A novel lipid-anchored A-kinase Anchoring Protein facilitates cAMP-responsive membrane events

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Introduction

The activation of signal transduction pathways that relay messages from the plasma membrane to specific effector molecules is the principal mechanism through which extracellular signals influence intracellular processes (Sutherland, 1972). Invariably these events involve changes in the phosphorylation state of target proteins through the activation of protein kinases and phosphatases (Krebs, 1985). The activity of both enzyme classes responds to fluctuations in the levels of second messengers such as calcium, phospholipid and cAMP. It is now evident that cross-talk between signaling pathways leads to the phosphorylation of individual substrates by several kinase classes (Houslay, 1991). To avoid indiscriminate phosphorylation events, it has been postulated that local activation of protein kinases or phosphatases is a regulatory mechanism that increases the level of specificity in these signaling pathways (Hubbard and Cohen, 1993; Faux and Scott, 1996b). At the molecular level subcellular targeting of these enzymes is achieved by association with targeting, anchoring or adaptor proteins that tether these enzymes to intracellular structures or organelles (Mochly-Rosen, 1995; Pawson and Scott, 1997).

Localization of the cAMP-dependent protein kinase (PKA) is achieved through the association of the PKA holoenzyme with A-kinase Anchoring Proteins (AKAPs) (Rubin, 1994; Dell’Acqua and Scott, 1997). All AKAPs contain a common structural motif which tethers PKA through interaction with the regulatory subunit (R) dimer of the kinase (Carr et al., 1991; Hausken et al., 1994, 1996b; Newlon et al., 1997). While all AKAPs contain a conserved RI binding domain, a second motif unique to each anchoring protein allows it to sequester the kinase to specific intracellular locations. For example, subcellular fractionation and immunohistochemical analyses have detected AKAPs at specific subcellular sites such as the cytoskeleton, endoplasmic reticulum, filopodia, golgi, microtubules, plasma membrane, postsynaptic density and secretory granules (Theurkauf and Vallee, 1982; De Camilli et al., 1986; Joachim and Schwoch, 1990; Salvatori et al., 1990; Carr et al., 1992b; Rios et al., 1992; McCartney et al., 1995; Dransfield et al., 1997; Nauert et al., 1997). Therefore, the role of AKAP targeting is to provide specificity in cAMP-responsive events by placing the anchored kinase close to specific substrates.

Several studies have demonstrated that one such group of PKA substrates are ion channels. Moreover, PKA anchoring seems to augment the rapid cAMP responses required for ion channel modulation. Microinjection of ‘anchoring inhibitor peptides’, which compete for the RI–AKAP interaction, displaces the kinase from anchoring sites and attenuates ion flow through AMPA-kainate glutamate receptor ion channels, skeletal and cardiac muscle L-type Ca2+ channels and Ca2+-activated potassium channels (Johnson et al., 1994; Rosenmund et al., 1994; Wang and Kotlikoff, 1996; Gao et al., 1997).

In this study we describe the cloning and characterization of a low-molecular weight anchoring protein, AKAP18, which targets PKA to the plasma membrane. Targeting is dependent on residues in the extreme N-terminus of AKAP18 which are lipid-modified through myristoylation and palmitoylation. Functional studies demonstrate that heterologous expression of AKAP18 in HEK-293 cells with the e9059 and c9064 subunits of the cardiac L-type Ca2+ channel enhances cAMP-responsive Ca2+ currents. Further studies show that localization of RI to the cell membrane by AKAP18 is also able to facilitate hormone-mediated insulin secretion in a pancreatic β cell line.
Fig. 1. Sequence of AKAP18. (A) Comparison of the human and mouse AKAP18 amino acid sequences. Common residues are indicated by a vertical line. (B) A human multiple tissue Northern blot probed with a 32P-radiolabelled 187 bp fragment of the AKAP18 coding sequence. mRNA size markers are shown in kilobases. (C) HEK-293 cell lysates expressing the cDNAs for human and mouse AKAP18. Protein (50 μg) was separated by SDS–PAGE (4–15%) and AKAP18 was detected by immunoblot using a polyclonal antibody raised against the recombinant human protein. (D) Immunoprecipitation of AKAP18 from transfected HEK-293 cells. An RII overlay of preimmune control and anti-AKAP18 immunoprecipitations is shown. (E) PKA activity is enriched in AKAP18 immunoprecipitates. PKA activity from preimmune (Pre-I) and AKAP18-specific (IP) immunoprecipitates is shown in the presence and absence of the PKA inhibitor, PKI. Data are representative of four experiments.

