are also crucial determinants of the reciprocal binding surface on the AKAP (31) it is possible that RII/AKAP docking may be analogous to the hydrophobic interactions that maintain a leucine zipper in transcription factors such as CEBP and CREB. However, it must be noted that the protein-protein interactions required for RII-AKAP interaction are more elaborate and involve three polypeptide chains, *i.e.* two RII protomers and a binding surface on the AKAP (Fig. 1). Furthermore, additional AKAP binding determinants have been mapped between residues 11 and 25 of each RII molecule (32).

An added level of specificity in PKA signaling may be achieved through the differential localization of RIIα or RIIβ by association with isoform-selective AKAPs. Several years ago, it was reported

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; R, regulatory; C, catalytic; PKI, protein kinase A inhibitor; AKAP, A-kinase-anchoring protein; MAP, microtubule-associated protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; PKC, protein kinase C; PP, protein phosphatase.

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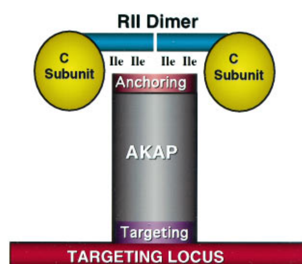

FIG. 1. **A PKA-anchoring model is depicted.** Binding surfaces on the AKAP for association with PKA (*Anchoring*) and for interaction with subcellular organelles or structures (*Targeting*) are indicated.

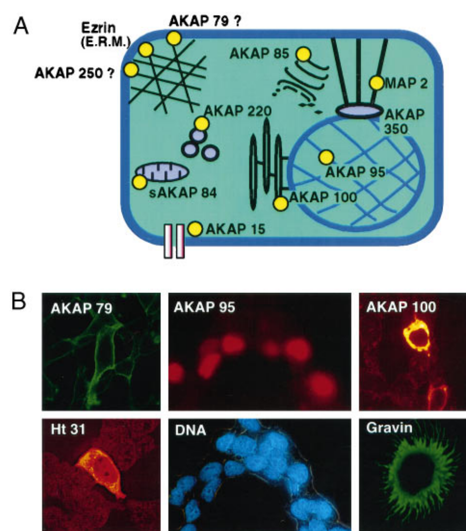

FIG. 2. **Subcellular targeting of AKAPs.** *A*, schematic diagram indicating the locations of known AKAPs in a prototypic cell. See text for details. *E.R.M.*, ezrin, radaxin, moesin. *B*, subcellular distribution of AKAPs expressed in cultured cell lines. Each *panel* shows immunofluorescent staining with antisera specific for the indicated AKAPs. Localization of AKAPs 79, 95, and 100 and an untargeted RII-binding fragment of Ht31 was visualized in transiently transfected HEK 293 cells. DNA in the nucleus was detected by Hoechst staining, and gravin expression was detected in human erythroleukemia cells treated with phorbol esters for 18 h.

that RIIα had a 6-fold preference for MAP2, whereas RIIβ had a 2-fold preference for AKAP75 (33). Recently it has been shown that follicle-stimulating hormone treatment of rat granulosa cells induces an 80-kDa RIIα-selective AKAP (34). Sequences of the first 10 amino acids of RIIα and RIIβ are almost identical except for a pair of prolines at positions 6 and 7 in RIIα that is not present in RIIβ. Removal of this proline pair impairs preferential interaction with RIIα-selective AKAPs (30). Although a precise structural explanation is not available for these observations, proline 6 may increase RIIα affinity for certain AKAPs through direct contact with the anchoring proteins, or alternatively, the greater rigidity of the imino peptide linkage may precisely orient other AKAP binding determinants. Nevertheless, it is clear that the orientation of the RII dimer (parallel or antiparallel) is another essential component of the AKAP-binding site as it controls the spatial geometry of isoleucines 3 and 5. Undoubtedly, the completion of studies to solve the structure of an RII fragment complexed with an AKAP peptide will resolve these issues.

