REVIEW ARTICLE

AKAPs (A-kinase anchoring proteins) and molecules that compose their G-protein-coupled receptor signalling complexes

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Cell signalling mediated via GPCRs (G-protein-coupled receptors) is a major paradigm in biology, involving the assembly of receptors, G-proteins, effectors and downstream elements into complexes that approach in design 'solid-state' signalling devices. Scaffold molecules, such as the AKAPs (A-kinase anchoring proteins), were discovered more than a decade ago and represent dynamic platforms, enabling multivalent signalling. AKAP79 and AKAP250 were the first to be shown to bind to membrane-embedded GPCRs, orchestrating the interactions of various protein kinases (including tyrosine kinases), protein phosphatases (e.g. calcineurin) and cytoskeletal elements with at least one member of the superfamily of GPCRs, the prototypical β_2-adrenergic receptor. In this review, the multivalent interactions of AKAP250 with the cell membrane, receptor, cytoskeleton and constituent components are detailed, providing a working model for AKAP-based GPCR signalling complexes. Dynamic regulation of the AKAP–receptor complex is mediated by ordered protein phosphorylation.

Key words: A-kinase anchoring protein (AKAP), β-adrenergic receptor, gravin, multivalent signalling complex, protein kinase A, protein kinase C, scaffold.

INTRODUCTION

The discoveries of PKA (protein kinase A) binding to MAP-2 (microtubule-associated protein 2) [1,2], to a bovine brain calmodulin-binding protein (p75; [3]), and to a family of 'anchoring' proteins that can bind the RIIα regulatory subunit of PKA [4,5], provided a critical conceptual leap in our understanding of the spatial dimension of cAMP signalling [6]. The identification of AKAPs (A-kinase anchor proteins) stimulated a provocative proposal in which local increases in the intracellular concentration of cAMP activated only those PKA molecules tethered in the vicinity by an AKAP. This spatial configuration would provide an explanation for the observed phosphorylation of only a subset of known PKA substrates within striking distance of the activated catalytic subunit homodimers of PKA, those released from the tethered homodimer of RII that constitute the inactive PKA tetramer bound to the AKAP. Although cAMP can regulate the activities of some molecules directly, PKA appears to be the major 'read-out' for cAMP to downstream signalling pathways. These downstream substrates include other protein kinases, protein phosphatases, other enzymes and ion channels. As additional signalling elements, including tyrosine kinases, protein phosphatases, GPCRs (G-protein-coupled receptors) and ion channels, were shown to be organized by AKAP signalling complexes, a more universal role of AKAPs as 'active' scaffolds in cellular signalling was envisioned. This role of AKAPs as scaffolds for signalling complexes likewise reflects the growing recognition that cellular signalling may best be envisioned as the output of 'solid-state' signalling devices of great complexity, providing spatial resolution and compartmentalization of signalling pathways. More than 30 mammalian AKAPs sharing the ability to associate tightly with PKA (RII- as well as some RI-containing forms) have been identified, and more will probably be discovered [7]. The tissue distribution of the AKAP family is wide, but selective. The expression of a family of three orthologues (AKAP75, AKAP79 and AKAP150 molecules, for example), is confined largely to neurons, where they are expressed in relatively high abundance [3,4,8,9].

MULTIVALENT SIGNALLING

Initially, there may have been good reason to expect AKAPs to be no more than bivalent, with an RI/II-binding domain located in the C-terminus of the AKAP, and a targeting domain located elsewhere in the molecule. AKAP-binding partners included the PKA RII subunit, MAP-2 [1], calmodulin [3] and cytoskeletal elements [10,11]. A ternary complex involving PKA, AKAP79 and protein phosphatase 2B [PP2B; also called calcineurin (CaN)] was identified in 1995 [12], establishing a multivalent paradigm for AKAPs that has been expanded over the past several years. This discovery was significant, not only because it revealed multivalency, but also because it demonstrated that both a protein kinase (PKA) and a protein phosphatase (CaN) can be tethered spatially to the same scaffold within the cell [12,13]. Particularly in synaptic densities, where ion channels are regulated by phosphorylation/dephosphorylation, the benefits of spatial organization of components by an AKAP seemed obvious. AKAPs have been localized to a variety of cellular structures, including synaptic densities [3,8], the cytoskeleton [11], Golgi [14], microtubule-organizing centres [15], mitotic spindles [16], microtubules [1] and the cell membrane [13,17–19]. In 1997, human AKAP250 (also known as AKAP12, gravin, and as the mouse orthologue SSeCKS [20]) was discovered, revealing for

Abbreviations used: AKAP, A-kinase anchoring protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; β_2AR, β_2-adrenergic receptor; BRET, bioluminescence resonance energy transfer; CaM, calmodulin; CaN, calcineurin; FK, forskolin; FRET, fluorescence resonance energy transfer; GPCR, G-protein-coupled receptor; GRK2, G-protein-coupled receptor kinase 2; MAP-2, microtubule-associated protein-2; MARCK(S), myristoylated alanine-rich C kinase (substrate); NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C; PP2B, protein phosphatase 2B.