Results

Cloning of AKAP18
cDNAs encoding RII binding proteins were isolated from a human fetal brain cDNA expression library using 32P-radiolabeled murine RIIα as a probe (Hausken et al., 1996a). Eight positive clones were identified from a screen of ~400 000 recombinants, one of which represented a novel RII-binding protein cDNA of 2726 bp called HFB-6. Nucleotide sequencing identified a short open reading frame of ~300 bp at the 5’ end of the clone. 5’-RACE was employed to isolate cDNAs with sequence upstream of HFB-6 and a further 150 bp of message was identified. This sequence contained upstream termination codons in all three reading frames, suggesting that the initiation codon was contained within the original HFB-6 sequence. An initiation codon was identified which lay within a viable Kozak consensus sequence, giving an open reading frame of 243 bp to encode a protein of 81 amino acids (Figure 1A: DDBJ/EMBL/GenBank accession No. AFO47715). Further database analysis identified an EST clone (mf17g02.r1/ DDBJ/EMBL/GenBank accession No. AA072273), which may represent a murine homolog of HFB-6. It encodes a protein of 81 amino acids with ~80% sequence identity to the human protein (Figure 1A: Murine GenBank Acc. No. AFO47716). Comparison of the human and murine DNA sequences provided further support for the proposed open reading frame of HFB-6 as the putative coding sequence thereafter was >80% identical (data not shown).

In order to establish the mRNA message size and tissue distribution of the novel RII-binding clone, a multiple tissue Northern blot was probed with a 32P-radiolabeled fragment of HFB-6 containing 187 bp of the coding sequence. Two mRNA species were detected: a prominent message of 2.9 kb in pancreas, brain and heart and a weaker band of 4.3 kb in heart and skeletal muscle (Figure 1B). The 2.9 kb message size in human brain confirms that the sequence of the novel clone is likely to represent a full-length transcript. On the basis of these findings we propose that the HFB-6 clone encodes an 81 amino acid A-kinase-anchoring protein with a predicted molecular weight of ~9 kDa. However, immunochemical detection of the full-length human and mouse cDNAs transiently expressed in HEK-293 cells detected proteins migrating at ~18 kDa on SDS–PAGE (Figure 1C). In accordance with the established nomenclature we have named the protein AKAP18 (Hirsch et al., 1992).

To determine whether AKAP18 can function as a PKA-anchoring protein inside cells, HEK-293 cells were transiently transfected with a mammalian expression vector encoding Myc.His-tagged AKAP18. Immunoprecipitation with an antibody raised against recombinant AKAP18 identified an RII-binding protein of ~22 kDa which was absent from control immunoprecipitates with preimmune serum (Figure 1D). The increase in apparent molecular weight of the AKAP18 protein is in agreement with the addition of fusion protein sequence in the Myc.His
expression construct. Measurement of PKA catalytic activity from the immunoprecipitates shown in Figure 1D demonstrates enrichment of kinase activity using the AKAP18 anti-serum (IP) which is absent in the preimmune (PI) control (Figure 1E). These results show that AKAP18 associates with the PKA holoenzyme inside cells.

**Mapping of the RII-binding site of AKAP18**

It has been well established that the RII-binding site of AKAPs contain a region of conserved secondary structure which has a high probability of forming an amphipathic helix (Carr et al., 1991, 1992a; Coghlan et al., 1994; Dransfield et al., 1997; Nauert et al., 1997). Therefore, studies were undertaken to identify the RII-binding domain of AKAP18. Computer predictions of secondary structure suggested that residues 29–42 exhibited a high probability supported by segregation of hydrophobic and hydrophilic side chains to opposite faces of a helical wheel configuration (Figure 2A). Also, residues 29–42 of AKAP18 showed some limited sequence similarity to the RII-binding regions of previously characterized AKAPs (Figure 2B).

In order to determine empirically whether this region represents the RII-binding site of AKAP18, the entire coding sequence was expressed in a bacterial expression vector (pET30; Novagen). Expression of the protein as a His.Tag fusion permitted efficient purification on a nickel affinity resin (Pharmacia) and a recognition site for protein-S facilitated detection of the recombinant protein. The recombinant AKAP18 fusion protein was detected on SDS–PAGE at an increased molecular weight of 21 kDa (Figure 2D) and it was shown to retain the ability to bind RII as assessed by an in vitro overlay assay (Figure 2E). It is noteworthy that the predicted molecular weight of the AKAP18 His.Tag/S.Tag fusion construct was only 14 kDa. The calculated pl of AKAP18 is 4.7 so it seems likely that the overall acidic nature of the protein causes it to migrate anomalously on SDS–PAGE and would explain the increased apparent size of the native protein in cell extracts.

The same expression system was used to purify His.Tag fusion proteins encompassing residues 1–30, 28–81, 1–45 and 43–81 of AKAP18 (Figure 2C). Only those fragments containing residues 29–42 bound RII in the overlay assay (Figure 2F). More conclusive evidence that residues 29–42 were sufficient for binding was provided by experiments with a peptide encompassing residues 25–48 of AKAP18 (DDAEVLRLSRLKVALKAVQYQ). This peptide effectively blocked all RII binding when used as an antagonist in the overlay assay (Figure 2F). Collectively, these results demonstrate that AKAP18 is a PKA-anchoring protein and that residues 29–42 represent the principal determinants for RII binding.