Complementary studies have mapped the corresponding site on AKAPs that binds the RII dimer. Deletion analyses located the RII-binding sequences of MAP2 and AKAP150, the murine homologue of AKAP75, to short continuous regions of amino acid sequences (35, 36). However, the nature of the RII-binding motif remained unclear until a human thyroid-anchoring protein called Ht31 was identified (17, 37). The RII-binding sequence of Ht31 exhibited sequence similarities to both MAP2 and AKAP150 and was predicted to form an α-helix. Helical wheel projections of all three sequences exhibited a striking segregation of hydrophobic and hydrophilic side chains. This led to the proposal that the RII-binding motif of Ht31 and other AKAPs involves an amphipathic helix (37). Subsequent studies performed on Ht31 and AKAP79, the human homolog of AKAPs 75 and 150, demonstrated a requirement for this region in RII binding (19, 37). The role of helical secondary structure in RII-AKAP interactions was supported by demonstrating that substitution of proline, a residue that perturbs helix formation, at various positions within the RII-binding domain abolished RII binding (19, 37). These findings were consolidated by the synthesis of peptides encompassing the predicted helical region of Ht31, which were shown to bind either RII or the type II PKA holoenzyme with nanomolar affinity (17). Although the involvement of an amphipathic helix has not been definitively proven, independent studies have confirmed that similar regions of several other AKAPs are essential determinants for RII binding (20, 23, 24). The high affinity of these interactions has important consequences for the intracellular localization of PKA. First, the K_D for the RII-AKAP interaction has been calculated from 1 to 11 nM by a variety of analytical methods. This affinity constant is well within the intracellular concentration ranges of RII and most AKAPs suggesting that the RII-AKAP complex will be favored *in situ*. Second, the PKA holoenzyme binds Ht31 with the same high affinity as the RII dimer. Thus, the PKA holoenzyme will be anchored in cells when cAMP is at basal levels.

Functional Consequences of PKA Anchoring

Knowledge of the RII-binding domains on several AKAPs has allowed the generation of reagents that alter PKA anchoring within cells. So far two experimental strategies have been used: overexpression of AKAPs or RII-binding fragments (Fig. 2*B*) and the introduction of peptides that disrupt anchoring. Overexpression of AKAP75 or its human homolog AKAP79 redirects RII and other enzymes to the periphery of HEK 293 cells (38, 39), whereas expression of an untargeted RII-binding fragment called AKAP45 prevents membrane targeting of RIIβ in thyroid-derived cell line FRTL-5 (40). The functional consequences of these studies are not yet clear although there is some evidence that expression of the AKAP45 fragment prevents nuclear accumulation of the C subunit and decreases phosphorylation of the nuclear transcription factor CREB (40), suggesting a role for anchored PKA in cAMP-mediated transcriptional regulation.

Peptides encompassing the amphipathic helix region of Ht31 (residues 493–515) effectively compete for RII-AKAP interaction *in vitro* and disrupt the subcellular localization of PKA inside cells (41). Perfusion of cultured hippocampal neurons with these "anchoring inhibitor peptides" caused a time-dependent decrease in AMPA/kainate-responsive currents, whereas perfusion of control peptides, which were unable to compete for RII binding, had no effect on channel activity (41). Additional controls confirmed that the effects emanated from PKA as perfusion of PKI peptides, which block kinase activity, caused a decrease in channel activity, whereas microinjection of excess C subunit overcame the anchoring inhibitor effect. Collectively, these findings suggested that the Ht31 peptide displaced PKA from anchored sites close to the AMPA/kainate channels, thereby decreasing the probability of channel phosphorylation. Parallel studies by Catterall and colleagues have subsequently shown that Ht31 peptide-mediated disruption of PKA anchoring modulates L-type Ca^{2+} channels in skeletal muscle (42). Recently, this group has postulated that the peptides may uncouple the association of RII with a low molecular weight AKAP (AKAP15) that co-purifies with the channel (43). Taken together, these studies provide convincing evidence that PKA anchoring may facilitate preferential modulation of physiological PKA substrates.

However, there are technical limitations associated with the introduction of bioactive peptides into cells. Although microinjection or microdialysis is suitable for peptide delivery into single cells, the uptake of lipid-soluble peptide analogues is necessary to affect many cells. Accordingly cell-permeant anchoring inhibitor peptides have been developed. Myristoylated Ht31 peptides have been used to demonstrate a role for PKA anchoring in the cAMP-induced attenuation of interleukin-2 transcription in Jurkat T cells,[2] and stearoylated Ht31 analogues have been shown to arrest the motility of mammalian sperm (44). An intriguing aspect of the

[2] M. Howard, J. D. Scott, and W. M. Gallatin, unpublished observations.

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latter study is that inhibition of the C subunit with PKI peptides does not mimic the “anchoring inhibitor peptide” effect. This has led to the proposal that the R subunit has a distinct function in the regulation of sperm motility that is independent of the C subunit (44).

AKAP Targeting

The PKA-anchoring model (Fig. 1) implies that AKAPs must contain a unique targeting site that is responsible for association with subcellular structures. The targeting domain is an essential feature of each AKAP as it confers specificity by tethering the anchored PKA complex to particular organelles. So far, immunochemical and subcellular fractionation techniques have identified AKAPs localized to centrosomes (AKAP350), the actin cytoskeleton (Ezrin/AKAP78, AKAP250, and AKAP75/79/150), the endoplasmic reticulum (AKAP100), the Golgi (AKAP85), microtubules (MAP2), mitochondria (sAKAP84), the nuclear matrix (AKAP95), the plasma membrane (AKAP15), and peroxisomes (AKAP220). The subcellular locations of these known AKAPs are indicated in Fig. 2*A*. Less characterized anchoring proteins have been identified in secretory granules, plasma membranes, and the flagella of mammalian sperm (3).