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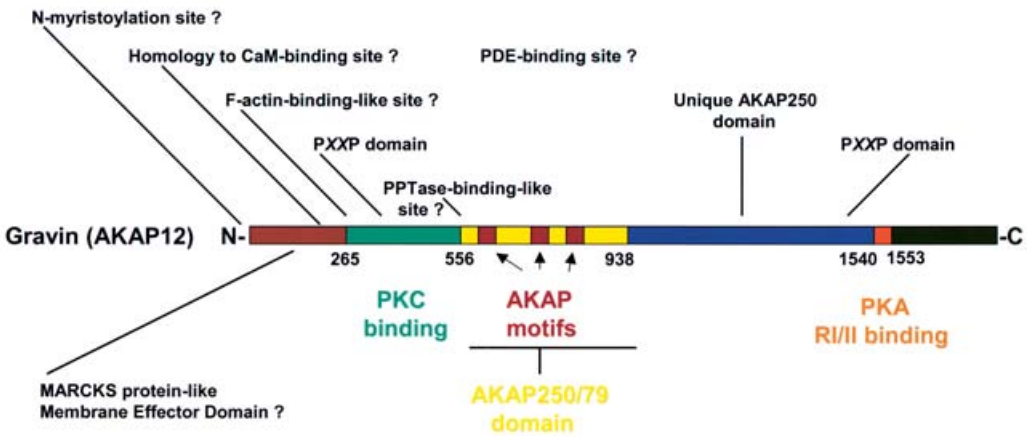

Figure 1 Working schematic representation of the AKAP250 topological map: identification of known and suspected protein–lipid and protein–protein interactions

The linear protein sequence of AKAP250 is displayed and annotated to show the demonstrated or probable protein–lipid (e.g. N-myristoylation site, MARCKS protein-like, membrane effector domain and scaffold–receptor interaction site) and protein–protein [e.g. binding sites for CaM, PKC, F-actin, SH3 Domain, protein phosphatases, phosphodiesterase (PDE) and RI/II subunits of PKA] interaction sites.

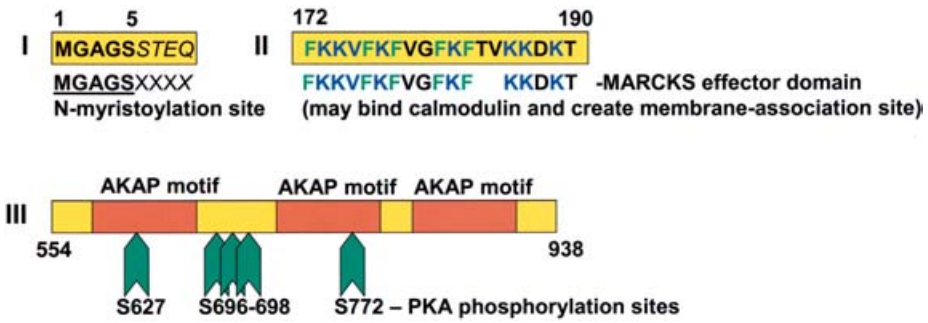

Figure 2 Three putative sites of regulated association of AKAP250 with the cell membrane

Three sites constitute the likely basis for interaction of the scaffold with the lipid bilayer: I, the N-myristoylation site; II, the MARCKS protein-like membrane effector domain; and III, the AKAP motifs through which the scaffold associates with the β_2AR. The site through which the scaffold interacts with the C-terminal 'tail' of the β_2AR includes several sites for phosphorylation by PKA.

the first time an AKAP with multivalency for two protein kinases, PKC (protein kinase C) and PKA [21]. The multivalency of AKAP250 has since increased with discovery of its association with a GPCR {the β_2-adrenergic receptor (β_2AR) [17–19,22,23]}, the non-receptor tyrosine kinase Src [24,25] and other proteins such as CaN (see below). Although for more than a decade CaN, PKA and PKC were known to be expressed in post-synaptic densities and to function in neural plasticity, only in the last several years has the pivotal role of AKAPs in providing a scaffold for these signalling elements been revealed.

Although many studies have revealed essential and key roles of AKAP250 and AKAP79 in multivalent signalling of GPCRs through serine/threonine protein kinases, tyrosine kinases, phosphatases, ion channels and other complex constituents, how these AKAPs organize signalling molecules, localize and traffic the complexes remains largely unexplored. Suppressing AKAP250/AKAP79 disrupts normal β_2AR-based signalling, and knock-out of the genes encoding either AKAP (*AKAP12* and *AKAP5* for AKAP250 and AKAP79 respectively) appears to be embryonic-lethal. Important differences exist between AKAP250 and AKAP79. This review seeks to examine the current knowledge on AKAP involved in GPCR signalling, to identify important structural and functional landmarks of AKAPs, and to thoughtfully delineate several key unresolved questions about these critically important scaffold molecules. To facilitate a methodical analysis of AKAP250, as well as a comparison with AKAP79, a schematic representation of the primary structure and landmarks of AKAP250 is shown (Figure 1).The analysis starts at the N-terminus of this scaffold molecule, and concludes at the C-terminus.

MEMBRANE ASSOCIATION

Careful analysis of the primary structure of AKAP250 reveals three domains critical to association of the scaffold with the membrane (Figure 2): a putative N-myristoylation site, an N-terminal sequence with high homology with the membrane effector domain of the MARCKS (myristoylated alanine-rich C kinase substrate) protein, and an AKAP domain conserved in AKAP250/79 that is critical to dynamic interactions between the β_2AR and the scaffold (the potential individual contribution of each of these domains is provocative with respect to understanding how AKAP250 associates dynamically with the lipid bilayer). N-myristoylation and the MARCKS protein membrane effector-like domain are discussed below, whereas the AKAP motifs

involved in binding AKAP250 to the plasma membrane-embedded β_2AR are discussed in a later section.

