Membrane-targeting sequences on AKAP79 bind phosphatidylinositol-4,5-bisphosphate

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Protein kinases and phosphatases are targeted through association with anchoring proteins that tether the enzymes to subcellular structures and organelles. Through in situ fluorescent techniques using a Green Fluorescent Protein tag, we have mapped membrane-targeting domains on AKAP79, a multivalent anchoring protein that binds the cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and protein phosphatase 2B, calcineurin (CaN). Three linear sequences termed region A (residues 31–52), region B (residues 76–101) and region C (residues 116–145) mediate targeting of AKAP79 in HEK-293 cells and cortical neurons. Analysis of these targeting sequences suggests that they contain putative phosphorylation sites for PKA and PKC and are rich in basic and hydrophobic amino acids similar to a class of membrane-targeting domains which bind acidic phospholipids and calmodulin. Accordingly, the AKAP79 basic regions mediate binding to membrane vesicles containing acidic phospholipids including phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P₂] and this binding is regulated by phosphorylation and calcium-calmodulin. Finally, AKAP79 was shown to be phosphorylated in HEK-293 cells following stimulation of PKA and PKC, and activation of PKC or calmodulin was shown to release AKAP79 from membrane particulate fractions. These findings suggest that AKAP79 might function in cells not only as an anchoring protein but also as a substrate and effector for the anchored kinases and phosphatases.

Introduction

Signaling through protein phosphorylation is a tightly regulated process that is controlled by the opposing actions of protein kinases and phosphatases. The targeting of these signaling enzymes to certain intracellular microenvironments provides a mechanism that influences the specificity of signal transduction events by dictating which substrates are phosphorylated. Accumulating evidence now demonstrates that pools of protein kinases and phosphatases are maintained in specific subcellular locations by anchoring and targeting proteins (Faux and Scott, 1996). For example, an increasing number of A-kinase-anchoring proteins (AKAPs) have been characterized which bind the type II regulatory subunit (RII) of the cAMP-dependent protein kinase (PKA) to target the kinase to various intracellular membranes and discrete subcellular structures (Dell’Acqua and Scott, 1997). Each AKAP contains a conserved helical region responsible for binding the R subunit as well as targeting determinants that are responsible for localizing individual PKA/AKAP complexes to distinct intracellular locations.

The neuronal anchoring protein, AKAP79, is enriched in postsynaptic density fractions and has been detected immunohistochemically in the cell bodies and dendrites of cortical and hippocampal neurons (Carr et al., 1992; Glantz et al., 1992; Klauck et al., 1996). AKAP-mediated targeting of PKA has been shown to be important for regulation of postsynaptic excitatory neurotransmitter receptors (Rosenmund et al., 1994). AKAP79 also binds the calcium calmodulin-dependent protein phosphatase-2B, calcineurin (CaN) and the calcium-and phospholipid-activated protein kinase C (PKC) (Coghlan et al., 1995; Klauck et al., 1996). Hence, it has been proposed that AKAP79 directs the postsynaptic targeting of a multi-enzyme signaling complex which is involved in coordinat- ing second messenger-responsive phosphorylation of synaptic proteins. Thus, it is of great importance to identify targeting sequences and understand the mechanism of subcellular targeting of AKAP79.

It has been shown previously that AKAP79 and its bovine homologue AKAP75 are targeted to the cell periphery and enriched in the particulate fractions of transfected HEK-293 cells (Glantz et al., 1993; Ndubuka et al., 1993; Klauck et al., 1996). Deletion analysis has implicated N-terminal portions of AKAP75 as determinants for submembrane targeting in HEK-293 cells; however, it is unknown if these regions are sufficient for targeting of the anchoring protein or whether additional targeting determinants exist (Glantz et al., 1993; Li et al., 1996). Moreover, it has not been shown that targeting determinants identified in HEK-293 cells also promote targeting of AKAP79 in neurons. Interestingly, corresponding N-terminal portions of AKAP79 have also been implicated in binding to CaN and PKC, suggesting that subcellular targeting might involve secondary interactions mediated by the phosphatase and kinase (Coghlan et al., 1995; Klauck et al., 1996). However, since very little is known about the mechanism of AKAP79 membrane targeting, these important questions remain unanswered.

In the current study we have significantly extended our understanding of the mechanism of AKAP79 targeting through a combination of in vitro biochemical and cellular...
approaches. A series of AKAP79 fragments fused to the Green Fluorescent Protein (GFP) was used to define regions in AKAP79 which are sufficient for membrane targeting. These studies identified three regions which are rich in basic amino acids and mediate peripheral targeting of AKAP79 in 293 cells. Microinjection of cDNAs encoding AKAP79–GFP fusion proteins into cortical neurons demonstrated that these basic regions are also responsible for targeting of AKAP79 in neurons. Analysis of the AKAP79 targeting sequences suggests that they contain putative phosphorylation sites for PKA and PKC and have a similar amino acid composition to a class of membrane-targeting domains found in the Myristoylated Alanine-Rich C-Kinase Substrate protein (MARCKS), GAP43/neuromodulin and neurogranin which bind acidic phospholipids and calmodulin (Houbre et al., 1991; Aderem, 1992; Blackshear, 1993; Lu and Chen, 1997). Accordingly, recombinant AKAP79 fragments encompassing these basic regions were shown to bind in a phosphorylation-dependent and calmodulin-dependent manner to membranes containing the acidic phospholipid phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2]. The membrane-targeting functions of these basic regions were shown to be separable from any requirements for RII, PKC and CaN binding to the anchoring protein, suggesting the possibility of additional mechanisms for regulation of second messenger signaling and subcellular localization for the AKAP79 complex. In support of this possibility, AKAP79 was shown to be phosphorylated in transfected HEK-293 cells following stimulation of PKA with forskolin and PKC with phorbol-12,13-myristate (PMA), and activation of PKC or incubation with calmodulin was shown to release AKAP79 from HEK-293 membrane particulate fractions.

Results

**AKAP79 is targeted to the plasma membrane in HEK-293 cells**

Heterologous expression of AKAP79 or its bovine homologue AKAP75 in HEK-293 cells has been shown previously to mediate redistribution of RII-PKA from the cytoplasm to the cell periphery, suggesting that the transfected AKAP protein is functional in this system (Ndubuka et al., 1993; Li et al., 1996; Gao et al., 1997). Accordingly, transfection of HEK-293 cells with an AKAP79 cDNA expression construct results in the detection by immunoblotting of a 79 kDa protein that is enriched in the cellular particulate fraction (Figure 1A). The overexpressed protein is functioning as AKAP, as shown by the detection of a 79 kDa RII-binding protein (Figure 1B) and enrichment of PKA activity in AKAP79 immunoprecipitates (Figure 1C). No kinase activity was detected in control precipitations with preimmune serum (Figure 1C). Although a small amount of endogenous AKAP79 was detected by RII-overlay in untransfected HEK-293 cell lysates and immunoprecipitates (Figure 1B), the levels of native anchoring protein were too low to be detected routinely by immunoblotting (Figure 1A).

AKAP79 was detected exclusively at the periphery of HEK-293 cells as visualized by immunochromical staining with anti-AKAP79 polyclonal antibodies (Figure 2A) (Klauck et al., 1996). This localization shows considerable overlap with the cortical actin cytoskeleton which was visualized by staining actin with rhodamine–phalloidin (Figure 2B and C). However, disruption of the actin cytoskeleton by cytochalasin D (CHD) treatment had no effect on peripheral localization of AKAP79 (Figure 2D–F). Similar results have been reported for CHD treatment of HEK-293 cells transfected with AKAP75 (Li et al., 1996). Although CHD treatment caused collapse of the cortical actin (Figure 2E) and pronounced rounding of the cells, staining for AKAP79 remained tightly associated with the plasma membrane (Figure 2D). Thus, we conclude that AKAP79 peripheral targeting is not mediated through direct binding to actin microfilaments but is more likely to involve attachment to the plasma membrane through either protein–protein interactions with other membrane proteins or direct protein–phospholipid binding.