**Identification of the targeting domain of AKAP18**

We have previously proposed that AKAPs influence the specificity of cAMP-responsive events by directing the anchored kinase to specific intracellular sites (Dell’Acqua and Scott, 1997). Accordingly, each AKAP must contain a unique targeting domain responsible for directing the PKA–AKAP complex to specific regions of the cell. Inspection of the AKAP18 sequence identified three putative signals for lipid modification: a myristoylation site at the N-terminal glycine residue and two palmitoylation sites at Cys4 and Cys5 (Figure 3A). We postulated therefore that protein–lipid interactions may promote association of AKAP18 with the plasma membrane. Evidence that these residues undergo lipid modification was derived from subcellular fractionation of HEK-293 cells transiently transfected with wild-type AKAP18. Cells fractionated in standard hypotonic buffer show that the heterologously expressed AKAP18 protein segregates exclusively with the particulate fraction (Figure 3B). However, when cells were fractionated in the presence of increasing concentrations of Triton X-100, 0.2% detergent was sufficient to re-localize a significant proportion of the AKAP18 from the particulate to the soluble fraction (Figure 3B). Further studies were carried out to determine directly whether myristate and palmitate were incorporated into AKAP18 transiently expressed in culture. HEK-293 cells were transfected with wild-type AKAP18 and a
that residues at the N-terminus of AKAP18 are responsible for membrane targeting. The N-terminal glycine residue was substituted by an alanine (G1A) and Cys4 and Cys5 were changed to serine (C4S,5S) (Figure 4A). Together with the triple mutant described earlier, localization of these AKAP18 mutants was first analyzed by transient expression in HEK-293 cells followed by subcellular fractionation. As noted previously, the wild-type AKAP18 protein partitioned exclusively to the particulate fraction (Figure 4B). Removal of the myristoylation signal alone appeared to have little effect on localization as the G1A mutant remained exclusively in the particulate fraction (Figure 4B). In contrast, removal of both palmitoylation signals caused a shift of ~50% of the C4S,5S mutant to the cytosol, while mutation of all three residues (triple mutant) caused a complete shift of anchoring protein from the particulate to the soluble fraction (Figure 4B). These findings supported the notion that lipid modification is involved in the localization of AKAP18 to the plasma membrane.

In order to analyze the subcellular localization of wild-type and mutant AKAP18 proteins inside cells, plasmids were constructed to heterologously express the proteins with a C-terminal Green Fluorescent Protein tag (GFP). HEK-293 cells transfected with GFP alone exhibited fluorescence throughout the cell, whereas expression of the AKAP18/GFP fusion clearly shows a peripheral staining pattern (Figure 4C). Control experiments demonstrated that the same peripheral localization was observed in cells transfected with an expression construct encoding the wild-type AKAP18 without a GFP tag (Figure 4C). In the cell shown, AKAP18 was detected by immunocytochemical staining with a polyclonal antibody raised against recombinant protein. An indistinguishable staining pattern was observed when the same protein was detected using a monoclonal antibody to the c-Myc epitope tag expressed with the triple mutant described earlier, localization of the full-length anchoring protein. In contrast, gradual delocalization of AKAP18 was apparent as one peripheral localization was observed in cells transfected with an expression construct encoding the wild-type AKAP18 without a GFP tag (Figure 4C). In the cell shown, AKAP18 was detected by immunocytochemical staining with a polyclonal antibody raised against the recombinant protein. An indistinguishable staining pattern was observed when the same protein was detected using a monoclonal antibody to the c-Myc epitope tag expressed at the C-terminus of the wild-type AKAP18 fusion protein (data not shown). These results confirm that AKAP18 is targeted to the cell membrane and that C-terminal fusion of the GFP moiety does not affect the membrane association of the full-length anchoring protein. In contrast, gradual delocalization of AKAP18 was apparent as one (G1A), two (C4S,5S) and then three (triple mutant) lipid modification signals were removed (Figure 4C).

Four additional AKAP18 mutants were constructed to determine if acylation of a particular residue was more significant than the others for membrane targeting (Figure 4A). Mutants were produced where each cysteine residue was mutated individually (C4S and C5S) and where the N-terminal glycine was altered in combination with a palmitoylation signal alone (G1A), two (C4,5S) and then three (triple mutant) lipid modification signals were removed (Figure 4C).

Further mutants were generated to test the hypothesis

mutant in which residues Gly1, Cys4 and Cys5 were substituted with Ala, Ser and Ser respectively (triple mutant). The cells were incubated in media containing [3H]myristic acid or [3H]palmitic acid, subjected to SDS–PAGE and fluorography. Figure 3C shows incorporation of both [3H]myristate and [3H]palmitate in cell culture. HEK-293 cells transfected with wild-type AKAP18 or a mutant with residues Gly1, Cys4 and Cys5 substituted with Ala, Ser and Ser, respectively (triple mutant) and labeled with [3H]myristate or [3H]palmitate. The presence of AKAP18 protein in immunoprecipitates was confirmed by RII overlay and 3H incorporation was detected by fluorography. Data shown are representative of four independent experiments.
The N-terminal sequence of AKAP18 is sufficient for membrane localization