N-myristoylation of AKAP250

The first region of considerable interest is the N-terminus of AKAP250, which, unlike its orthologue mouse SSeCKS, possesses the N-terminal sequence H_2N-**MGAGS**STEQR, the signal sequence of N-myristoylation (where the residues in bold represent the minimum consensus sequence). N-myristoylation is the post-translational, covalent linkage of myristic acid ($C_{14:0}$) via an amide bond to the N-terminal glycine residue of a protein, following removal of the initiator methionine by an aminopeptidase. The biology and enzymology of N-myristoylation of proteins is well known [26,27]. The targets for N-myristoylation are diverse, but include other members of signalling cascades, such as heterotrimeric G-proteins (e.g. $G_i\alpha$ family members), non-receptor tyrosine kinases and the MARCKS protein [28], and may work in combination with other fatty acylation modifications [27]. This essentially irreversible post-translational modification affords weak and reversible protein–membrane (and protein–protein) interactions. Although AKAP250 displays the canonical N-terminal sequence targeting proteins for N-myristoylation, N-myristoylation of AKAP250 has not been demonstrated. From studies in unilamellar lipid vesicles, it is clear that the addition of the C-14 myristoyl group can increase the local concentration of a model peptide/protein at the membrane by a factor of ≈ 1000 [29]. Two additional forces driving the interaction between the scaffold and the membrane are well-known: a region equivalent to the 'effector-membrane domain' of the MARCKS protein [21,30] and a region by which AKAP250 binds to the membrane-embedded β_2AR [25]. If AKAP250 is N-myristoylated *in vivo*, and it is likely that it is, the functional role of this post-translational modification, its role in dictating membrane association and the effect of the loss of this fatty acylation on scaffold function remain to be established.

MARCKS protein membrane effector-like domain

Moving C-terminally along the AKAP250 sequence, the next region of major interest is the MARCKS protein-like 'membrane effector domain' (F^{172}KKVFKFVGFKFTVKK^{187}), corresponding to the C-terminal region of MARCKS protein and a nearly identical region in SSeCKS (Figure 3; the membrane-penetrating residues are shown in green, and the basic charged residues are in blue). The proteins of the MARCKS family are ubiquitous, abundant (especially in brain tissue), prominent substrates for PKC that are localized to the plasma membrane in quiescent cells.

The membrane effector domain of the MARCKS protein [31,32], a protein that, like AKAPs in general, is phosphorylated by PKA as well as PKC, and provides positive electrostatic potential that can act as a target for attracting multivalent acid lipids, including PtdIns(4,5)P_2 [33]. For the MARCKs protein, this domain affords regulatable membrane association. Like AKAP250, MARCKS protein is N-myristoylated and the proteins show significant similarity (compare Figures 3A and 3B). For MARCKS protein, this membrane effector region has been studied in detail, and has been shown to provide binding to lipid bilayers in a manner reversible by CaM (calmodulin) binding [34]. The membrane effector domain, displaying mutually exclusive interactions among its multivalent partners (e.g. CaM and CaN; see below), appears to function as an integrator for multivalent signalling. As noted earlier, there has been speculation that CaM binds to AKAP250/79/SSeCKS [35], but its role in regulation of these proteins remains to be established. It is tantalizing to speculate that the MARCKS-like sequence in AKAP250/79/SSeCKS is involved in membrane association, and is critical to directing AKAP250 to the cell membrane and to providing a CaM-sensitive mechanism to diminish that membrane association.

Although this region is poorly understood in AKAPs, the MARCKS protein-like domain has the potential for providing strong binding to membrane phospholipids, may sequester PtdIns(4,5)P_2, and, as mentioned above, is certainly a putative binding site of CaM influenced by protein N-myristoylation of MARCKS protein [36]. In AKAP79, the sequence K^{31}ASMLC-FK**RR**KKAAKALKPKAG^{52} (where the bold residues represent the dibasic residue motif) also has been implicated as a CaM-binding domain, as well as a weak PKCβII-binding site [37]. The first 150 residues of AKAP79 include several clusters of hydrophobic and basic charged residues proposed to function as a 'polybasic membrane-targeting domain' [7]. As AKAP79 lacks an N-myristoylation signal sequence, this polybasic region in the N-terminus and a putative β_2AR-binding sequence may constitute the likely forces driving association of AKAP79 scaffold to the cell membrane.

Detailed analyses of the MARCKS membrane effector domain (including X-ray crystallography [34]) have been performed, providing insight into the nature of the association [32,38]. The *Drosophila* AKAP200 (involved in development) also has a similar MARCK-protein-like region [39], but with two fewer aromatic residues than either MARCKS or AKAP250; each of these display five aromatic residues in this region [21,31]. The molar partition coefficient (K_D) for this region of these proteins into the lipid bilayer is increased 100–1000-fold for the AKAP250 and MARCKS proteins (containing five aromatic residues) compared with *Drosophila* AKAP200 (three aromatic residues).

PROTEIN PHOSPHATASE ASSOCIATION

The protein phosphatase CaN has been shown to be associated with AKAP79 [12] and with β_2AR-based signalling complexes [19,40–42]. CaN associates with either AKAP, and appears to be 'silenced' (i.e. enzymic activity suppressed) through binding, rather than simply tethered to the scaffold molecules [12,40]. Recognition that AKAP association with CaN is able to suppress phosphatase activity expanded our understanding of the function of AKAPs. Although it can be said that the binding of the PKA catalytic domains to the R subunits has the same type of effect, in the case of CaN, it is its association with the β_2AR-based signalling complex that silences its phosphatase activity. For PKA, in contrast, it is the association of the catalytic subunits to the regulatory subunits of PKA tethered to the AKAP that provides the silencing. Thus scaffolds such as AKAP250/79 are not simple templates functioning passively to increase the local density of signalling elements (e.g. kinases and phosphatases) in restricted spatial domains of the cell, but rather the dynamic regulators capable of both tethering and regulating enzymic activity of associated signalling molecules.