**AKAP79 membrane targeting in HEK-293 cells is mediated by three N-terminal regions which are rich in basic amino acids**

In order to investigate further the mechanism of AKAP79 membrane targeting, we sought to identify domains on
Membrane targeting of AKAP79 in transfected 293 cells is not affected by disruption of the actin cytoskeleton. Untreated AKAP79-293 cells (–CHD, panels A–C) and AKAP79-293 cells treated with 5 μM cytochalasin D for 4 h (+CHD, panels D–F), were stained immunochemically to visualize both AKAP79 (A and D, Anti-79) and F-actin (B and E, Phalloidin). Fluorescent staining was imaged by laser-scanning confocal microscopy. The images shown in (A–B) and (D–E) are for single-representative focal planes. Areas with overlapping anti-79 (FITC-green) and phalloidin (rhodamine-red) staining are seen as yellow in (C) and (F) (Composite).

AKAP79 which are sufficient to mediate targeting to the cell periphery. We addressed this question by generating a series of constructs encoding C-terminal GFP fusions of either full-length (1–427) AKAP79 protein or various AKAP79 fragments (Figure 3). By utilizing the GFP fusion tag we avoided the shortcomings of indirect immunochemical detection methods as we were able to detect the intracellular location of all AKAP79 fragments equally by excitation at 490 nm. Initial experiments confirmed that expression of the full-length AKAP79–GFP fusion in 293 cells results in a pattern of membrane localization (Figure 4C) which is indistinguishable from the localization of the untagged anchoring protein as detected by indirect immunofluorescent staining (Figure 4B). In contrast, control cells transfected with GFP alone exhibited fluorescence throughout the cytoplasm and nucleus (Figure 4A). These results indicate that C-terminal GFP fusion does not adversely affect the subcellular targeting of AKAP79 and confirm the utility of the fluorescent tag approach to map targeting determinants in the anchoring protein.

The C-terminal two-thirds of AKAP79 contains the structurally conserved RII-binding domain (residues 388–409) and is rich in acidic amino acids (Carr et al., 1992). In contrast, the N-terminal third of the AKAP79 protein contains three distinct regions of primary structure which are extremely rich in basic amino acids: region A, residues 31–52; region B, residues 76–101; and region C, residues 116–145 (Figure 3). Previous studies indicated that sequences present in the N-terminal portion of AKAP75, termed T1 and T2, which overlap with the A and B basic regions of AKAP79, were involved in targeting in HEK-293 cells (Glantz et al., 1993; Li et al., 1996). However, we have previously shown that the A region contains determinants for binding to calmodulin and PKC (Klauck et al., 1996; Faux and Scott, 1997) while the B region might interact with CaN (Coghlan et al., 1995). In order to resolve this issue, we generated AKAP79–GFP fusion proteins containing different combinations of the three basic regions to examine their possible roles in AKAP79 membrane targeting (Figures 3 and 4). A fragment encompassing all three basic regions, 1–153, was found to mediate targeting of
GFP to the cell periphery, indicating that the N-terminal basic regions alone were sufficient for membrane targeting (Figure 4D). Likewise, additional constructs containing any combination of two basic regions, A+B (1–108; Figure 4E), B+C (75–153; Figure 4F; 75–427; Figure 4G) and A+C (1–ΔB–153; Figure 4H; AKAP79-ΔB; Figure 4I) targeted GFP to the cell periphery. In contrast, constructs containing any single basic region failed to target GFP effectively to the plasma membrane (Figure 4J–L). A fragment expressing the A region (1–75) was distributed in the cytoplasm and the nucleus in a pattern that was similar to the GFP control (Figure 4J). However, a larger fragment (108–427) encompassing the C region was exclusively cytoplasmic (Figure 4L). Interestingly, expression of the B region alone (75–108) allowed partial targeting to the plasma membrane although a significant proportion of the fragment remained in the cytoplasm (Figure 4K). These results are summarized in Figure 3 and suggest that any two of the three AKAP79 basic regions are necessary and sufficient for submembrane targeting in HEK-293 cells.
AKAP79 targeting in neurons is also mediated by the N-terminal basic regions

AKAP79 is a neuronal protein which is highly expressed in the brain and is enriched in neuronal postsynaptic density fractions (Carr et al., 1992). The rat homologue of AKAP79 (AKAP150) was detected at the periphery of neuronal cell bodies and in dendritic regions by immunocytochemistry (Glantz et al., 1992; Klauck et al., 1996). Therefore, we reasoned that it was important to determine whether the poly-basic targeting sequences identified in HEK-293 cells also mediated subcellular targeting of AKAP79 in neurons. Selected AKAP79–GFP fragments were expressed in primary cultures of mouse cortical neurons by direct nuclear microinjection. Microinjection of a control plasmid encoding GFP resulted in a uniform distribution of fluorescence throughout the neuron including the nucleus, the cytoplasm of the neuronal cell body and the dendritic processes as depicted in three confocal sections of the same cell (Figure 5A–C). In contrast, full-length AKAP79–GFP was targeted to the periphery of the cell body and highly enriched in discrete dendritic regions (Figure 5D–F). This localization pattern (Figures 5D–F and 6C) is very similar to that detected immunocytochemically for the endogenous anchoring protein in cultured neurons (Figure 6B) (Klauck et al., 1996). These findings indicate that microinjection-based expression of the AKAP79–GFP fusion constructs is a viable method for studying subcellular targeting of AKAP79 in neurons. AKAP79–GFP fusions containing any two basic regions, 75–153 (B+C) (Figure 6D); 1–ΔB–153 (A+C) (Figure 6E); 1–108 (A+B) (data not shown), all displayed normal neuronal targeting, whereas a fragment encoding only the C region, 108–427, failed to target correctly (Figure 6F) and was found delocalized throughout the cell bodies and dendrites in a manner more similar to the distribution of GFP alone (Figure 6A). However, similar to the findings in 293 cells, GFP alone was present both in the nucleus and cytoplasm of neuronal cell bodies while the larger 108–427 fusion protein was excluded from the nucleus and delocalized in the cytoplasm. These microinjection studies confirm our transfection studies in HEK-293 cells and show that any two AKAP79 basic regions are sufficient for correct subcellular targeting of AKAP79 in neurons.

Calcineurin binding determinants on AKAP79 are functionally distinct from the basic membrane-targeting domains

In light of the mapping studies described above it is clear that three poly-basic sequences govern the submembrane targeting of AKAP79. Interestingly, two of these regions overlap with sequences that have been implicated in binding PKC and CaN. Therefore, it was plausible that membrane targeting of the AKAP79 could be achieved through secondary interactions mediated by PKC and CaN (Coghlan et al., 1995; Klauck et al., 1996). Further support for this view is provided by evidence that PKC is attached to membranes through protein–phospholipid interactions and that the B subunit of CaN contains a myristoylation signal that often participates in membrane anchoring (Klee et al., 1988; Newton, 1995). Although we have extensively characterized AKAP79–PKC interaction by mapping determinants in the A region that are responsible for...
Regulation of AKAP79 membrane targeting

Fig. 6. Neuronal targeting of AKAP79 is mediated by the N-terminal basic regions. Control neurons expressing GFP alone (A), AKAP79–GFP (C), or stained immunochemically with rabbit polyclonal anti-150 and FITC-secondary antibodies to visualize endogenous AKAP79/150 (B) are shown. The pattern of cellular GFP fluorescence was imaged in microinjected neurons expressing the 75–153 (D), 1ΔB–153 (E) and 108–427 (F) GFP fusion proteins. The images shown correspond to a single representative focal plane which shows portions of both the cell body and dendritic processes for each neuron. These images are representative of data obtained from multiple injected neurons for each GFP fusion protein.

binding and inhibition of the kinase, there is less experimental evidence to suggest that the B region binds CaN. Therefore, we sought to characterize the AKAP79–CaN interaction in more detail.

