Subcellular targeting of kinases and phosphatases by association with bifunctional anchoring proteins

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Introduction

Since the discovery by Sutherland and colleagues [1–3] of cyclic AMP as an intracellular second messenger for hormone-mediated events, considerable research has focused on understanding its action. A key step in this process was the discovery by Fischer and Krebs that cyclic AMP, Mg^{2+} and ATP were factors affecting the activity of phosphorylase *b* kinase [4], which led to the discovery of a cyclic-AMP-dependent protein kinase (PKA) [5]. Since then, it has been demonstrated that 20 or more different hormones activate PKA through a common signalling pathway that elevates intracellular cyclic AMP [6–8].

PKA is a multifunctional enzyme with a broad substrate specificity. It is therefore hard to imagine how the kinase is able to phosphorylate the correct target proteins rapidly and preferentially in response to activation by individual hormones. This is particularly true if one considers PKA as a soluble enzyme freely diffusing throughout the cytoplasm. Under these conditions, elevation of cyclic AMP concentration would cause an indiscriminate burst of phosphorylation. Thus a preferred hypothesis is that PKA is compartmentalized and individual hormones preferentially activate specific pools of kinase that are co-localized with substrate proteins (Figure 1). Consequently, the localization of PKA may be a key regulatory event in determining the intracellular sites of hormone action. Support for PKA compartmentalization is provided by three lines of evidence: different hormones activate specific PKA subtypes [9], cyclic AMP accumulates in distinct cellular compartments [10], and PKA subunits are detected in different cellular compartments [11,12]. Moreover, a body of work over the past 10 years has shown that the type II PKA holoenzyme (2R2C) can be tethered at specific subcellular locations through the interaction of its regulatory (RII) subunit with A-kinase anchor proteins (AKAPs) [13–16]. The scope of this paper is to highlight our recent progress on three features of PKA anchoring indicated in Figure 1. These are: (1) the identification of AKAP-binding determinants located on each RII protomer, (2) the use of AKAP-derived peptides to disrupt PKA anchoring and antagonize cyclic-AMP-responsive events, and (3) the use of yeast two-hybrid screening strategies to identify additional AKAP-binding proteins including the phosphatase 2B, calcineurin.

Abbreviations used: AKAP, A-kinase anchoring protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; CaN, calcineurin; PKA, cyclic-AMP-dependent protein kinase; PSD, postsynaptic density; RII, regulatory subunit of type II PKA holoenzyme.

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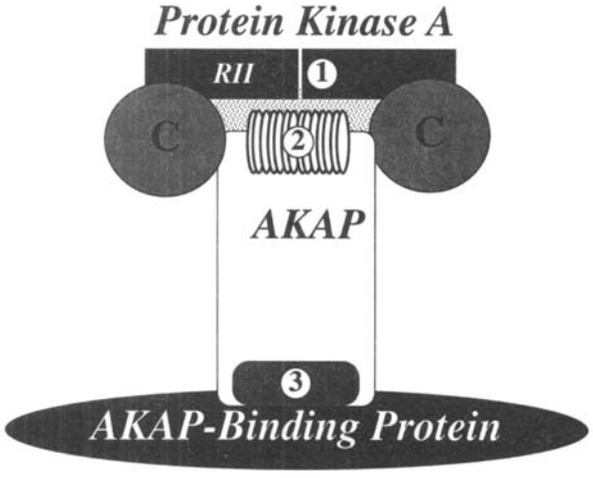
AKAP-binding determinants on RII

Initially, PKA anchoring proteins were identified by Rubin and colleagues as contaminating proteins that co-purified with RII after affinity chromatography on cyclic-AMP agarose [13,14]; however, detailed study of AKAPs was made possible by the original observation of Lohmann [17] that many AKAPs retain their ability to bind RII after transfer

Figure 1

Topology of the anchored protein kinase A complex

A schematic representation of a model for protein kinase A targeting through association with AKAPs. The three sites of protein–protein interaction discussed in the text are indicated (1, AKAP-binding determinants on RII; 2, conserved kinase-binding domain; 3, unique targeting domain). RII denotes the type II regulatory subunit and C denotes the catalytic subunit of protein kinase A.