The above experiments demonstrate that Gly1, Cys4 and Cys5 are essential for efficient targeting of AKAP18 to the cell membrane. In addition, four of the first 10 residues in AKAP18 are hydrophobic (Figure 3A), suggesting that after lipid modification this region of the anchoring protein may insert into the plasma membrane. To determine if a short N-terminal sequence is sufficient for targeting, constructs were prepared containing amino acids 1–10 of AKAP18 fused to either a c-Myc epitope tag or to GFP (Figure 5A). Transient transfection in HEK-293 cells shows that residues 1–10 fused to GFP targeted relatively poorly, whereas with a shorter c-Myc epitope tag targeting was more comparable with wild-type AKAP18 (Figure 5B). These data suggest that residues 1–10 are sufficient to localize AKAP18 to the cell membrane but that attachment of GFP immediately proximal to this sequence somehow impairs targeting. Accordingly, a construct containing residues 1–25 effectively localized GFP to the cell membrane when transiently transfected into HEK-293 cells (Figure 5B). Collectively, these results suggest that the first 10 amino acids of AKAP18 form a minimal membrane-targeting domain that includes a myristoylation signal and tandem palmitoylation signals to facilitate the membrane attachment of the anchoring protein.

Effect of AKAP18 on L-type Ca\(^{2+}\) currents

It is reasonable to postulate that membrane targeting of AKAP18 could mediate the localization of PKA in close proximity to transmembrane substrates. Previous studies have suggested that pools of PKA are localized close to skeletal muscle L-type Ca\(^{2+}\) channels in order to facilitate rapid and efficient channel phosphorylation (Salvatori et al., 1990), and more recent reports have shown that AKAP targeting of the kinase contributes to this process (Johnson et al., 1994, 1997; Burton et al., 1997; Gao et al., 1997). In keeping with this hypothesis, it has been proposed that a low-molecular weight AKAP serves to maintain a pool of PKA close to the L-type Ca\(^{2+}\) channel (Gray et al., 1997). Given our biochemical evidence that AKAP18 is targeted through protein–lipid interactions to the plasma membrane, we postulated that this anchoring protein may be a physiological partner of the L-type Ca\(^{2+}\) channel. A recently established model to test this hypothesis is the reconstitution of PKA modulation of L-type Ca\(^{2+}\) channels in HEK-293 or tsa-20 cells (Gao et al., 1997; Johnson et al., 1997).

Accordingly, whole-cell Ca\(^{2+}\) currents were recorded from HEK-293 cells transfected with the cardiac \(\alpha_1c\) and \(\beta_2a\) Ca\(^{2+}\) channel subunits. Okadaic acid (1 μM) was present in the external bath solution and in the pipette solution in order to prevent attenuation of Ca\(^{2+}\) current...
response to cAMP by endogenous phosphatase activity (Gao et al., 1997). Using barium (10 mM) as charge carrier, currents were evoked by depolarization from a holding potential of −80 mV. Whole-cell barium currents activated from −30 mV and peaked at +10 to +20 mV. Bath application of the cell-permeant cAMP analogue 8-CPT-cAMP (1 mM) significantly increased the barium current of cells co-transfected with AKAP18 compared with controls (18.4 ± 6.5%; n = 12, P < 0.05) (Figure 6A and C). Current augmentation >10% was observed in nine of 17 cells co-transfected with AKAP18 (34.4 ± 9.3%; n = 9), while only one of 12 control cells displayed an augmentation. Cells transfected with the triple mutant were not significantly different from controls (−0.3 ± 1.6%; n = 11), with 0 of 11 responding positively to 8-CPT-cAMP (Figure 6B and C). Mean whole-cell currents were different between conditions (control 124 ± 25 pA; n = 12, AKAP18 229 ± 55 pA; n = 17, triple mutant 255 ± 85 pA; n = 11); however, the difference was not significant (P > 0.14). The larger currents could be attributed to two cells in each of the latter two groups that had unusually large currents (>700 pA). Currents of similarly large amplitudes have been observed in separate experiments using these control cells (S.J.Tavalin and N.V.Marrion, unpublished data)
AKAP18 alters GLP-1-mediated insulin secretion in RINm5F cells

A recent report has shown that subcellular targeting of PKA by AKAPs is required for efficient hormone-mediated insulin secretion in pancreatic β cells (Lester et al., 1997). Moreover, this study suggests that a significant site of PKA anchoring could be at, or proximal to, the L-type Ca$^{2+}$ channel, which has previously been implicated as a key mediator of the insulin secretion pathway (Bokvist et al., 1995; Gromada et al., 1997; Safayhi et al., 1997; Suga, 1997). We evaluated the effect of AKAP18 on the process of hormone-mediated insulin secretion. A clonal insulin-secreting rat β-cell line, RINm5F, was transfected with plasmids encoding wild-type AKAP18 and the untargeted triple mutant. As expected, immunocytochemical analysis showed that AKAP18 was concentrated at the periphery of the RINm5F cells whereas the untargeted triple mutant exhibited a more uniform cytoplasmic staining pattern (Figure 7A). Importantly, co-staining with RII showed that wild-type AKAP18 was able to mediate a redistribution of PKA to the plasma membrane (Figure 7A). In untransfected cells lacking exogenous AKAP18 staining, RII exhibits a perinuclear staining pattern (Figure 7A). As no significant AKAP18 staining is observed in these cells, it would appear that RINm5F cells do not contain endogenous AKAP18. The staining pattern for RII was more diffuse in RINm5F cells expressing the untargeted AKAP18 triple mutant (Figure 7A), in keeping with analogous experiments where PKA anchoring was disrupted using a cytoplasmic RII-binding protein (Lester et al., 1997). Wild-type AKAP18, RII and the catalytic (C) subunit of PKA exhibited overlapping subcellular distribution at the plasma membrane although the C subunit was also detected in the cytoplasm and perinuclear regions (Figure 7B). No evidence of membrane localization of the C subunit was observed in cells transfected with the AKAP18 untargeted triple mutant (data not shown). A more uniform distribution for the C subunit is to be expected considering that localized increases in cAMP
release the C subunit from the anchored PKA holoenzyme complex and a significant proportion of the protein could be bound to the type I regulatory subunit or to the heat-stable inhibitor of the kinase, PKI. Control experiments confirmed the expression of both wild-type AKAP18 and the triple mutant in transfected cells by immunoblot and RII overlay (Figure 7C).