AKAP79 binding inhibits phosphatase activity, hence we examined the inhibitory potency of various purified recombinant AKAP79 fragments toward the CaN holoenzyme using a PKA-phosphorylated peptide as a substrate (Figure 7). Both full-length AKAP79 protein and the 108–427 (C) fragment of AKAP79 displayed strong and dose-dependent inhibition of CaN activity from 0.1–10 μM with IC50 values of 2.1 ± 0.2 μM and 0.43 ± 0.04 μM (n = 3), respectively (Figure 7A). In contrast, the 1–108 (A+B) fragment was unable to inhibit the phosphatase even at 10 μM (Figure 7A). Attempts to map more precisely the CaN inhibitory determinants within residues 108–427 relative to the C basic region were less conclusive. Additional fragments encompassing residues 75–153, 154–291, 108–291 and 154–427 only partially inhibited phosphatase activity with inhibition values ranging from 50–70% CaN activity at concentrations of 10 μM (Figure 7B). Consequently, these results indicate that the principal determinants in AKAP79 for inhibition of CaN are C-terminal to residue 108 and do not include the B basic region as originally proposed (Coghlan et al., 1995).

Our previous studies indicated that the primary mode of interaction with AKAP79 was through the CaN A subunit (Coghlan et al., 1995). To confirm independently that residues contained in 108–427, and not 1–108, participate in the AKAP79–CaN interaction, we examined the ability of these fragments to bind directly to CaN A. Full-length AKAP79, 1–108 or 108–427, were incubated in the presence or absence of biotinylated CaN A and precipitated with streptavidin–agarose beads. AKAP79 and 108–427 were precipitated with the biotinylated CaN A protein as detected by immunoblot, whereas the 1–108 fragment was not precipitated (Figure 7C). Control experiments demonstrated that each AKAP fragment did not interact significantly with streptavidin–agarose alone (Figure 7C). These binding assay results further suggest that CaN binding to AKAP79 does not require the B region but do not rule out a role for the C region.

**AKAP79 basic targeting regions bind to phosphatidylinositol-4,5-bisphosphate**

Even though binding determinants for PKC are contained in the A region and those for CaN may overlap with the C region, the results do not support a direct correlation between enzyme binding and membrane targeting. For example, fragments 75–153 and 1–108 both contain the B region and target to the cell periphery (Figure 4), yet lack binding determinants for PKC and CaN, respectively. Conversely, fragments 1–75 and 108–427 contain the determinants for binding PKC and CaN, respectively, yet fail to target to the cell periphery (Figure 4). Therefore, we concluded that another mechanism must participate in the submembrane targeting of AKAP79. In support of this suggestion, the AKAP79 poly-basic regions have amino acid compositions which are similar to those of the membrane-targeting/acidic phospholipid-binding domain of the MARCKS protein, an important cytoskeletal regulatory protein which is modulated by both PKC phosphorylation and calmodulin (Figure 8A) (Aderem, 1992; Blackshear, 1993; McLaughlin and Aderem, 1995). In addition, the B region of AKAP79 also has limited sequence homology with phospholipid- and calmodulin-binding regions found in two other prominent neuronal PKC substrates, GAP43/neuromodulin and neurogranin
including PtdIns(4,5)P2 and phosphatidylinositol-3,4,5-
trisphosphate [PtdIns(3,4,5)P3] (Lu and Chen, 1997). Based on the similarities to the phospholipid-binding
domains of MARCKS and neurogranin, we investigated
whether the AKAP79 basic regions could also bind directly
to acidic phospholipids.

The phosphoinositide-binding activity of purified
recombinant AKAP79 and fragments was assessed using a
lipid–protein co-sedimentation assay (Mosior and Newton,
1995). This assay monitors the recruitment of protein
from solution (supernatant fraction) to phosphatidylycholine
(PC) vesicles (pellet fraction) that contain increasing
concentrations of PtdIns(4,5)P2. Detection of AKAP79 in
the lipid-bound/pellet and unbound/supernatant was by
immunoblotting. Specific binding to PtdIns(4,5)P2 was
observed as a decrease in supernatant fraction and an
increase in pellet fraction AKAP79 immunoreactivity with
the increasing molar percentage of PtdIns(4,5)P2 relative
to the 0% (100% PC) control. AKAP79 shifted from the
supernatant to the pellet at concentrations of PtdIns(4,5)P2
as low as 1–2.5% or 1–2.5 μM (Figure 8C). These binding
data for AKAP79 suggest an affinity for PtdIns(4,5)P2 in
the low micromolar range which would be very similar
to affinity measurements of PtdIns(4,5)P2 binding to neuro-
grainin (Lu and Chen, 1997). The 1–108 (A+B) and 75–
153 (B+C) fragments exhibited PtdIns(4,5)P2 binding
activity which was comparable with that of full-length
AKAP79 (Figure 8C). In contrast, the 108–247 (C) frag-
ment showed little or no binding activity even at 10%
PtdIns(4,5)P2 (Figure 8C). A similar lack of binding
activity was also seen for the 1–75 (A) fragment (data not
shown). Interestingly, the 1–ΔB–153 (A+C) fragment,
which lacks the B region, shifted to the pellet to a lesser
to the other binding fragments and remained
partially in the supernatant fraction even in the presence
of 10% PtdIns(4,5)P2 (Figure 8C). These results suggest
that combinations of two or more basic regions are able
to mediate AKAP79 binding to PtdIns(4,5)P2. However,
the observation that the 1–ΔB–153 fragment displays
some weaker lipid-binding activity might suggest a
more specific role for the AKAP79 B basic region in
recognition of PtdIns(4,5)P2.

The binding of full-length AKAP79 to several other
acidic phospholipids was evaluated in order to address
whether the binding of the basic regions to membrane
vesicles containing acidic phospholipids is purely electro-
static in nature or whether there is any specificity for
PtdIns(4,5)P2. Over the same concentration range as
PtdIns(4,5)P2 (1–10%), similar AKAP79 binding was seen
for PtdIns(4)P while no binding was seen for PtdIns(PI)
(Figure 8D), thus suggesting some specificity for recogni-
tion of the 4-phosphate on the inositol ring. However,
AKAP79 binding to a structurally unrelated acidic
phospholipid, phosphatidylserine (PS), was also detected,
albeit at much higher concentrations (20–40 μM) than for the phosphoinositides tested (Figure 8D).
Similar phospholipid binding specificities were seen for
the 1–153, 1–108 and 75–153 fragments of AKAP79 (data
not shown). Taken together these results suggest that
AKAP79 binding to acidic phospholipids might involve
non-specific electrostatic interactions as well as a higher-
affinity preference for certain phosphoinositides.

Phosphorylation of AKAP79 regulates PtdIns(4,5)P2
binding

In an attempt to elucidate a mechanism for the regulation
of AKAP79 targeting we focused on a potential role for

![Image](https://example.com/image.png)
Regulation of AKAP79 membrane targeting

Fig. 8. AKAP79 basic targeting regions bind to phosphatidylinositol-4,5-bisphosphate. AKAP79 membrane-targeting sequences are similar to calmodulin and acidic phospholipid binding domains in MARCKS, GAP43 and neurogranin. (A) Amino acid sequences of the A, B and C membrane-targeting regions of AKAP79 and the calmodulin- and phospholipid-binding domain of MARCKS are shown. The numerous basic residues within these sequences are shown in bold type. Potential PKA and PKC phosphorylation sites are underlined for AKAP79 (PKA: T87, S92, S94; PKC: S81, T87, S96, S117, S123, S142). (B) Homology within the AKAP79 B region and the calmodulin- and phospholipid-binding domains of GAP43/neurogranin and neurogranin. The corresponding sequences for the bovine (AKAP75) and rat (AKAP150) homologues of human AKAP79 are also included in the alignment. (C) AKAP79 and fragments were analyzed for binding to vesicles containing the indicated concentrations of the acidic phospholipid PtdIns(4,5)P2. (D) AKAP79 was analyzed for binding to the vesicles containing the indicated percentages of additional acidic phospholipids: PtdIns(4)P [PI(4)P], PtdIns (PI) and phosphatidylserine (PS). In (C) and (D), a lipid–protein co-sedimentation assay was used (see Materials and methods). Proteins were detected in the resulting lipid-bound (pellet) and unbound (supernatant) fractions by immunoblotting with an anti-79 polyclonal antibody [immunoblots shown in (C) and (D)]. Note that because in these experiments the pellet fraction contains both the sedimented lipid vesicles as well as 25% of the supernatant fraction, there is AKAP79 immunoreactivity present in both the S and P fractions even in the absence of lipid vesicles (nl) or in the presence of neutral lipid vesicles containing 100% PC (0% acidic lipid). Thus, specific binding of the proteins to PtdIns(4,5)P2 or other acidic phospholipids is observed as a decrease in S fraction and an increase in P fraction AKAP79 immunoreactivity with the increasing molar percentage (1–10%) relative to the 0% control. The data shown are representative of similar results obtained in at least two additional experiments.