1. AKAP-binding determinants on RII
2. Conserved kinase-binding domain
3. Unique targeting-domain

to nitrocellulose membranes. As a result, the standard method for detecting AKAPs is an overlay technique that is essentially a modification of the Western blot procedure [17,18]. Using the RII overlay we were able to screen a family of RII deletion mutants to map the AKAP-binding region [19]. Our early studies narrowed the AKAP-binding region to sites within the first 79 amino acids of RIIα [19,20] while, at the same time, Erlichman et al. [21] had demonstrated that the first 50 residues of RIIβ participate in anchoring. Comparison of the murine RIIα and RIIβ sequences revealed a high degree of homology (65% identity) in the first 40 residues, whereas both sequences diverged from residues 40 to 75 [22]. On the assumption that conserved structure often reflects conserved function, we concluded that the AKAP-binding region must be located between residues 1 and 40. To narrow the AKAP-binding region of RIIα, a family of C-terminal truncations and N-terminal deletions were constructed within the first 40 amino acids.

Truncated RII forms containing residues 1–40, 1–35 and 1–30 of murine RIIα bound AKAPs as assessed by the RII overlay, indicating that the AKAP-binding site is located within the first 30 residues of RIIα. Previous studies have shown that this region is also important for dimerization of RII [19]. To define more closely the AKAP-binding and dimerization domains, amino-terminal deletion mutants lacking the first five or ten amino acids of RIIα were constructed and were assayed for their ability to bind AKAPs and to dimerize. Both mutants were unable to bind AKAPs as assessed by the RII overlay and only RIIα des 5 retained the ability to form a dimer [23]. Thus determinants for AKAP binding are located within the first five residues of each RII protomer (Ser-His-Ile-Gln-Ile-), whereas determinants for dimerization are located further downstream. By a process of elimination, residues 3–5 were considered the most likely to be important for association with AKAPs because recombinant RIIα retains wild-type AKAP-binding activity although it contains a glycine residue in place of serine at position 1 and the histidine residue at position 2 is not conserved in the RIIβ isoform. To identify their contribution to AKAP binding, residues 3–5 were systematically replaced with alanine and the binding properties of the mutant proteins were compared with wild-type RIIα by equilibrium dialysis. Substitution of alanine for their Ile^3 or Ile^5 decreased AKAP binding to approx. 60% of wild-type activity, whereas substitution of alanine for glutamine at position 4 had no significant effect. The double mutant, with substitutions at both positions 3 and 5, had only approx. 15% of wild-type binding activity. The structural integrity of each mutant protein was retained, as assessed by circular dichroism, binding to cyclic-AMP agarose and dimer formation. Collectively, these results suggest that isoleucine residues at positions 3 and 5 play a major role in the high-affinity interaction of RII with AKAPs.

Anchoring inhibitor peptides antagonize cyclic-AMP-responsive events

In complementary studies we have investigated the regions within AKAPs that interact with RII. Because several AKAPs bind to the same or overlapping sites on RII, we hypothesized that all AKAPs would share a conserved RII-binding domain. A comparison of several AKAP sequences showed no striking homology, leading us to examine the RII-binding site of each AKAP for a conserved secondary structure motif. Computer-aided analysis of these sequences predicted that they all had a high probability for formation of an amphipathic α-helix (Figure 1). Disruption of secondary structure within this region by introduction of proline into the α-helix conformation of Ht31, a human thyroid AKAP, abolished RII-binding, whereas substitution of proline at sites outside the putative helical region had no effect [24,25]. Furthermore a synthetic peptide encompassing the putative helical region blocked all RII–AKAP interaction (as assessed by RII overlay) and bound RII or the type II holoenzyme with nanomolar affinity [25]. Putative amphipathic helices have now been identified in all AKAPs and we proposed that they form a conserved RII-binding site [15,24–28].

The development of 'anchoring inhibitor peptides' provided us with reagents to test whether disruption of PKA anchoring in cells could antagonize certain cyclic-AMP-responsive events. The test system for these studies were cultured hippocampal neurons where type II PKA is tethered at postsynaptic sites through association with AKAP79. We have previously shown that AKAP79 is associated with a cytoskeletal-like structure known as the postsynaptic density (PSD) located just below the postsynaptic membrane [26]. We have proposed that this anchor protein positions the kinase close to the ionotrophic glutamate receptors, which are ion channels known to be modulated by cyclic-AMP-dependent phosphorylation [29–31].