The region R^{88}RRKRSESSKQQKPL^{101} of AKAP79 initially appeared to be a likely site for CaN binding [45]. Subsequent analysis revealed, however, the C-terminal sequence E^{330}ESKR-ME**PI**A**III****T**DTEISEFDVTKSKN^{357}, displaying a '**PI**x**I**x**IT**' motif found in other CaN-interacting proteins, to be the dominant anchoring site for CaN binding [43]. Synthetic peptides corresponding to the amino acid sequence of either of these regions can block CaN binding to AKAP79. The peptide comprising residues 330–357 is 50-fold more potent, however,

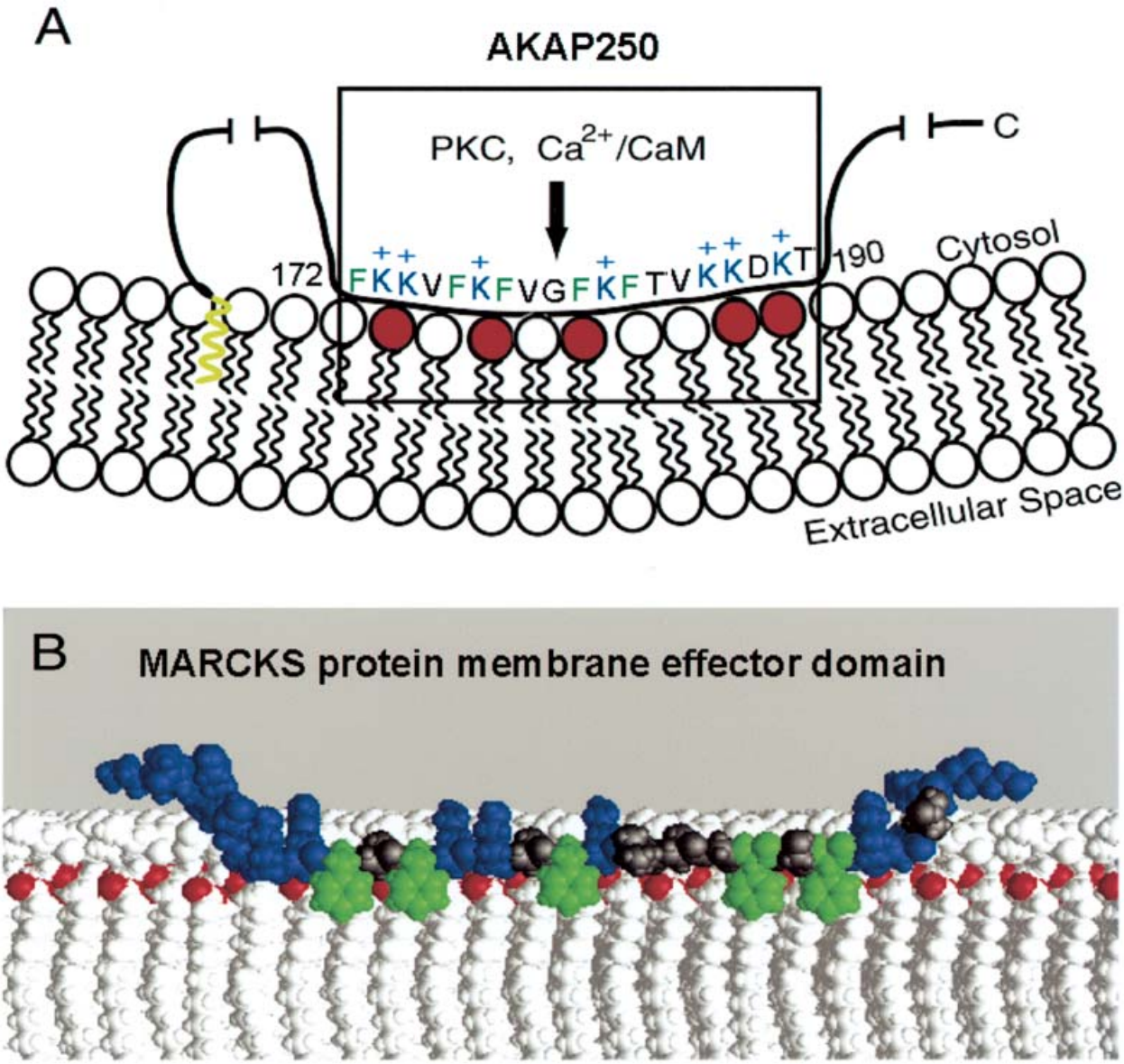

Figure 3 Model of the MARCKS protein-like membrane effector domain found in AKAP250

The scaffold displays an N-terminal region (**A**) that is highly analogous to the membrane effector domain of the MARCKS protein (**B**). Shown is the proposed interaction of the positively charged (blue) and polar (green) residues of this domain with the negatively charged phospholipids (red) constituting the lipid bilayer, displaying polar head groups of residues embedded by electrostatic charges into the inner leaflet of the bilayer (**A**,**B**). The interaction of the MARCKS protein membrane effector domain can be largely neutralized by increases in Ca^{2+} and the concentration of CaM or by the action of PKC. The authors propose the same combination of physical forces and regulation by Ca^{2+}, CaM and PKC are operating in the regulation of AKAP250-lipid bilayer interactions. Reproduced with permission from A. Gambhir, G. Hangyás-Mihályné, I. Zaitseva, D. S. Cafiso, J. Wang, D. Murray, S. N. Pantyala, S. O. Smith and S. McLaughlin (2004) Electrostatic sequestration of PIP_2 on phospholipid membranes by basic/aromatic regions of proteins, Biophys. J. in the press. © The Biophysical Society.

than the peptide of AKAP79 comprising residues 88–101 [43]. Although association with the β_2AR-based signalling complex clearly silences CaN [40], the binding sites for CaN on AKAP250 have not been mapped. There are no regions in AKAP250 with high similarity to either the dominant (residues 330–357) or lesser (residues 88–101) sequences of AKAP79. For the SSeCKS orthologue, a protein-phosphatase-binding site (shown subsequently in bold) has been proposed in the region corresponding to the AKAP250 sequence K^{602}REGVT**PWAS-FKKMVTPKKRVRR**PSESDKED^{632}. This region does include the dibasic residue motifs found in other protein-phosphatase-interacting proteins [35].