PKA and PKC phosphorylation. Although the A region does not contain any phosphorylation sites (Figure 8A) and is not a substrate for PKA or PKC (Klauck et al., 1996; Faux and Scott, 1997), the B and C regions contain several potential phosphorylation sites for either kinase (Figure 8A). In fact, all of the potential sites for phosphorylation by PKA and PKC in AKAP79 are contained within the 75–153 (B,C) fragment. Accordingly, AKAP79 and the 1–153 (A,B,C) fragment are substrates for PKA and PKC in vitro (Figure 9A). Likewise, the 75–153 (B,C) fragment is also phosphorylated by both kinases (data not shown). Since PKC phosphorylation of the phospholipid-binding domains in MARCKS, GAP43/neurogranin and neurogranin inhibits phospholipid binding and regulates membrane localization (Thelen et al., 1991; Kim et al., 1994a,b; Seykora et al., 1996; Swierczynski and Blackshear, 1996), we tested whether PKA and PKC phosphorylation was able to regulate AKAP79 binding to PtdIns(4,5)P2. The binding properties of phosphorylated AKAP79 or 1–153 fragment were compared with those of unphosphorylated control proteins by the lipid–protein co-sedimentation assay in the presence of 5% PtdIns(4,5)P2 vesicles. Unphosphorylated AKAP79 partitions almost exclusively to the lipid-bound pellet fraction (Figure 9B). In contrast, phosphorylation of AKAP79 by either PKA or PKC decreased PtdIns(4,5)P2 binding as demonstrated by a shift in AKAP79 immunoreactivity from the lipid pellet fraction to the unbound supernatant fraction (Figure 9B). Phosphorylation by both kinases inhibited PtdIns(4,5)P2 binding further, as seen in a near-equal distribution of AKAP79 between the supernatant and pellet fractions similar to 0% PtdIns(4,5)P2 (100% PC).
controls (Figure 8C and D and data not shown). Similar inhibition of PtdIns(4,5)P2 binding was observed for the 1–153 fragment following PKA and PKC phosphorylation (Figure 9B). Thus, these results suggest that phosphorylation at sites within the AKAP79 targeting domains negatively regulates binding to acidic phospholipids.

**Regulation of AKAP79 PtdIns(4,5)P2 binding by calcium-calmodulin**

In addition to regulation by PKC phosphorylation, the phospholipid-binding domains of MARCKS, GAP43 and neurogranin are also regulated by the direct binding of calcium-calmodulin (Houbre et al., 1991; Aderem, 1992; Blackshear, 1993; McLaughlin and Aderem, 1995). Significantly, AKAP79 is also a calmodulin-binding protein, and Ca\(^{2+}\)-calmodulin has been shown to regulate interaction of PKC with the A region (Carr et al., 1992; Faux and Scott, 1997). The affinity of AKAP79 for Ca\(^{2+}\)-calmodulin has been determined by surface plasmon resonance to be ~28 nM for the highest affinity site with analysis of the kinetic data indicating the likelihood of multiple calmodulin binding sites (Faux and Scott, 1997). When these observations are taken together with the homology to the phospholipid- and calmodulin-binding basic domains of GAP43 and neurogranin (Figure 8B), it suggests that the AKAP79 A and B regions are both likely to bind calmodulin. Thus, we determined whether calmodulin might also regulate binding of AKAP79 to PtdIns(4,5)P2. Full-length AKAP79 and the 1–153 N-terminal fragment were assayed for binding to 5% PtdIns(4,5)P2 vesicles in the presence of increasing concentrations of calmodulin (0.1–10 μM) and 0.1 mM Ca\(^{2+}\). For both proteins, calmodulin inhibited PtdIns(4,5)P2 binding in a dose-dependent manner as seen by a shift in AKAP immunoreactivity from the lipid pellet fraction to the supernatant fraction (Figure 9C). At 10 μM calmodulin, inhibition of PtdIns(4,5)P2 binding was complete when compared with 0% PtdIns(4,5)P2 (100% PC) controls (data not shown). However, consistent with previous affinity measurements (Faux and Scott, 1997), as little as 100 nM calmodulin caused measurable inhibition of AKAP79 PtdIns(4,5)P2 binding (Figure 9C). This inhibitory effect of calmodulin was entirely dependent on Ca\(^{2+}\) as shown by normal binding of AKAP79 or 1–153 to PtdIns(4,5)P2 in the presence of the calcium chelator, EGTA and 10 μM calmodulin (Figure 9C). These results suggest that, in addition to protein phosphorylation, AKAP79 binding to acidic phospholipids may also be regulated by binding of Ca\(^{2+}\)-calmodulin to the N-terminal basic regions.

**Phosphorylation of AKAP79 in HEK-293 cells and regulation of membrane binding by PKC and calcium-calmodulin**

To investigate a possible role for PKA and PKC phosphorylation in regulation of AKAP79 membrane targeting in a cellular system, we first set out to determine if AKAP79 is a substrate for PKA and PKC in living cells. The HEK-293 cells expressing AKAP79 which were characterized in Figure 1 were metabolically labeled with [\(^{32}\)P]orthophosphate and stimulated with forskolin to activate PKA or phorbol-12,13-myristate (PMA) to activate PKC. Total cell lysates were prepared and subjected to immunoprecipitation with anti-AKAP79 polyclonal antiserum; the washed immunoprecipitates were subsequently analyzed by SDS–PAGE and autoradiography to visualize \(^{32}\)P-labeling of AKAP79. A 79 kDa protein labeled with \(^{32}\)P was immunoprecipitated from unstimulated control cells, and cellular stimulation of PKA with forskolin or PKC with PMA both increased the phosphate labeling of this protein over that seen for the unstimulated controls (Figure 10A). The identification of this 79 kDa protein as AKAP79 was supported by control immunoprecipitations with preimmune serum from AKAP79-transfected cells and anti-79 polyclonal antiserum from...
Fig. 10. Phosphorylation of AKAP79 in HEK-293 cells and regulation of membrane binding by PKC and Ca$^{2+}$-calmodulin. (A) AKAP79 is phosphorylated in HEK-293 cells in response to stimulation of PKA and PKC. AKAP79 was immunoprecipitated in duplicate from cell lysates of transfected AKAP79-293 cells metabolically labeled with $[^{32}P]$orthophosphate. Phosphorylated AKAP79 precipitated from unstimulated cells (control) and cells stimulated with forskolin to activate PKA or PMA to activate PKC was detected by SDS–PAGE and autoradiography. The position of AKAP79 and the molecular weight standards (kDa) are shown on the autoradiograph in (A). (B) Activation of PKC in HEK-293 cells causes redistribution of AKAP79 from the membrane fraction to the soluble fraction. Membrane pellet and soluble supernatant fractions were prepared by centrifugation (100 000 $g$) from lysates of transfected AKAP79-293 cells either untreated (control) or treated with forskolin and PMA as indicated. The distribution of AKAP79 in these fractions was detected by $[^{32}P]$RII overlay blotting. (C) Incubation with purified PKC releases AKAP79 from cell membranes. Membrane pellet fractions prepared by centrifugation (100 000 $g$) from transfected AKAP79-293 cells were incubated as indicated with PKA and PKC without ATP (control), PKA with ATP (PKA), PKC with ATP (PKC) or PKA and PKC with ATP (PKA&PKC) and then pelleted again (100 000 $g$). The supernatant fraction containing soluble (released) proteins and the fraction containing proteins still associated with the pellet (membranes) were analyzed by AKAP79 immunoblotting. (D) Incubation with Ca$^{2+}$-calmodulin releases AKAP79 from cell membranes. AKAP79-293 membranes were incubated with calcium (1 mM), calmodulin (10 $\mu$M) or EGTA (5 mM) as indicated and then assayed for release of AKAP79 as described for (B). Note: the AKAP79 released by Ca$^{2+}$-calmodulin does not run as a tight band, most likely due to the high concentration of calcium ions present in this sample. The data shown in (A–D) are representative of similar results obtained in two or more additional experiments.

untransfected cells which both failed to precipitate this 79 kDa band (data not shown). Consistent with our in vitro phosphorylation studies (Figure 9A), we see greater $^{32}$P labeling of AKAP79 in response to PKC activation with PMA than following PKA stimulation by forskolin (Figure 10A). Also in agreement with the in vitro labeling studies, activation of both PKA and PKC resulted in greater $^{32}$P labeling of AKAP79 than with activation of either kinase alone (Figure 10A). These results suggest that AKAP79 is a substrate for PKA and PKC not only in vitro but also in a cellular environment.