Whole-cell electrophysiology recording techniques were used to show that anchoring of PKA

by AKAPs is required for modulation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) kainate responsive glutamate currents. This was achieved by using the microelectrode as a delivery system to introduce the anchoring inhibitor peptides into hippocampal neurons [32]. Anchoring inhibitor peptides (1 μM) derived from Ht31 or AKAP79 blocked AMPA/kainate responsive currents to the same extent (64.9 ± 3.2%, $n = 12$, and 68.3 ± 3.3%, $n = 12$, respectively) as the PKI peptide (61.8 ± 4.1%, $n = 12$), a reagent that specifically inhibits the C subunit of PKA [33,34]. The effects of PKI and the anchoring inhibitor peptides were not additive. However, the action of the anchoring inhibitor peptides was overcome by addition of C subunit of PKA (0.3 μM), suggesting that the anchoring inhibitor peptide interfered with PKA-dependent phosphorylation but did not directly inhibit the kinase. In addition, a control peptide unable to block RII/AKAP interaction had no significant effect on kainate currents (85 ± 4.1%, $n = 7$). This was the first demonstration of an altered cyclic-AMP-responsive function as a result of disrupted PKA localization. However, Catterall and colleagues [35] have recently used the same anchoring inhibitor peptides to demonstrate that compartmentalization of PKA close to the L-type Ca^{2+} channels is required to maintain a voltage-dependent influx of calcium into skeletal muscle cells. This observation provides additional credence to the notion that PKA anchoring is a physiologically relevant regulatory mechanism to ensure the rapid and preferential phosphorylation of key substrates.

Additional AKAP-binding proteins: phosphatase 2B

The 'targeting hypothesis' of Hubbard and Cohen [36] stipulates that a targeting subunit directs protein kinases and phosphatases to an organelle, membrane or cytoskeletal component. Accordingly, AKAPs must contain two binding sites for localization of PKA: an amphipathic helix for association with RII, and a targeting site for attachment to subcellular structures [37]. Therefore a principal aim of the laboratory was, and still remains, the characterization of binding proteins that serve to target the AKAP (Figure 1). In an effort to identify such proteins, we used AKAP79 as the 'bait' in the yeast-two-hybrid cloning method popularized by Durfee et al. [38]. We initially screened a mouse T-cell library and demonstrated the validity of this strategy because we were able to retrieve numerous clones encoding the R subunit. However, to our surprise, one strongly positive clone contained cDNA encoding the catalytic subunit of the β isoform of the phosphatase 2B, calcineurin (CaN). A series of control experiments with the yeast constructs demonstrated that CaN specifically interacted with AKAP79 but not with RII alone, and other controls confirmed that AKAP79 bound RII in yeast. Collectively these findings suggested the occurrence of a ternary complex between CaN, the AKAP and PKA. Indeed, such ternary complexes were identified in bovine brain by using two separate biochemical fractionation methods [39]. First, CaN was immunoprecipitated from purified bovine brain extracts and was shown to co-precipitate with both AKAP75 (the bovine homologue of AKAP79) and PKA. Secondly, PKA R subunit was purified from brain extracts by affinity chromatography on cyclic-AMP-agarose. AKAP75 and the CaN holoenzyme were co-purified with R subunit in these experiments, and were specifically eluted from the affinity column with the PKA anchoring inhibitor peptide. This demonstrates that the CaN holoenzyme associates with PKA via the AKAP. Formation of this complex is supported by earlier work of Klee et al. [40], who showed that purified bovine brain CaN preparations contained the PKA holoenzyme and an unidentified 75 kDa protein.

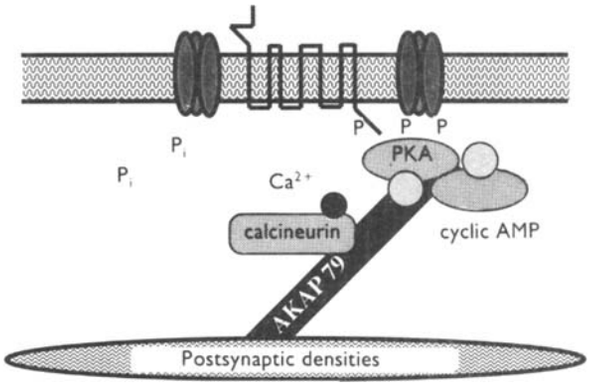
One important observation made during the isolation of ternary complexes was that the phosphatase recovered in these experiments was inactive. Using recombinant forms of CaN and AKAP79 in kinetic experiments, we found the AKAP to be a non-competitive inhibitor of the phosphatase ($K_i = 4.2 \pm 1.8$ μM) with respect to phosphorylated substrate peptide [39]. Comparison of the sequence of AKAP79 with those of other known inhibitors of CaN identified a region within the AKAP, distinct from the PKA-binding site, similar to the CaN-binding region found in the immunophilin FKBP-12. A synthetic peptide encompassing this region of the AKAP is also a specific inhibitor of CaN [39]. Unlike the immunophilins, however, inhibition of CaN by AKAP79 does not require the presence of immunosuppressant drugs. These results are intriguing because they represent the first report of a direct, endogenous inhibitor of CaN.