MULTIVALENT PKC/CaM/CaN-BINDING SITE

PKC plays a central role in cell signalling, especially for signalling via GPCRs [44]. PKC has been shown by pull-down assays to bind to, and to be silenced by, its association with AKAP79 [45] and AKAP250 [19]. AKAP79, by virtue of its binding of PKC, PKA and CaN, was the scaffold shown first to co-ordinate three key signalling enzymes [45]. The AKAP79 sequence K^{31}ASMLCFKRRKKAAKALKPKAG^{52}, implicated in CaM binding as well as CaN binding (minor site; [43]), also has been mapped as the PKC-binding site for AKAP79. For AKAP250, *in vitro* studies of the ability of various regions

of this scaffold to bind purified PKCβII in a phospholipid-dependent manner identified PKC-binding somewhere within residues 265–556 of AKAP250. The possibility that PKC, CaN [37] and, perhaps, CaM can compete for binding to a common region is extremely provocative. Detailed study of the interactions of AKAP79/250 with these potentially competing signalling molecules remains an important goal for the investigation of this tantalizing hypothesis. Recently, it has been shown in human model neurons (NT2-N neurons) that PKCα and PKCβII can differentially interact with AKAP250, the former requiring the presence of Ca^{2+} for docking, whereas the binding of the latter (as well as PKA) was independent of Ca^{2+}. Inhibition of PKC activity increased PKCα binding, but did not affect that of PKCβII [46]. Thus overlap of docking sites, as well as changes in local concentration of Ca^{2+}, may dictate the character of the composition and activities of AKAP-based signalling complexes for GPCRs.

AKAP MOTIFS AND GPCRs

The demonstration that both AKAP79 [47] and AKAP250 [40] scaffolds bind to the β_2AR, a prototypical GPCR [44], achieved another important milestone in our understanding of the roles of AKAPs in multivalent signalling complexes [44]. Intuitively, one can appreciate why there are spatial advantages provided by an AKAP scaffold, localizing PKA, PKC and CaN in close proximity to a GPCR that couples downstream to cAMP production. The β_2AR, like many GPCRs, is a well-known substrate for PKA, PKC and CaN [44]. The determinants of the β_2AR critical to binding AKAP250 were mapped by mutagenesis to region Arg^{329}–Leu^{413} of the C-terminal cytoplasmic tail, whereas the cytoplasmic loops 1, 2 and 3 appear to contribute little to the AKAP receptor binding [25]. AKAP79 binding to the β_2AR, however, not only requires the C-terminus, but also some portion of intracellular, cytoplasmic loop 3 (*i*Loop3; [47]).

The binding of AKAP250 to β_2AR is reversible, dynamically regulated by activation of the receptor (Figure 4) and essential for the normal subsequent sequellae in the desensitization, sequestration, resensitization and recycling of the β_2AR [25,48]. For AKAP79, β_2AR binding appears to be largely constitutive. More detailed analysis of this observation [47] and the cellular context, however, will be needed to fully explore this topic. The suppression of AKAP250 expression by antisense technology leads to a disruption of β_2AR trafficking and resensitization, demonstrating the central role of this scaffold in AKAP79/250-based β_2AR signalling complexes [19,25,47,48]. *In silico* analysis of AKAP250 revealed three 'AKAP motifs' at residue positions 603–633, 752–782 and 797–827. Deletion/mutagenesis of these AKAP motifs led to loss of β_2AR-binding capability of AKAP250 [50]. The two C-terminal motifs (TEG**EG**VST**WES**F**KRLVT**P**RKKS**K**S**SK**LEE**K** and EPG**KEESW**-VSIK**KFIPG**RRKKRPD, where the bold residues show identity between AKAP79 and AKAP250) are found also in AKAP79, suggesting that the region comprising residues 753–827 is essential for AKAP250 binding to β_2ARs. The first N-terminal AKAP motif of AKAP250 is lacking in AKAP79, and by itself fails to confer β_2AR-binding capacity to the AKAP [50]. Deletion of AKAP79 sequence 108–427 (containing the AKAP motifs) does appear to abolish β_2AR binding [47]. This same domain appears to provide AKAP79 binding to NMDA (*N*-methyl-D-aspartate) receptors and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) $GluR_1$ receptors via the MAGUK (membrane-associated guanylate kinase) proteins PSD-95 (post synaptic density protein-95) and SAP97 (synapse-associated protein 97) respectively [49].

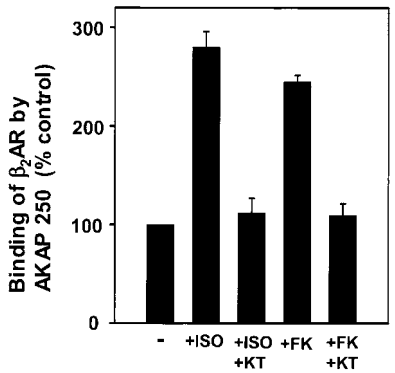

Figure 4 Binding of AKAP250 to the cell-membrane-embedded β_2AR is regulated in response to agonist (isoproterenol) or to elevation of cAMP by the plant diterpene FK

Pull-down assays of the scaffold from lysates of A431 cells treated for 30 min with or without 10 μM isoproterenol (ISO) or 10 μM FK. The pull-down samples were subjected to SDS/PAGE and immunoblotting, stained with antibody against the β_2AR. Both ISO and FK stimulate scaffold–receptor interaction. Addition of the PKA inhibitor KT5720 (KT; 0.1 μM) blocks the increased association stimulated by either ISO or FK, demonstrating the central role of cAMP and PKA in regulating the interaction.