To address whether phosphorylation of AKAP79 might regulate membrane localization in transfected HEK-293 cells, we treated serum-starved cells with forskolin, PMA or both forskolin and PMA, as described for the phosphate-labeling experiments. After these treatments, the cells were fractionated into soluble and membrane/particulate fractions. In unstimulated control cells AKAP79 is enriched in membrane/particulate fractions as visualized by $[^{32}P]$RII overlay blotting (Figure 10B) or immunoblotting (Figure 1A). Stimulation of the cells with PMA but not forskolin resulted in redistribution of a small but reproducible amount of AKAP79 from the membrane fraction to the soluble fraction (Figure 10B). Treatment of cells with both forskolin and PMA did not increase the amount of AKAP79 found in the soluble fraction over that obtained with PMA alone. These results suggest that phosphorylation by PKC but not PKA may regulate membrane targeting of the anchoring protein in living cells.

To confirm independently this specific effect of PKC on AKAP79 membrane targeting and to determine if calcium-calmodulin might also regulate AKAP79 localization to cell membranes, we incubated membrane/particulate fractions from transfected HEK-293 cells with exogenous PKC, PKA or calmodulin and assayed for release of soluble AKAP79. Consistent with our results for PKC activation in intact cells, incubation of AKAP79-293 membranes in the presence of ATP with PKC but not PKA resulted in the release of a small but reproducible amount of AKAP79 into the soluble supernatant fraction (Figure 10C). Likewise, incubation with both PKA and PKC did not increase the amount of AKAP79 released
over that obtained with PKC alone. The ability of PKC to release AKAP79 from the membrane pellet is likely to be due to protein phosphorylation, as control incubation with both PKC and PKA but no ATP failed to produce any soluble AKAP79 (Figure 10C). Incubation of these same membrane pellet fractions with 1 mM Ca\(^{2+}\) and 10 μM calmodulin also led to release of AKAP79 into the soluble fraction (Figure 10D). This effect of calmodulin on AKAP79 membrane localization was dependent on calcium as control incubations with 1 mM Ca\(^{2+}\) alone or 10 μM calmodulin plus 5 mM EGTA did not release any AKAP79 from the pellet (Figure 10D). These findings demonstrate that PKC phosphorylation as well as calcium-calmodulin are both able to regulate the binding of AKAP79 to cell membranes.

**Discussion**

Through the use of GFP as a tag for cellular fluorescence microscopy we have mapped three distinct membrane-targeting sequences in the N-terminus of AKAP79. These sequences termed A (31–52), B (76–101) and C (116–145) are rich in basic and hydrophobic amino acids. The A and B regions characterized in this study correspond to the T1 and T2 domains previously defined as targeting domains in 293 cells for the bovine homologue AKAP75 (Glantz et al., 1993; Li et al., 1996). However, our current studies arrive at different conclusions and significantly extend previous studies. Rubin and colleagues suggested that deletion of either T1 or T2 resulted in a loss of AKAP75 targeting to the cortical membrane-cytoskeleton (Li et al., 1996). We have identified a third downstream targeting sequence (the C region) and have demonstrated that deletion of any individual targeting sequence does not affect subcellular targeting. Moreover, combinations of any two basic regions were shown to be sufficient for proper targeting of AKAP79 in HEK-293 cells. Some of these differences may be due in part to the lower sensitivity of indirect immunoochemical detection methods used in the previous study. In contrast, the GFP tag is a very sensitive indicator of subcellular location and does not suffer from the shortcomings of immunoochemical detection methods such as differential recognition of protein fragments. Thus, the use of GFP fusion proteins allowed us to map more precisely the AKAP79 targeting determinants by focusing on regions of the molecule that were both necessary and sufficient for submembrane targeting.

The basic-rich targeting regions identified by our mapping studies in 293 cells also mediate proper targeting of AKAP79 in neurons. This finding is significant because AKAP79 is highly expressed in the brain where it is found enriched both at the periphery of neuronal cell bodies and associated with elements of the dendritic cytoskeleton (Carr et al., 1992; Glantz et al., 1992; Klauck et al., 1996). Moreover, the targeting functions of the basic-rich domains are separable from any structural requirements for the binding of RI, PKC and CaN to the anchoring protein. Our previous studies mapped the RI binding site to residues 388–409 and the PKC binding site to residues 31–52 of the A region (Carr et al., 1992; Klauck et al., 1996). Earlier peptide inhibition studies suggested that residues 81–102 contained within the B region might participate in binding CaN (Coghlan et al., 1995). However, our current inhibition and binding studies, which analyzed a more complete set of AKAP79 fragments, collectively indicate that CaN binding determinants are not contained within any single short linear sequence of the anchoring protein. Instead, they suggest that CaN binding is more likely to depend on tertiary structure and involve multiple sites of contact within the entire 108–427 fragment of AKAP79.

The separation of targeting and enzyme binding functions is especially relevant for one of the proposed functions of AKAP79 in neurons, which is to mediate the postsynaptic localization of a kinase/phosphatase signaling complex (Klauck et al., 1996). Localization of such a multienzyme signaling scaffold would allow coordinated regulation of second messenger-dependent phosphorylation of synaptic substrates such as ion channels and neurotransmitter receptors (Rosenmund et al., 1994; Gao et al., 1997). In this model the enzymes are bound to the anchoring protein in the inactive state and are then released in response to second messenger stimulation. For example, active PKA catalytic subunit is released from the PKA holoenzyme by cAMP binding to the AKAP-anchored inhibitory R subunits, while PKC is most likely released from inhibition by the anchoring protein in response to intracellular calcium signaling via the competitive binding of calmodulin to AKAP79 (Faux and Scott, 1997). For this an anchoring model to function properly, it would be important for the anchoring protein to be localized at postsynaptic membranes regardless of the docking of individual enzymes to complex.

Mapping the AKAP79 basic targeting determinants has allowed us to investigate further the molecular mechanism of AKAP79 membrane targeting. Earlier studies proposed that subcellular targeting of AKAP75/79 was mediated primarily through interactions with cytoskeletal proteins (Glantz et al., 1993; Li et al., 1996). While our current studies do not rule out interaction of AKAP79 with cytoskeletal components, they suggest that AKAP79 is likely to be targeted to the plasma membrane through direct binding of the basic targeting domains to acidic phospholipids. Because of the structural similarities of the AKAP79 targeting regions with the phospholipid-binding domains of MARCKS, GAP43/neuromodulin and neurogranin (Figure 8A and B), we hypothesized that AKAP79 targeting might involve binding to acidic membrane phospholipids. In support of this hypothesis, we demonstrated that the AKAP79 basic regions are able to bind to the acidic phospholipids, including the phosphoinositide PtdIns(4,5)P\(_2\). This observation further supports our conclusions that membrane targeting does not depend on cortical actin or require PKC and CaN to be bound to the anchoring protein. Similar to our analyses of membrane targeting in 293 cells and neurons, the results suggested that multiple AKAP79 basic regions might cooperate to efficiently bind PtdIns(4,5)P\(_2\) (Figure 11). Such cooperative protein–phospholipid binding interactions are reminiscent of the mechanism of MARCKS binding to phospholipids in which the basic-rich phosphorylation site domain and the N-terminal myristoyl group each contribute to proper membrane localization (McLaughlin and Aderem, 1995). By analogy, the combined electrostatic interactions of at least two of the three AKAP79 basic regions with acidic phospholipids might be needed for
Regulation of AKAP79 membrane targeting

Fig. 11. A model for regulated membrane targeting of the AKAP79 signaling scaffold. A schematic diagram that indicates the potential domain organization of the membrane-targeted AKAP79 signaling complex. Potential positive (+) targeting interactions between the acidic phospholipids, phosphatidylinositol (PS) and phosphatidylinositol 4,5-bisphosphate (PIP2), with PKC and the AKAP79 basic regions (A,B,C) are shown. Possible negative (−) regulation of targeting by protein phosphorylation (PKC and PKA) and calcium signaling (calmodulin) are also depicted.

proper membrane localization, thus explaining why AKAP79−GFP constructs containing two basic domains target to the plasma membrane while those containing single basic domains do not.