A hypothetical model for AKAP-mediated localization of PKA and CaN in neurons is presented in Figure 2. In this model both the kinase and phosphatase are optimally positioned at the PSD for modulating ion channels. Under conditions of elevated cyclic AMP, the channel becomes phosphorylated by localized PKA. The event is subsequently terminated through dephosphorylation of

Figure 2

Targeting of protein kinase A and calcineurin to the PSDs through association with AKAP79

The figure indicates our current thinking about the putative function of AKAP79, which simultaneously targets protein kinase A (PKA) and the phosphatase 2B (calcineurin) to the PSDs in neurons. Targeting of PKA and calcineurin at this site is likely to facilitate their preferential activation in response to the diffusible second messengers cyclic AMP and Ca^{2+}, thereby permitting modulation of the phosphorylation state of ion channels and transmembrane receptors located in the post-synaptic membrane (at top). Protein-bound phosphate groups are indicated (P) and inorganic phosphate released by calcineurin is denoted (P_i)



the channel by co-localized CaN. While the factors that activate localized CaN have not been determined, they presumably include Ca^{2+} and calmodulin to release the enzyme from auto-inhibition. A Ca^{2+}-dependent mechanism for dephosphorylation would be especially convenient for deactivation of Ca^{2+} channels, which would be shut down as part of a feedback mechanism. However, results from experiments *in vitro* in our laboratory have suggested a more complex mechanism for activation of localized CaN because inhibition of the phosphatase by AKAP79 is Ca^{2+}-independent [39].

Conclusions

The subcellular distributions of kinases and phosphatases are directed through their association with targeting proteins. In this paper we have reviewed studies that identify molecular determinants in RII. These determinants contribute to its function as a targeting subunit that binds to AKAPs and localizes the PKA holoenzyme near specific substrates. Until recently the AKAPs were thought to function exclusively in the localization of PKA. However, this view has been modified to include recent findings that demonstrate that certain AKAPs also function to localize the phosphatase 2B, calcineurin. Co-localization of kinases and phosphatases may account for the exquisite modulation of certain phosphorylation events that are necessary for maintaining cellular homoeostasis (Figure 2).

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Interaction of protein kinase C with RACK1, a receptor for activated C-kinase: a role in β protein kinase C mediated signal transduction

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Introduction

Protein kinase C (PKC) isoenzymes translocated from the cell soluble fraction to the cell particulate fraction after activation *in vivo* [1]. Evidence from a number of laboratories, including our own, has demonstrated that the activated enzyme is associated with cytoskeletal proteins [2-7] and that, within the same cell, each activated isoenzyme is differentially localized after activation [8-11]. Several proteins were found to bind PKC *in vitro* [12-17]. However, a role for these binding proteins in PKC-mediated signal transduction has been shown only for one group of proteins, which we have termed receptors for activated C-kinase (RACKs) [17-20]. This paper describes the results indicating that binding of PKC to RACKS is required for PKC-mediated signal transduction.

Abbreviations used: PKC, protein kinase C; cPKC, conventional PKC; RACK, receptor for activated C-kinase; WD40, 40 amino acid repeat sequence.

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RACKs are intracellular receptor proteins that bind activated PKC

Our recent studies suggest that translocation of PKC is due to binding of the activated enzyme to specific anchoring proteins, RACKs, at the cell particulate fraction [17-20]. These are proteins present in the cell particulate fraction that bind only activated PKC. Binding of PKC is specific for this kinase and is saturable [18]. In addition, we found that PKC does not bind to RACKs via the substrate binding site; an excess of substrate peptide does not inhibit PKC binding to RACKs [18]. Moreover, PKC activity is not inhibited by RACKs. Indeed, substrate phosphorylation is increased in the presence of limiting concentrations of cofactors. On