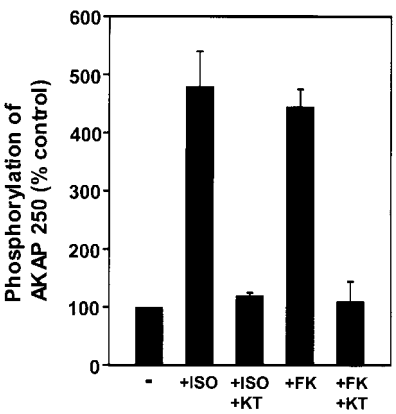

Figure 5 Protein phosphorylation of AKAP250 is regulated in response to agonist (isoproterenol) or to elevation of cAMP by the plant diterpene FK

Pull-down assays of the scaffold from lysates of A431 cells metabolically labelled with $[^{32}P]P_i$ and then treated for 30 min with or without 10 μM isoproterenol (ISO) or 10 μM FK. The pull-down samples were subjected to SDS/PAGE and immunoblotting (stained with antibody to the β_2AR to establish equivalent loading) and then autoradiography. Both isoproterenol and FK stimulate phosphorylation of the AKAP250. Addition of the PKA inhibitor KT5720 (KT; 0.1 μM) blocks the increased phosphorylation stimulated by either ISO or FK, demonstrating the central role of cAMP and PKA in regulating the phosphorylation of the scaffold (shown) and the receptor (not shown), as well as their interaction.

That the interactions between the β_2AR and the scaffold are dynamic in character and can be regulated by phosphorylation (Figure 5) was discovered only recently [50]. Although the full composition of the AKAP-based β_2AR signalling complexes remains to be established, two critical elements have been targeted for an exhaustive analysis of dynamic phosphorylation: the AKAP250 scaffold and the receptor itself. The recent discovery of the central role of AKAP phosphorylation by PKA in controlling receptor–scaffold interactions deserves some comment. Using pull-downs of AKAP250 (haemagglutinin-tagged, C-terminal) to assay for association of β_2AR (by immunoblotting with anti-β_2AR antibody) with the scaffold, it was observed that receptor–scaffold association increased

> 2.5-fold in response to agonist (Figure 4). Treatment with the plant diterpene adenylate cyclase activator forskolin (FK; 10 μM) mimics agonist, whereas the PKA inhibitor KT5720 (0.1 μM) blocks the increased association stimulated by isoproterenol or FK. Analysis of the phosphorylation of AKAP250, under the same design, revealed a 4–5-fold increase in phosphorylation in response to agonist or FK, and both of these response are blocked by KT5720 (Figure 5). Establishing the sites of phosphorylation on AKAP250 that are regulated by agonist required a fusion of proteomics and mutagenesis. Serine residues at amino acid positions 627, 696–698 and 772 in the AKAP motifs of the β_2AR-binding domain of AKAP250 were established as sites of phosphorylation. Mutagenesis studies demonstrated that Ser^{627} was not involved in regulating AKAP–β_2AR association, i.e. Ser^{627} could be mutated to alanine without effect. Residues $Ser^{696/697/698}$ as well as Ser^{772}, in contrast, are essential in the dynamic association of scaffold to receptor. The Ser^{772} residue appears to be constitutively phosphorylated in the absence of agonist activation, when the PKA activity ratio is typically $\approx$20%. The phosphorylation of $Ser^{696/697/698}$, however, occurs rapidly following agonist stimulation of the β_2AR, and this phosphorylation is required for enhanced scaffold–receptor interactions.

Thus this region of AKAPs may be central to receptor interactions. The AKAP250/β_2AR signalling complex appears to represent a 'mobile scaffold', since confocal microscopy reveals the sequestration of the β_2AR–AKAP250 complex in response to agonist stimulation [25]. As appealing as these data are, more detailed analysis of the trafficking of β_2AR and AKAP scaffold will require additional strategies, e.g. two-photon laser scanning confocal capabilities and energy-transfer measurements among the signalling complex components using FRET (fluorescence resonance energy transfer) [51], or even the more powerful $BRET^2$ (second generation bioluminescence resonance energy transfer) technology [52]. Likewise, when used in combination with site-specific mutagenesis of the potential binding sites for AKAPs on β_2AR, energy transfer and the increased confocality of the two-photon scope to begin to provide detailed analysis of scaffold–receptor interactions as the 'meso'-scale [49].

'INTERVENING' SEQUENCE BETWEEN AKAP MOTIFS AND THE RII-BINDING DOMAIN

Extending from residue 938 to 1540 of AKAP250 is a region in which no major protein binding module or association has been identified. When one compares the paucity of known interactions for this rather large region of AKAP250 (larger than many AKAPs) to the density of interacting proteins implicated in the slightly longer N-terminus of the molecule (Figure 1), it seems likely that new functions of AKAP250 remain to be discovered. Application of the yeast two-hybrid screen using regions of AKAP250 as 'bait' will probably identify new 'prey', i.e. additional constituents to AKAP-based signalling complexes. The Alliance for Cell Signaling (http://www.signaling-gateway.org/) has selected AKAP250 as one of its primary targets for yeast two-hybrid screening, a process that has already identified several new 'prey'.