It is not clear whether the cooperative actions of the AKAP79 basic regions in binding to acidic phospholipids are purely electrostatic in nature or whether there is any specific preference for PtdIns(4,5)P2. AKAP79 exhibited half-maximal membrane binding at ~2.5 μM PtdIns(4,5)P2, which is in the same concentration range as PtdIns(4,5)P2 affinity measurements determined for neurogranin (Lu and Chen, 1997) and another class of proteins which bind phosphoinositides through pleckstrin homology (PH) domains (Rameh et al., 1997). Over the same concentration range tested for PtdIns(4,5)P2 (1–10 μM), similar AKAP79 binding was seen for PtdIns(4)P while no binding was seen for phosphatidylinositol (PI) or phosphatidylyserine (PS). However, some AKAP79 binding was observed with PS at much higher concentrations (20–40 μM). Low-affinity binding of AKAP79 to PS could be of some relevance not only in membrane targeting, because PS is an abundant membrane lipid, but also in the mechanism of PKC binding to the anchoring protein which involves both PS and the A basic region (Figure 11) (Klauck et al., 1996; Faux and Scott, 1997). Collectively, these studies suggest that AKAP79 membrane binding might involve some component of non-specific electrostatic interactions as seen by the binding to PS at high concentrations as well as a higher-affinity preference for certain phosphoinositides.

Interestingly, a peptide corresponding to the phospholipid-binding domain of neurogranin, which shows some sequence homology to the B basic region of AKAP79 (Figure 8B), binds phosphoinositides with the following order of specificity: PtdIns(3,4,5)P3 > PtdIns(4,5)P2 >> PtdIns(3,4)P2 (Lu and Chen, 1997). Therefore, if AKAP79 does have a preference for binding certain phospholipids with high affinity it might be a function of the B basic region. This postulate is consistent with evidence that a deletion fragment lacking the B region displayed weaker binding activity relative to other PtdIns(4,5)P2-binding fragments. Further support for this view is provided by data indicating that the (75–108)−GFP fragment containing only the B region, but not those fragments encoding the A or C regions alone, was partially targeted to the cell periphery (Figure 4K). Future studies examining the preferences of the different AKAP79 fragments for various phosphoinositides should help address these issues and explore how phospholipid kinases and phospholipases might influence, or be influenced by, AKAP79 localization within cells.

Because of the similarities to MARCKS, neuromodulin and neurogranin, AKAP79 phospholipid binding might be regulated by protein phosphorylation and Ca2+−calmodulin. Calmodulin binding and PKC phosphorylation of serine residues within the basic domains of all three proteins has been shown to regulate phospholipid-binding activity and membrane localization for these proteins (Thelen et al., 1991; Kim et al., 1994a,b; Seykora et al., 1996; Swierzynski and Blackshear, 1996). It is believed that phosphorylation of these serine residues inhibits acidic phospholipid binding through introduction of negatively charged phosphate groups which neutralize the basic electrostatic environment of the domains, thus composing an ‘electrostatic switch’ (McLaughlin and Aderem, 1995; Seykora et al., 1996; Swierzynski and Blackshear, 1996). Likewise, calmodulin binding to these same basic and hydrophobic domains blocks both phospholipid binding and PKC phosphorylation of the regulatory serine residues.

According to our mapping studies, a minimum of two AKAP79 basic domains are needed for membrane targeting and PtdIns(4,5)P2 binding. Thus, negative regulatory mechanisms affecting at least two of the three basic domains would be predicted to inhibit membrane binding. This model of regulation (Figure 11) is consistent with our observations that both protein phosphorylation and Ca2+−calmodulin are able to negatively regulate PtdIns(4,5)P2 binding for full-length AKAP79 and 1–153 which contain all three basic regions. To this end, the AKAP79 B and C regions both contain multiple sites for phosphorylation by PKC or PKA, and the A and B regions are both likely to bind calmodulin. In fact, a conserved serine residue in the calmodulin/phospholipid-binding regions of neurogranin and neuromodulin which is phosphorylated by PKC is also present in the B region of AKAP79 (Figure 8B). Phosphorylation of this residue in AKAP79 could account for the observed inhibition of phosphoinositide binding by PKC; however, phosphorylation of other residues within the B and C regions by PKC or PKA might also be involved in this process. The participation of multiple phosphorylation sites in regulation of lipid binding is supported by the observation that phosphorylation of AKAP79 by both PKA and PKC has a stronger inhibitory effect on PtdIns(4,5)P2 binding than phosphorylation by either enzyme alone. The involvement of multiple phosphorylation sites is also seen for PKC regulation of MARCKS membrane localization. Mapping and mutagenesis of PKA and PKC phosphorylation sites within the B and C basic regions of AKAP79 should help address this issue of whether multiple sites of phosphorylation are required for regulation of phosphoinositide binding and membrane targeting.

Based on functional analogy with the MARCKS ‘electrostatic switch’ which allows reversible translocation
between the membrane and cytosol in response to PKC phosphorylation or Ca\(^{2+}\)-calmodulin (McLaughlin and Aderem, 1995), it is interesting to speculate that membrane targeting of AKAP79 could also be regulated by phosphorylation and calcium signaling in cells. In support of this possibility, AKAP79 was shown to be phosphorylated in transfected HEK-293 cells following stimulation of PKA and PKC, and activation of PKC or incubation with Ca\(^{2+}\)-calmodulin was shown to release AKAP79 from cell membranes. Somewhat in contrast to our \textit{in vitro} lipid vesicle binding studies, PKA activation failed to release any AKAP79 from HEK-293 cell membranes. However, this result is not surprising considering that there are more potential PKC sites than PKA sites present within the B and C basic regions, AKAP79 is phosphorylated better by PKC than PKA both \textit{in vitro} and even more so in HEK-293 cells, and PKC activation released only a small fraction of the AKAP79 from membranes. Nonetheless, it is clearly possible that PKC plays a greater role than PKA in regulation of AKAP79 membrane targeting in cells.

If an AKAP79 ‘electrostatic switch’ operates within cells, then AKAP79 might function in second messenger signaling not only as a static kinase and phosphatase-anchoring protein but also as a substrate and cellular effector for the anchored kinases and phosphatases. For instance, through regulation of phosphoinositide binding AKAP79 could effect second messenger signaling-mediated remodeling of the membrane cytoskeleton similar to MARCKS. Regulation of the membrane localization of AKAP79 in response to prolonged second messenger activation of PKA and PKC or elevation of intracellular calcium could also function in cells as an adaptive negative feedback mechanism which would limit the strength or duration of kinase signaling by repositioning the entire AKAP79 scaffold relative to the locations of substrates or second messenger generation. Alternatively, phosphorylation or Ca\(^{2+}\)-calmodulin-mediated redistribution of AKAP79 could act as an amplification mechanism which would remove the inhibitory anchoring protein from close proximity to the released active enzymes, thus favoring maintenance of the activated state. It will be of great interest in the future to characterize further the cellular role of phosphoinositides, protein phosphorylation and calmodulin in regulation of AKAP79 signaling.