To determine if membrane targeting of PKA by AKAP18 influences hormone-mediated signaling events, insulin secretion was measured upon application of the insulinotropic hormone glucagon-like peptide 1 (GLP-1). Insulin secretion from RINm5F cell lines expressing AKAP18 or the untargeted triple mutant was assessed by radioimmunoassay (Drucker et al., 1987; Yaekura et al., 1996). The increase in insulin secretion over basal levels in response to GLP-1 was significantly higher in cells expressing AKAP18 (34.7 ± 8 pmol/min/10^6 cells, n = 9) than in pcDNA-transfected controls (16.3 ± 2.6 pmol/min/10^6 cells, n = 6) (Figure 7D). Furthermore, expression of the untargeted triple mutant of AKAP18 resulted in a markedly lower level of GLP-1-stimulated secretion relative to controls (10.5 ± 0.5 pmol/min/10^6 cells, n = 9) (Figure 7D). These results suggest that membrane targeting of PKA through its interactions with AKAP18 can facilitate GLP-1-mediated insulin secretion. This is consistent with the hypothesis that PKA-mediated membrane events, such as phosphorylation of L-type Ca^{2+} channels, are required for hormone-mediated insulin secretion (Bokvist et al., 1995; Gromada et al., 1997; Safayhi et al., 1997; Suga, 1997).

Discussion

In this study we have cloned and characterized a new A-kinase Anchoring Protein, AKAP18, which we propose functions to localize PKA to the plasma membrane. Bringing the kinase to this site permits the modulation of L-type Ca^{2+} channels and augments physiological processes that require Ca^{2+} influx such as hormone-mediated insulin secretion in pancreatic β cells. These studies provide compelling evidence to support a targeting hypothesis which suggests that second messenger-mediated signaling events are not only controlled by the catalytic activities of kinases and phosphatases but also by where these enzymes are localized within the cell (Hubbard and Cohen, 1993; Faux and Scott, 1996b).

Recently, Murphy and colleagues reported partial purification of a low-molecular weight AKAP that co-purifies with the rabbit skeletal muscle L-type Ca^{2+} channel (Gray et al., 1997). They named this protein AKAP15 and proposed that it bound directly to the channel to promote PKA-dependent modulation of Ca^{2+} currents. Considering the ability of AKAP18 to facilitate the cAMP-responsive potentiation of L-type Ca^{2+} currents in this study, it is likely that our brain-derived protein is a human homolog of AKAP15, especially given that anchoring proteins can show size variation between species, as has been shown for the AKAP 75/79/150 family (Bregman et al., 1989; Carr et al., 1992b; Hirsch et al., 1992). However, in contrast to the previous suggestion that AKAP15 may be associated with the Ca^{2+} channel, we show that AKAP18 is directly anchored to the plasma membrane. This is supported by two lines of evidence. First, in HEK-293 cells which do not contain endogenous L-type Ca^{2+} channels, AKAP18 is still able to localize efficiently to the cell periphery. Second, if direct binding to the Ca^{2+} channel existed as a subsidiary mechanism to membrane association, one would predict that the delocalized triple mutant of AKAP18 would exhibit some membrane attachment in cells expressing the L-type Ca^{2+} channel. However, when the AKAP18 triple mutant was co-expressed in HEK-293 cells with the γ3s and β3s subunits of the L-type Ca^{2+} channel, the mutant was still unable to localize to the periphery of the cell (I.D.C. Fraser, L.K. Langeberg and J.D. Scott, unpublished observation).