Although an increasing number of compartment-specific AKAPs are now identified, targeting sequences have been extensively mapped for only two proteins. A C-terminal octadecapeptide repeat sequence targets the microtubule-associated protein MAP2 to microtubules (45), whereas two non-contiguous N-terminal basic regions, called T1 and T2, facilitate submembrane attachment of AKAP75/79 to the cortical cytoskeleton in HEK 293 cells (38). At this time the extent of AKAP75/79 targeting interactions is not clear as the T1 region has been independently identified as a site of contact with another signaling enzyme, protein kinase C (PKC) (39). This raises the intriguing possibility that AKAP79 could be targeted, in part, through protein-lipid interactions between PKC and the plasma membrane. However, AKAP79 targeting could also involve a third signaling enzyme as the phosphatase 2B (PP-2B), calcineurin, also associates with AKAP79 (46). Thus, submembrane targeting of AKAP79 could be mediated partly through the myristoyl moiety on the B subunit of the PP-2B holoenzyme. Conceivably, the T1 and T2 regions may represent two distinct targeting signals since AKAP79 is localized to both the cell bodies and dendrites in neurons (39).

Three other AKAPs appear to have distinct targeting sequences but have been not been analyzed in depth. AKAP220 may be targeted to peroxisomes (22) as the last 3 residues of the protein, Cys-Arg-Leu, conform to a peroxisomal targeting signal 1 motif, which is thought to facilitate the attachment of proteins to the lipid matrix of the peroxisome (47). AKAP250 is a component of the membrane/cytoskeleton, which is enriched in the filopodia of adherent human erythroleukemia cells (24). The N terminus of AKAP250 contains a consensus myristoylation signal, as well as other structural regions that bear some resemblance to actin-binding proteins such as MARCKS and GAP-43 (48). Likewise, an anchoring protein previously called AKAP78 has now been identified as the cytoskeletal component ezrin (49). In fact, ezrin and its two close relatives, radaxin and moesin, bind RII in the overlay assay. All three proteins (ezrin, radaxin, moesin) are members of the band 4.1 superfamily of proteins, which link the membrane and the cytoskeleton. Therefore, cross-linking from the membrane to the cytoskeleton might be a shared function of anchoring proteins such as AKAP79, AKAP250, and ezrin, radaxin, moesin (Fig. 2*A*).

AKAP Signaling Complexes

Although the principal function of the AKAPs is undoubtedly to target PKA, a fascinating new aspect has been the discovery of anchoring proteins, which simultaneously bind more than one signaling enzyme. It is thought that these multivalent AKAPs serve as scaffolds for the assembly of signaling complexes consisting of several kinases and phosphatases (50). This is an appealing variation on the anchoring theme as it provides a model for reversible phosphorylation in which the opposing effects of kinase and phosphatase action are co-localized in a multiprotein transduction complex (3, 51).

In neurons AKAP79 targets PKA to postsynaptic densities, cytoskeletal-like dendritic structures located on the internal face of excitatory synapses. Biochemical studies have shown that AKAP79 also binds PP-2B (46) and PKC (39). The structure of AKAP79 is modular as peptide studies and co-precipitation techniques have demonstrated that each enzyme binds to a distinct region of the anchoring protein. This has led to the proposal that AKAP79 coordinates the location of three signaling enzymes at postsynaptic sites (Fig. 3*A*). Potential substrates for this signaling complex are likely to be postsynaptic AMPA/kainate receptors or Ca^{2+} channels, which can be modulated by AKAP-targeted PKA (41), and *N*-methyl-D-aspartate receptors, which are activated by PKA or PKC and attenuated by PP-2B. A potential role for AKAP79 in the coordination of synaptic signaling is similar to the function of sterile 5 (STE5), a yeast scaffold protein that organizes three protein kinases that govern the pheromone mating pathway (52). There are, however, important differences between these two signaling scaffolds; AKAP79 dictates the subcellular location of two protein kinases of broad specificity and a phosphatase, whereas STE5 organizes the localization and sequential activation of a MAP kinase cascade. Another distinction is that the AKAP79 scaffold integrates signals from three second messengers, cAMP, Ca^{2+}/phospholipid, and Ca^{2+}/calmodulin, which activate three independent enzymes. In contrast, a single upstream event, the activation of STE20, is sufficient to transduce a signal from one kinase to the next in the STE5 signaling scaffold. Nevertheless, the similarities between AKAP79 and STE5 provide an opportunity to speculate that other anchoring proteins may also function as signaling scaffolds.