PKA RI/II BINDING DOMAIN

The best understood motif in AKAPs remains the binding site for the regulatory subunits (RI/II) of PKA, located in the C-terminal region of most AKAPs. In AKAP250, the sequence E^{1540}LETKSSKLVQNII^{1553} constitutes the binding site for PKA RII subunits. In AKAP79, a homologous sequence occurs in residues 392–405. These sequences are amphipathic helices that provide a hydrophobic pocket for the binding of PKA R-subunit dimers [13,53]. The affinity of the R subunits for binding to these regions is in the nanomolar range: 0.5 nM for RI and 4.5 nM for RII [54].This sequence provides a basis for the Ht-31 blocking peptide (residues 493–515) [4], which has been shown to block the interaction of the β_2AR with AKAP79 [47] and AKAP250 [19].

The role that the PKA tethered to AKAP79/250 has in terms of the regulation of the molecules of the β_2AR signalling complex is only beginning to be appreciated. Mutation of the binding site for the RII subunit of PKA was shown recently to abolish the ability of PKA to phosphorylate only those AKAP molecules lacking the RII-binding site [50]. When performed in A431 cells expressing both the wild-type and RII-deficient AKAPs, in contrast, the RII-deficient AKAPs are no longer phosphorylated in response to agonist, demonstrating a new dimension in our understanding of spatial constraints on signalling complexes. One would have presumed initially that, with a mixture of wild-type and mutant AKAPs being expressed in the same cells, the wild-type scaffold would donate the PKA catalytic activity and still phosphorylate the RII-deficient AKAPs, but this was not the case [50].

Many of the signalling molecules constituting the complex are known to be substrates for PKA (e.g. β_2AR, AKAP79 and PKC) and are functionally regulated by PKA-catalysed phosphorylation. How protein kinase phosphorylation of complex members is ordered and how the phosphorylation contributes to defining the composition/interactions of the complex remains an enigma.

SCAFFOLDS AND CYTOSKELETAL INTERACTIONS

Central to understanding the dynamic role of AKAP79/250 is discerning how scaffold-based signalling complexes interact with cytoskeletal elements. The trafficking of β_2ARs appears to require cytoskeletal elements for membrane organization and transiting various endosomal compartments [55]. The interaction of GPCR signalling complexes with cytoskeletal elements has been recognized for some time [56,57]. Cytoskeletal interactions have been reported for AKAP79 [51] and AKAP250 [35,58]. The N-terminal region of AKAP250 includes a putative 'F-actin-binding domain', sequence Glu^{191}–Ala^{250}, that has been studied in some detail [35]. The sequestration of the AKAP250/β_2AR signalling complex can be stimulated by β-agonist or by treatment with insulin, a hormone that counter-regulates β-catecholamine action at several levels, including β_2AR internalization [59–62]. The sequestration of β_2AR signalling complexes in response to stimulation by β-agonist requires microtubule integrity, whereas the ability of insulin to sequester the β_2AR signalling complex requires F-actin dynamics [22]. Such dependence of GPCR trafficking on cytoskeletal elements is well known. Establishing whether or not these interactions are direct protein–protein contacts of cytoskeletal elements with the scaffold, the receptor, the G-protein, or these and other proteins in combination remains to be elucidated.

PxxP DOMAINS AND Src

Other protein motifs for molecules known to be associated with the scaffold and to participate in the downstream signalling pathway are present in AKAP250/79. The PxxP sequence that functions as an SH3 domain (Src homology 3 domain) are found

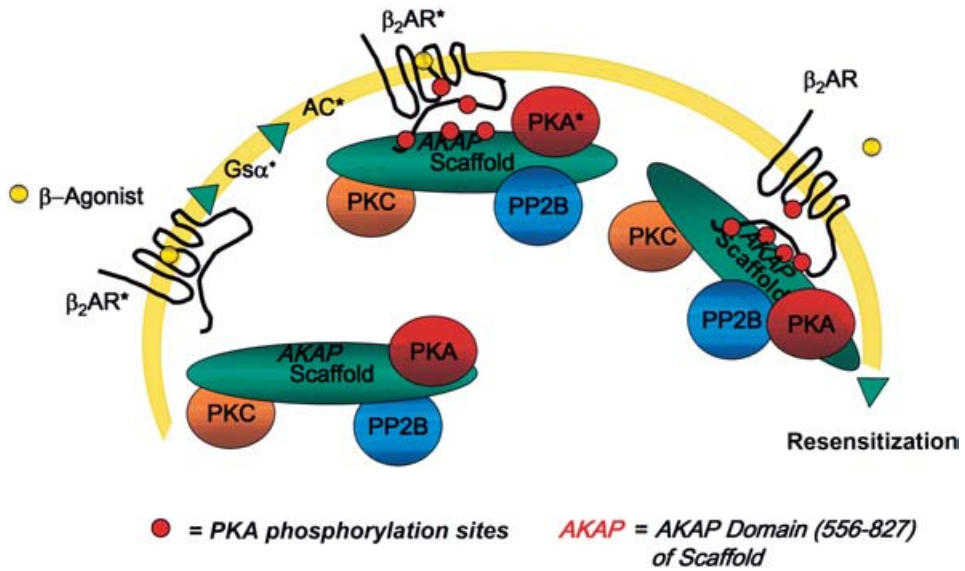

Figure 6 Schematic representation of the intramolecular phosphorylation of the AKAP domain of AKAP250 in response to and mediated by the association with β_2AR

The β_2AR is localized to the cell membrane via its seven transmembrane-spanning segments, providing three intracellular loops and a C-terminal 'tail' in the cytoplasm. The activation of the receptor (β_2AR*) by binding of an agonist provokes the activation of G_s ($G_s\alpha$*) and activation of adenylate cyclase (AC*), leading to the production of cAMP and the activation of PKA (PKA*). In the absence of receptor activation, AKAP250 docks PKC, PP2B and PKA. Upon activation of the receptor-mediated pathway and activation of PKA, both the receptor and the scaffold are phosphorylated. The phosphorylation of the receptor occurs on serine/threonine residues in the intracellular loop and C-terminal tail. AKAP250 undergoes phosphorylation by PKA, confined largely to the AKAP domain. The AKAP domain is shown in the current study to be the site by which the scaffold binds dynamically to the receptor. PKA-catalysed phosphorylation enhances the docking of AKAP250 to the receptor and is essential for the resensitization process which follows, a process dependent upon the protein phosphatase PP2B.