Our studies show that myristoylation of the N-terminal glycine residue and palmitoylation of Cys4 and Cys5 in AKAP18 are involved in attaching the anchoring protein to the cytoplasmic face of the plasma membrane. Post-translational modification of proteins by acyl groups is now well established as a mechanism for membrane association of signaling proteins (Schlesinger, 1993). One common theme seems to be the presence of multiple sites of lipid modification on the acceptor protein. For example, the Src family of tyrosine kinases contain an N-terminal myristoyl group and one or two palmitoyl groups attached to cysteine residues which contribute to membrane targeting (Resh, 1994; Kabouridis et al., 1997). Likewise, several of the α subunits of heterotrimeric G-proteins are subject to dual acylation by myristoyl and palmitoyl (Milligan et al., 1995). Our studies with point mutations of AKAP18 suggest that any two of the three lipid side chains are sufficient to mediate membrane association. The inability of a single lipid moiety to sustain membrane targeting of AKAP18 is in agreement with reports that one acyl group is insufficient to mediate stable attachment of a protein to a lipid bilayer (Resh, 1994). In fact, membrane-associated proteins which are singly acylated are only able to mediate their attachment when another interaction accompanies the lipid modification. For example, the Myristoylated Alamine-Rich C-Kinase Substrate protein (MARCKS), is membrane targeted through an N-terminal myristoyl group and a polybasic region which binds acidic phospholipids (Aderem, 1992; Blackshear, 1993), while a recently identified Grb2/Sos binding protein, FRS-2, is myristoylated and has been proposed to bind to the FGF receptor through a PTB domain (Kouhara et al., 1997).

In addition to the three residues which undergo lipid modification there are also several hydrophobic amino acids in the first 10 residues of AKAP18. Therefore, we propose that the increased hydrophobicity provided by the lipid moieties may permit the insertion of the N-terminus of the protein into the lipid bilayer. The first 10 residues seem to be sufficient for membrane targeting when fused to a c-Myc epitope tag but not when attached to the larger GFP moiety. The inefficient targeting of the latter construct may be due to steric hindrance by GFP, especially if the N-terminus of the AKAP does indeed insert into the
plasma membrane. This is supported by our studies showing that the presence of additional AKAP18 sequence (residues 11–25) permitted efficient membrane localization of the GFP fusion construct.

In spite of considerable progress in establishing a role for A-kinase-anchoring proteins in cAMP-mediated events, many AKAPs remain poorly characterized with respect to their functional importance or physiological targets. One of the better characterized functions is the control of the rapid cAMP-responsive events required for modulation of ion channels. Most of the early cell-based experiments in this field were performed with anchoring inhibitor peptides which compete for the RII–AKAP interaction and displace the kinase from anchoring sites in vivo (Carr et al., 1992a). Electrophysiological studies have shown that peptide displacement of anchored PKA pools accelerates the rundown of AMPA/kainate channels (Rosenmund et al., 1994). Application of these peptides also attenuates the cAMP-responsive augmentation of skeletal and cardiac L-type Ca²⁺ channels (Johnson et al., 1994, 1997; Gao et al., 1997), and Ca²⁺-activated K channels (Wang and Kotlikoff, 1996). More recently it has been shown that membrane targeting of PKA through association with another anchoring protein, AKAP79, also promotes cAMP-responsive modulation of cardiac L-type Ca²⁺ channels (Gao et al., 1997). While AKAP79 and AKAP18 are both capable of targeting PKA to the Ca²⁺ channel in a heterologous expression system, there are several reasons why AKAP18 is more likely to be the physiological partner to the Ca²⁺ channel. The tissue distribution of AKAP18 is more consistent with the expression pattern of the L-type Ca²⁺ channel as Northern blot analysis detected AKAP18 mRNA in pancreas, brain, heart and skeletal muscle. In contrast, AKAP79 is predominantly a neuronal protein and is concentrated in the cerebellum and enriched in hippocampal and cortical neurons (Glantz et al., 1992; Klauck et al., 1996). Furthermore, AKAP79 serves as a scaffold for the assembly of three signaling enzymes, PKA, PKC and calcineurin, suggesting a more complex role for this anchoring protein in the control of membrane phosphorylation events (Coghlan et al., 1995; Faux and Scott, 1996a; Klauck et al., 1996). Although AKAP79 and AKAP18 target PKA to the cell periphery, they do so through different molecular mechanisms. AKAP79 has been shown to mediate membrane binding by direct association with acidic phospholipids (Dell’Acqua et al., 1998), whereas covalent addition of fatty acid side chains to AKAP18 is responsible for its association with the plasma membrane. It is therefore tempting to speculate that both AKAPs may maintain distinct pools of anchored PKA in particular microdomains of the membrane.

In pancreatic β cells, increased insulin secretion in response to hormonal stimulation involves a number of metabolic factors including elevation of cAMP (Ammala et al., 1992; Sjöholm et al., 1995). We have previously shown that the cAMP-dependent pathways in this process require anchored pools of PKA as introduction of anchoring inhibitor peptides or soluble AKAP fragments decreases insulin release (Lester et al., 1997). In this study, expression of the untargeted AKAP18 mutant further supports this conclusion as the intracellular location of RII is disrupted and is accompanied by a decrease in the stimulation of insulin secretion by GLP-1.