AKAP250, the anchoring protein enriched in the filopodia of adherent megakaryocytes (Fig. 2*B*), also contains binding sites for both RII and PKC (24). It was first noticed that the C-terminal third of AKAP250 was identical in sequence to that of a previously cloned protein called gravin, which was identified as a cytoplasmic antigen recognized by sera from patients with myasthenia gravis (53). Gravin/AKAP250 is depicted in Fig. 3*B* as a scaffold linking an actin target site and the cell membrane. When the complete amino acid sequence of gravin/AKAP250 was available it became apparent that distinct regions were similar to other AKAPs and to a PKC substrate/binding protein called SSECKs or clone 72 (54, 55). The PKA-binding site on gravin was mapped to a region of sequence in which 10 of 14 residues are identical to those in the corresponding region of AKAP79. This RII-binding sequence is also present in SSECKs/clone 72. The similarity of all three sequences was rather surprising as it had been proposed that a lack of sequence identity among AKAPs is due to a conservation of secondary, rather than primary, structure in the RII-binding motif (14). Therefore, gravin, SSECKs/clone 72, and AKAP79 may be members of a structurally related subfamily of anchoring proteins, each of which bind more than one kinase or phosphatase and are targeted to the membrane cytoskeleton.

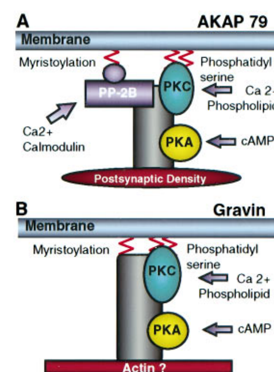

FIG. 3. **AKAP signaling complexes.** Models are shown for the AKAP79 signaling scaffold (*A*) and the proposed gravin (AKAP250) signaling scaffold (*B*). The enzymes and second messenger systems coordinated by these AKAP complexes are shown. The potential subcellular targeting functions of protein-lipid interactions involving PKC, PP-2B, or N-terminal myristoylation and protein-protein interactions involving cytoskeletal components are also shown for AKAP79 and gravin.

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Future Directions

The cloning of additional AKAPs should provide a more complete data base of sequences. This information should establish whether the AKAPs merely represent a convergent group of proteins sharing a common RII-binding motif or whether there are families of anchoring protein genes. Although most of the current data supports the first view, it is noteworthy that sAKAP84 is expressed in several forms due to alternative splicing; one splice variant, dAKAP1, was isolated in a two-hybrid screen using an RIα fragment as bait (56). Independent studies have shown that AKAP79 also binds RIα, albeit with a 100-fold lower affinity than it does RIIα.[2] This raises the intriguing possibility that certain AKAPs are dual function anchoring proteins, which bind both RI and RII. Not only do these findings provide a molecular mechanism for the compartmentalization of RI (57), but they also significantly expand the original anchoring hypothesis to include all PKA holoenzymes. Undoubtedly, future research will elucidate the nature of the RI-AKAP interactions and establish the physiological significance of type I PKA anchoring.

Despite the finding that both PKA holoenzyme subtypes may associate with AKAPs, it remains unclear why a significant fraction of PKA in most cell types is soluble. When this observation is considered together with evidence that the number of available anchoring sites in cells is in excess over the kinase, it seems likely that R-AKAP interactions are regulated in some manner. Although phosphorylation of RIIβ by CDC2 kinase prevents its association with MAP2, this does not occur with RIIα or RI (58). No doubt, future studies will focus on regulation of PKA anchoring. Another area of future emphasis will be the characterization of AKAP-targeting domains. AKAPs such as ezrin, radaxin, moesin, AKAP79, and gravin appear to be anchoring proteins that are linked to both the plasma membrane and the actin cytoskeleton. This suggests that highly localized PKA phosphorylation events may regulate cell shape and motility. Finally, it is likely that additional AKAP multienzyme signaling complexes, analogous to those that include AKAP79 and gravin, will be identified. For example, the Tau protein associates with PP-2A, and it is likely that MAP2, which is very similar to Tau and an AKAP, will bind PP-2A as well (59). It is also conceivable that AKAP transduction complexes could contribute to the specificity of cAMP action by bringing PKA together not only with phosphatases but also with the cAMP phosphodiesterases responsible for signal termination. These types of macromolecular organization mediated by anchoring proteins would not only place PKA close to certain substrates but also cluster the kinase with enzymes that regulate its activation state and enzymes that control the dephosphorylation state of the substrate. The challenge now is to pinpoint which important cellular phosphorylation events are regulated by the anchored kinases and phosphatases.

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[3] While every effort has been made to appropriately recognize the contributions of all those investigators in this field, due to space constraints it was necessary to limit the number of primary references.

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