Materials and methods

\textbf{Construction of cDNA expression plasmids}

The coding sequence for AKAP79 was amplified by PCR using specific synthetic oligonucleotide primers to introduce a 5’ \textit{Hind}III site, followed by a consensus ribosome binding site, an \textit{Nco}I site at the ATG initiation codon, and a stop codon followed by a \textit{Bam}HI site at the 3’ end of the coding region. This 1.3 kb insert was then cloned as a \textit{Hind}III–\textit{Bam}HI fragment into pcDNA3 (Invitrogen) for expression of full-length AKAP79 in transfected HEK-293 cells for the experiments in Figures 1, 2, 4B and 10. For construction of the AKAP79–GFP fusion proteins used in the HEK-293 transfection experiments (Figures 3 and 4), the coding sequence of the S65T bright mutant of GFP (provided by Dr R.Tsen, HHMI, UCSD) was amplified by PCR using specific synthetic oligonucleotide primers which placed a \textit{Bam}HI at the 5’ end of the GFP coding sequence followed by a \textit{Bgl}II site at the 3’ end. The resulting 800 bp \textit{Bam}HI–\textit{Bgl}II fragment was then cloned into \textit{Bam}HI-digested pCDNA3 to generate pCDNA3–GFP(S65T). \textit{Hind}III–\textit{Bam}HI fragments encoding full-length AKAP79 or analogous fragments encoding regions of AKAP79 were amplified by PCR and then fused in-frame to GFP by ligation into \textit{Hind}III–\textit{Bam}HI-digested pCDNA3–GFP(S65T). For the neuron microinjection experiments, the coding sequences for the various AKAP79 fragments were excised from pCDNA3–GFP(S65T) as HindIII–\textit{Bam}HI fragments and ligated into \textit{Hind}III–\textit{Bam}HI-digested pEGFPN1 (Clontech) to generate C-terminal fusions to EGFP. The EGFP sequence contains brightness-enhancing mutations (S65T, F64L) and has been codon usage optimized for expression in mammalian cells resulting in higher expression levels and brighter fluorescence than GFP(S65T). The increased expression and fluorescence of EGFP was necessary for optimal visualization of AKAP79–GFP in the microinjected neurons.

\textbf{HEK-293 cell transfection}

HEK-293 cells at 20–50% confluency were transfected by calcium phosphate precipitation with the various plasmid cDNA expression constructs (5–10 µg) overnight for 16 h under 5% CO\(_2\) at 37°C. Cells were then washed with phosphate-buffered saline (PBS), fed with normal growth medium (DMEM, 10% fetal bovine serum, 1% penicillin/ streptomycin) and grown to confluency (48 h). For Figures 1, 2 and 10, stable AKAP79 transfectants were selected in 0.5 mg/ml G418. Confluent transfected cells were either harvested for subcellular fractionation or immunoprecipitation or split and plated on glass coverslips in six-well dishes for immunocytochemistry.

\textbf{Subcellular fractionation and immunoprecipitation}

For analysis of AKAP79 expression in the transfected HEK-293 cells by immunoblotting or immunoprecipitation, the cells were washed twice in PBS prior to harvesting. To determine the subcellular distribution of the expressed AKAP79 in the soluble and membrane/particle fractions (Figure 1A), cell lysates were prepared by lysis in ice-cold hypotonic lysis buffer containing 0.2% Triton X-100 [HLBT: 20 mM HEPES pH 7.4, 20 mM NaCl, 5 mM EDTA, 0.2% Triton X-100 (w/v), 1 mM DTT, 0.5 mg/ml leupeptin/pepsstatin, 1 mM benzamidine, 1 mM AEBSF] followed by Dounce homogenization. The homogenates were fractionated into soluble (S = supernatant) and particulate (P = pellet) fractions by centrifugation at 40 000 g for 30 min. For the experiments in Figure 10B–D, AKAP79-transfected HEK-293 cells were lysed in the same hypotonic buffer as above except without Triton X-100 (for Figure 10B, the buffer also contained phosphatase inhibitors: 50 mM NaF, 30 mM NaPi, 1 µM microcystin-LR, and 5 mM EGTA) and then the soluble and membrane/particle fractions were obtained by centrifugation at 100 000 g for 30 min. For immunoprecipitation of AKAP79 (Figure 1B and C), control HEK-293 cells or HEK-293 cells transfected with AKAP79 were lysed in Triton Lysis Buffer [TLB: 0.5% Triton X-100 (w/v), 20 mM HEPES pH 7.4, 20 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 µg/ml leupeptin/pepsstatin, 1 mM benzamidine, 1 mM AEBSF] followed by Dounce homogenization and clarification of the lysate by microcentrifugation (14 000 r.p.m., 15 min). 500 µg of cell lysate was then incubated overnight at 4°C with 5 µg of either affinity-purified rabbit polyclonal anti-79 918I or preimmune IgG followed by 1 h incubation with protein-A-Sepharose (100 µl, 10% slurry equilibrated in TLB). The immunocomplexes were pelleted by microcentrifugation (3000 g, 1 min) and the beads were washed in TLB + 1 M NaCl (3×1 ml) and TLB (3×1 ml). The immunocomplexes were then eluted either with 1 mM cAMP or SDS–PAGE sample buffer. The 1 mM cAMP elutions were assayed for PKA catalytic activity using Kemptide as substrate (Corbin and Reimann, 1974). The SDS–PAGE sample buffer elutions were analyzed by SDS–PAGE and \textsuperscript{32}PIRII overlay blotting as described below.

\textbf{Immunoblotting and \textsuperscript{32}PIRII overlay blotting}

Proteins in cell lysates, subcellular fractions (50 µg) or immunoprecipitates were separated by SDS–PAGE and electrotransferred to nitrocellulose membrane filters (Schleicher & Schuell). The filters were then blocked overnight in Blotto + 0.1% BSA (bovine serum albumin) in Tris-buffered saline (TBS: 10 mM Tris pH 7.4, 140 mM NaCl and 5% non-fat dry milk). For immunoblotting, the filters were then probed with the appropriate dilution of the primary antibody (1:1000 anti-AKAP79 mouse monoclonal 183C) in TTBS (TBS + 0.05% Tween-20) for 2–3 h, washed with TTBS, probed with secondary goat anti-mouse antibody–horseradish peroxidase conjugates (Amersham) in TTBS for 1–2 h, washed with TTBS, and finally washed with TBS. The washed immunoblots were then visualized using enhanced chemiluminescence (ECL; Pierce) and Kodak X-OMAT AR film. For \textsuperscript{32}PIRII overlay blotting the blocked filters were probed with 100 000 c.p.m./ml of PKA phosphorylated \textsuperscript{32}PIRII in Blotto + 1% BSA for 4–16 h (Carr and Scott, 1992). The radioactive blots were then washed extensively in TTBS and visualized by autoradiography (Kodak X-OMAT AR film).
Fluorescence microscopy
Transfected 293 cells (48 h post-transfection for transients) were seeded on glass coverslips in growth media 24–48 h prior to fixation for microscopy. The coverslips were washed twice in PBS, fixed in 3.7% formaldehyde/PBS, and permeabilized with acetone for 1 min. For AKAP79–GFP-transfected cells, the fixed coverslips were mounted directly on glass slides for microscopy. For immunochemical staining of AKAP79 or rhodamine–phalloidin staining of F-actin, the fixed and permeabilized cells were washed with PBS and blocked in PBS + BSA (0.1%) for 30 min. The primary antibodies (1:250 rabbit polyclonal anti-AKAP79) were incubated for 1 h followed by washing in PBS + BSA. Next, the coverslips were incubated for 1 h with fluorescent secondary antibodies (goat anti-rabbit antibody–FITC) and rhodamine–phalloidin (Molecular Probes) followed by washing in PBS + BSA. The washed coverslips were mounted on glass slides, and either the specific indirect immunofluorescent staining or intrinsic GFP fluorescence was detected in successive focal planes using a laser-scanning confocal microscope (Leitz).