Interestingly, expression of wild-type AKAP18 is able to enhance GLP-1-mediated insulin secretion to a level significantly higher than that in control cells. Since previous reports have proposed that hormone-mediated insulin secretion involves the activation of L-type Ca²⁺ channels (Gromada et al., 1997, 1998; Suga, 1997), our studies support a model where targeting of PKA to this channel contributes to the signaling events that promote insulin secretion (Bokvist et al., 1995; Safayhi et al., 1997). However, it must be stressed that a co-localization of PKA with the channel is likely to represent only one of several anchored PKA pools that participate in this process as multiple AKAPs have been identified in β cells (L.B.Lester, unpublished observation). PKA substrates involved in the insulin secretion pathway such as the Glut-2 glucose transporter and the GLP-1 receptor and intracellular Ca²⁺ release sites such as the IP₃ receptor and the ryanodine receptor may have their own pools of anchored PKA (Gromada et al., 1995; Widmann et al., 1996). Furthermore, cAMP-mediated insulin secretion by hormones such as GLP-1 has been shown to affect both calcium influx and late-stage calcium-independent secretion (Amamla et al., 1993; Gromada et al., 1998). Accordingly, overexpression of AKAP18 would tend to sequester RII away from other sites of action and may limit the potential for increased insulin secretion when the kinase is redistributed to the membrane. Nevertheless, the inhibitory effect on insulin secretion caused by widespread disruption of PKA anchoring is reversed by directing the kinase to the plasma membrane. This supports our hypothesis that targeting of PKA to the membrane with substrates such as the L-type Ca²⁺ channel is fundamental to the specificity of hormone-mediated responses that utilize the second messenger cAMP.

Materials and methods

Cloning of AKAP18 cDNA
A 2726 bp cDNA clone encoding an RII-binding protein was isolated from a λZAPII human fetal brain cDNA expression library using an interaction cloning strategy using [²²P]RIP as a probe (Hausken et al., 1996a). To identify sequence upstream of the HFB-6 clone, 5’–RACE was carried out using a ‘Marathon-Ready cDNA’ kit (Clontech). Double-stranded DNA sequencing was carried out using an Applied Biosystems sequencer. A Northern blot containing immobilized samples of mRNA from several human tissues (Clontech) was probed with a fragment containing the 3’ 187 bp of the coding sequence of AKAP18.

Bacterial expression of AKAP18 proteins
An Ncol–EcoRI 942 bp fragment comprising the entire coding sequence of AKAP18 and 698 bp of downstream sequence was subcloned directly onto the pET30b bacterial expression vector (Novagen). Using a SpeI restriction enzyme site 85 bp downstream from the Ncol site, the 1–45, 43–81 and 28–81 fragments were amplified by PCR using synthetic oligonucleotide primers to introduce restriction enzyme sites at the appropriate positions in the AKAP18 sequence. 1–45 was subcloned Ncol–EcoRI after introduction of a termination codon and an EcoRI site, while 43–81 and 28–81 were also subcloned Ncol–EcoRI using Ncol sites incorporated by PCR and the same downstream EcoRI site used for the full-length expression construct. All constructs prepared from PCR-amplified fragments were sequenced prior to expression. Recombinant AKAP18 proteins were expressed from the pET30 vector in the BL21/DE3 strain of Escherichia coli and purified as N-terminal His.Tag fusions using Ni–agarose chromatography (Pharmacia).

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Construction of AKAP18 mammalian expression plasmids
A synthetic oligonucleotide primer was designed to remove the AKAP18 termination codon while introducing a BamHI site at the 3’ end of the coding sequence. Following PCR amplification, wild-type AKAP18 was subcloned EcoRI-BamHI (using an EcoRI site ~50 bp upstream of the initiation codon) into the pEGFP-N3 (Clontech) and pcDNA3.1/Myc.His (Invitrogen) mammalian expression vectors. Similar plasmids were constructed where primers were designed to leave the AKAP18 termination codon intact in both the human and mouse clones while incorporating a BamHI site immediately downstream of the termination codon. Upon EcoRI–BamHI subcloning into the pcDNA vector, this allowed expression of the coding sequence of AKAP18 without additional fusion protein sequence. Further primers were designed to introduce a KpnI site upstream of the AKAP18 initiation ATG codon while incorporating point mutations into the first five residues of the coding sequence. All such primers were designed to include at least 9 bp of native AKAP18 sequence upstream of the initiation codon in order to provide a ribosome binding site for mammalian expression. AKAP18 mutants were PCR-amplified using these specific upstream primers in conjunction with the same primer used to remove the termination codon in the wild-type AKAP18 expression construct. All the AKAP18 mutants were then subcloned KpnI–BamHI into the pEGFP-N3 vector. The 1–10 AKAP18 constructs were prepared using two oligonucleotides with complementary sequences which, when annealed, represented the upstream ribosome binding sequence and the coding sequence for residues 1–10. This was flanked by KpnI and BamHI restriction sites for subcloning into both the pEGFP-N3 and pcDNA3.1/Myc.His vectors. The 1–25 AKAP18 construct was prepared using an oligonucleotide primer designed to introduce a BamHI site immediately after the codon for amino acid 25. This fragment was PCR-amplified and subcloned EcoRI–BamHI into the pEGFP-N3 vector using the same strategy as for wild-type AKAP18. All mammalian expression constructs prepared from PCR-amplified fragments were sequenced prior to transfection into cells.