in AKAPs. The non-receptor tyrosine kinase Src (and probably Src family members) regulates a myriad of signalling pathways, including those of GPCRs [63,64]. Src has been shown to be critical to β_2AR sequestration in response to stimulation by either β-agonist [65–67] or insulin [24]. There are several potential docking sites for Src within the AKAP250/β_2AR signalling complex. Upon phosphorylation in response to insulin, the Tyr^{350} residue of the β_2AR located in the C-terminal, cytoplasmic tail of the receptor is transformed to a SH2-binding domain that can interact with Grb2, dynamin, PI3K (phosphoinositide 3-kinase) regulatory subunit, and Src [18,22,62]. AKAP79 and AKAP250 likewise possess 'PxxP' motifs that are SH3-binding domains and WW (Trp-Trp) domains. For AKAP250, the PxxP motifs are found at sequence residues 282–285 and 1474–1477, whereas a single PxxP motif is found at sequence 266–269 in AKAP79. These motifs are found in several other AKAPs. In addition, Src appears to bind, phosphorylate and activate the GPCR kinase GRK2 (G-protein-coupled receptor kinase 2) in cells [68], providing an additional role and possible docking site in the β_2AR signalling complexes [22]. The roles played by Src in β_2AR signalling complexes in β-agonist-stimulated desensitization/internalization compared with insulin-stimulated counter-regulation/internalization are likely to be different, based upon the differences noted in many features of those modes of β_2AR regulation [22,24].

PROBING FOR NOVEL AKAP-ASSOCIATED PROTEINS

Is the full complement of AKAP79/250-based signalling complexes known? In addition to compelling data for inclusion of PKA, PKC, CaN, β_2AR, Src and GRK2 [69] in the AKAP79/250 signalling complexes, others have reported the association of AMPA/kainate-sensitive glutamate receptors [49,70], NMDA receptors [71], the Kir2.1 ion channel [72], L-type calcium channels [73], $GABA_A$ (γ-aminobutyric acid A) receptors [74], as well as cytoskeletal elements [35,71] in these complexes. It is likely that GPCRs other than the β_2AR make use of AKAP-based signalling complexes, although the reagents available for dependable screening of the multitude of GPCRs are far more limited. At least for AKAP79, α_{2A}-adrenergic receptors, AT_{1A}-angiotensin receptors and VIPRs (vasoactive intestinal peptide receptors) do not appear to bind the scaffold [69]. The analyses most likely to yield new members of these multivalent signalling complexes are the yeast two-hybrid screens and the newer high-throughput proteomic screens, discussed above.

PROBING PROTEIN–PROTEIN INTERACTIONS OF AKAP250 VIA FRET/BRET ANALYSIS

Validating suspected protein–protein interactions requires convergent technologies. The yeast two-hybrid screen and high-throughput proteomic screens provide invaluable leads, but benefit greatly from complementary studies by confocal microscopy and fluorescence/bioluminescence energy transfer measurements among members in the complex. Confocal microscopy of auto-fluorescent fusion proteins of AKAP250 and β_2AR enabled demonstration of close association and trafficking of this receptor–scaffold during desensitization, sequestration, resensitization and recycling of β_2AR [25]. FRET has been applied to the imaging of AKAP79 interactions with CaN catalytic subunit and PKA RII subunits, including data suggesting formation of the ternary complex of scaffold, CaN and PKA [51]. Neither FRET spectroscopy nor the more sensitive and advanced $BRET^2$ spectroscopy [52,75] has been applied to defining the distances between the scaffold and other molecules in the signalling complex. Furthermore, it will be very exciting to define

the temporal, dynamic associations and distances between AKAP79/250, the β_2AR and other members of the signalling complex.

WORKING MODEL OF DYNAMIC INTERACTIONS OF AKAP250 AND THE GPCR

These many observations from a spectrum of approaches provide a compelling argument for the tight spatial activation of PKA that occurs in this well-known paradigm of G-protein-mediated signalling (Figure 6). Gravin (AKAP250) not only co-ordinates the multivalent signalling complexes to the cell membrane by dynamic association with GPCRs, but also ensures that the activation of the PKA proceeds within a highly restricted spatial domain. Furthermore, the function of the AKAP domains in AKAP250 is now known. The AKAP domain provides a reversible, dynamic docking site for the β_2AR and, presumably, other GPCRs. This would explain the basis for the association of AKAP250 as well as AKAP79 with β_2AR [48,69]. We propose that, for AKAP79/250, this AKAP domain might be better considered as a 'GPCR-binding domain', reflecting its role in docking the β_2AR and probably other GPCRs. Future analyses of AKAP-co-ordinated, multivalent signalling complexes might include screens for other GPCRs to test further the range of receptors that might provide localization of these multivalent signalling complexes to the cell membrane. Finally, the loss of AKAP disrupts the ability of the GPCR-docked signalling complexes to resensitize the β_2AR, a process catalysed in part by PP2B present in the complex [43,51]. The PKA-mediated phosphorylation and enhanced association of gravin with the β_2AR may be required for the proper environment in which PP2B dephosphorylates both the receptor and the scaffold. Understanding the operation of these solid-state, multivalent signalling complexes would benefit from higher resolution of their structures, particularly in the regions of the AKAP responsible for the reversible docking of protein kinases, phosphatases and receptors.

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