Microinjection of cortical neurons in culture
Cortical neurons from neonatal mouse brains were cultured (Twyman et al., 1995) for 3–4 weeks on glass coverslips prior to nuclear microinjection of plasmid DNA (0.5 μg/ml in 0.5% PBS) using an automatic microinjection system (Eppendorf) (Alberts et al., 1993). At 24 h post injection the cells were fixed in 3.7% formaldehyde and analyzed for GFP fluorescence by laser-scanning confocal microscopy. For immunocytochemical visualization of endogenous AKAP79 (AKAP150), the cultured neurons were fixed on coverslips with 3.7% formaldehyde, permeabilized with acetone and stained with rabbit polyclonal anti-AKAP150 primary antibody and goat anti-rabbit–FITC secondary antibody (Molecular Probes) as described above.

Bacterial expression and purification of recombinant AKAP79 fragments
For the calcineurin (Figure 7) and lipid binding studies (Figures 8 and 9) recombinant AKAP79 and fragments were expressed as N-terminal His6-tagged fusions using PET vectors (Novagen) in bacteria (BL21DE3) and purified by Ni–agarose chromatography (Pharmacia) followed by extensive dialysis to remove imidazole. AKAP79, 1–75, 1–108 and 108–427 were cloned as Ndel–BanHI fragments into PET16 for bacterial expression while 75–153, 1–153, 1–153, 1–153, 154–291, 154–291 and 154–427 were cloned as Ncol–BanHI fragments into PET30 for bacterial expression. PET30, in addition to the His6-tag, also has an N-terminal S-Tag fusion sequence.

Calcineurin activity assays
Calcineurin was assayed in triplicate as described (Blumenthal et al., 1986) in a 20 μm reaction containing 40 mM Tris–HCl pH 7.5, 0.1 M KC1, 0.1 mM CaCl2, 6 mM magnesium acetate, 0.5 mM DTT, 0.1 mg/ml BSA, calmodulin (1.5 μM) and [32P]RII peptide (20 μM) as substrate by extensive dialysis to remove imidazole. AKAP79, 1–75, 1–108 and 108–427 were cloned as Ndel–BanHI fragments into PET16 for bacterial expression while 75–153, 1–153, 1–153, 1–153, 154–291, 154–291 and 154–427 were cloned as Ncol–BanHI fragments into PET30 for bacterial expression. PET30, in addition to the His6-tag, also has an N-terminal S-Tag fusion sequence.

Phosphatidylinositol-4,5-bisphosphate binding assay
Approximately 1 μg of the various purified recombinant AKAP79 fragments (200 nM–1 μM) in 100 μl total volume of buffer (20 mM Hepes pH 7.4, 100 mM KCl, 1.5 mM MgCl2, 0.1% Nonidet P-40, 0.1% BSA, 1 mM DTT) were incubated alone (no lipid = n) or with washed (20 μM HEPES pH 7.4, 100 mM KC1) sucrose (170 M)-loaded phosphatidylinositolcholine (PC, Avanti) lipid vesicles (100 μM final total lipid concentration) (Mosior and Newton, 1995). Vesicles were made to contain increasing molar percentages of Ptdlns(4,5)P2 (Sigma) (Figure 8C): [0% (100% PC), 1% (99% PC), 2% (97.5% PC), 5% (95% PC) and 10% (90% PC)] or the indicated concentrations of PtdIns(4)P (Sigma), PtdIns (PI, Avanti) or phosphatidylserine (PS, Avanti) (Figure 8D). The lipid vesicles were pelleted by centrifugation at 100 000 g and 80 μl was removed (supernatant fraction) while the remaining 20 μl of supernatant containing the lipid pellet (pellet fraction) was resuspended to 80 μl total volume. The distribution of the AKAP79 protein fragments between the supernatant (unbound) and pellet (lipid-bound) fractions for each concentration of acidic lipid was then determined by 4–15% SDS–PAGE followed by immunoblotting with rabbit-anti-79 918I (1:1000 in Blotto + 0.1% BSA) as described above. For studies of the effects of Ca2+-calmodulin on Ptdlns(4,5)P2 binding (Figure 9C), the indicated concentrations of calcium and calmodulin (purified from bovine brain) were preincubated with AKAP79 or 1–153 for 10 min prior to the addition of the lipid vesicles.

In vitro phosphorylation of AKAP79 and 1–153
Purified recombinant AKAP79 (8 μg) or 1–153 fragment (3 μg) was phosphorylated by PKA or PKC; phosphorylation reactions (50 μl total reaction volume) were performed by incubating, at 30°C for 30 min. AKAP79, 1–153 or 1–153 for 10 min prior to the addition of the PKA or PKC double phosphorylation in Figure 9A and B, the PKC buffer conditions were used. For all phosphorylation reactions the final concentration of ATP was 0.5 μM. For the autoradiogram shown in Figure 9A, 600 c.p.m./pmol [γ-32P]ATP (NEN) was included and 25 μl of the reaction volume was analyzed by SDS–PAGE (4–15% gel) followed by autoradiography (1 h, Kodak XR-OMAT AR film). For phosphorylation of proteins to be used in the phosphohilipid binding assays (Figure 9B), 5 μg protein and unlabeled ATP were used, and 10 μl (1 μg protein) of the reactions were included in the binding assay.

Cellular phosphorylation of AKAP79
For Figure 10A, HEK-293 cells stably transfected with AKAP79 (or untransfected control cells) were grown to near confluency and then starved for 4 h in serum-free, phosphate-free DMEM (37°C, 5% CO2). The cells were then incubated with ~1.5–2.5 mCi/10 cm plate (0.2–0.35 μCi/ml) [32P]orthophosphate (ICN) for 2 h prior to stimulation with agonists for 15–20 min. For PKA phosphorylation cells were stimulated in the presence of the adenylate cyclase activator forskolin (40 μM) plus phosphodiesterase (100 μM IBMX) and phosphatase (100 μM okadaic acid) inhibitors. For PKC phosphorylation cells were treated with the PKC activator PMA (100 nM) plus phosphatase inhibitor (100 μM okadaic acid). After agonist stimulation the media was removed and the plates were washed with ice-cold PBS. The cells were then harvested in ice-cold lysis buffer containing phosphatase and protease inhibitors (1% Triton X-100, 50 mM Tris pH 7.5, 0.5 mM NaCl, 30 mM NaPi, 50 μM Naf, 1 μM microcystin-LR, 5 mM EDTA, 5 mM EGTA, 2 μg/ml leupeptin/pepsatin, 1 mM benzamidine, 1 mM AEBSF). AKAP79 was then immunoprecipitated in duplicate for each agonist treatment with anti-79 918I or preimmune (control) sera from the cell lysates as described for Figure 1B and C except for the change of lysis buffer as detailed above. The final washed immunoprecipitates were eluted in sample buffer and analyzed by SDS–PAGE and autoradiography to visualize [32P]-labeled proteins.

AKAP79 membrane release assays
For the experiments in Figures 10A and B, infected AKAP79-293 cells were serum-starved for 3 h in DMEM and then treated with forskolin and PMA as described for Figure 10A. After these treatments the cells were fractionated (100 000 g) into crude plasma membranes/particulate pellet and soluble supernatant fractions as described above. For the [γ-32P]RII overlay blot shown in Figure 10B, 25 μg of each fraction was used for SDS–PAGE. For the experiments in Figure 10C and D, 50 μg (10 μl of
5 mg/ml) of crude AKAP79-293 plasma membranes (100 000 g pellet) were incubated with PKA (plus 5 μM myristoycin-LR), PKC or both PKA and PKC (plus 5 μM myristoycin LR, 100 nM PMA) with or without ATP (10 μM) for 30 min at 30°C as described above and then centrifuged at 100 000 g for 30 min to re-pellet the membranes. The supernatant (released) and pellet (membranes) fractions were then analyzed by immunoblotting with anti-79 monoclonal antibody 183C. Membranes were also analyzed in this manner following 30 min, 30°C incubations with calcium (1 mM), calmodulin (10 mM) and EGTA (5 mM) for the experiments shown in Figure 10C.

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References


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