Solid phase overlays and immunoblots
RII overlays were carried out using murine [32P]RII and immunoblots were performed with a variety of antibodies as previously described (Carr and Scott, 1992). Antibodies to AKAP18 were raised in rabbits against the baculovirus expressed recombinant protein (Bethyl Laboratories, Montgomery, TX) and were affinity-purified using antigen coupled to Affigel-15 (Bio-Rad). Polyclonal antibodies to the GFP protein (Clontech), monoclonal antibodies to the ε-Myc epitope tag (Santa Cruz Biotechnology) and HRP-conjugated S-protein (Novagen) were all obtained commercially.

Transfection of HEK-293 cells
HEK-293 cells at ~30% confluency were transfected with 2–5 µg of the AKAP18 mammalian expression CDNA by Ca2+ phosphate precipitate.

Subcellular fractionation and immunoprecipitation
Transfected HEK-293 cells at ~100% confluency were resuspended in 500 µl of ice-cold hypotonic buffer (20 mM HEPES pH 7.4, 20 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM MgCl2, 10 mM HEPES (Na), 10 mM glucose and 1 µM okadaic acid (pH 7.4). Patch pipettes (2–4 MΩ) were pulled from borosilicate glass (KG-33; Friedrich & Dimmock) and filled with a solution containing 125 mM NaCl, 5 mM KCl, 20 mM TEA-Cl, 10 mM BaCl2, 1 mM MgCl2, 10 mM HEPES (Na), 10 mM glucose and 1 µM okadaic acid (pH 7.4). Patch pipettes were then pulled from borosilicate glass (KG-33; Friedrich & Dimmock) and filled with a solution containing 120 mM Cs methanesulfonate, 20 mM TEA-Cl, 1 mM MgCl2, 5 mM ATP(βγS), 10 mM HEPES, 10 mM BAPTA and 1 µM okadaic acid (pH 7.4). Data were acquired at 10 kHz on a Macintosh Quadra 800 (Apple Computer, Cupertino, CA) using an Instrutech ITC-16 computer interface (Instrutech, Great Neck, NY) and Pulse software (Heka, distributed by Instrutech). Currents were filtered at 2 kHz using an 8-pole Bessel filter (Frequency Devices) prior to digitization. Voltage pulses (50 ms duration) were applied at 0.2 Hz and generated from a holding potential of ~80 mV. Currents were baseline-subtracted and measured between 5 ms after the start of the pulse and the end of the pulse. Current–voltage (I–V) relationships were generated by measuring the difference between the pulse current and the leak current at the potentials indicated. The leak current was determined by linear regression analysis of the data points between ~80 and ~40 mV and extrapolating the fit throughout the entire voltage range. For display purposes and determination of the effect of 8-CPT-cAMP, currents were leak- and capacity-subtracted using a P4 pulse protocol. P4 pulses were from ~80 mV. In a few cells, a contaminating outward current was observed above ~30 mV. This current was monitored by shifts in the zero current potential on the ascending limb of the I–V. Only cells in which this current did not change during the experiment were included in the analysis. Current rundown was observed in nine of 40 cells. To compensate for rundown of current, control current was estimated by fitting a linear function through the time course prior to 8-CPT-cAMP application and extended through the rest of the experiment. Cells in which rundown exceeded 25% of the current within 1–2 min prior to application of 8-CPT-cAMP were not included for analysis. Cells with <50 pA of current were not included in the analysis. Cells were perfused in the external solution for at least 10 min prior to recording. Responses to 8-CPT-cAMP were measured 2–3 min after the application of currents in which a 10% enhancement of amplitude occurred were considered as positive responses. Data are expressed as mean ± SE. Student’s t-test was used to determine statistical significance.

Transfection of RINm5F cells and insulin secretion assay
RINm5F cells at passage 5–7 were transfected with the mammalian expression vectors pcDNA3, pcDNA3.1(Myc.His)/AKAP18 and pcDNA3.1(Myc.His)/AKAP18 triple mutant by the lipofection method as previously described (Lester et al., 1997). Cell lines were plated at 1×105 cells per 35 mm well. After 24–48 h in culture, the cells were rinsed in low-glucose KRBB and incubated for 30 min with high-glucose KRBB. Insulin secretion was measured by incubating the transfected β cells with KRBB containing 16.7 mM glucose, 10 nM GIP-L1 (7–37) and 10 µM IBMX for 30 min at 37°C. Media were collected and
centrifuged at 16 000 g for 10 min. Insulin content was determined by radioimmunoassay using rat insulin as a standard (Linco, St. Charles, MO).

Acknowledgements

We thank Marlene Hosey for providing cDNA constructs for the c α subunit and β2 subunits of the cardiac L-type Ca2+ channel. We are grateful to Max Hallin and Gregory Scott for technical assistance and to Jodi Engstrom for assistance with confocal microscopy. We also thank Mark Dell’Acqua for helpful discussions and colleagues in the Vollum Institute for critical reading of the manuscript. This work was supported by a Wellcome Trust fellowship to J.D.C. (No. 040907/Z/96/N), National Institutes of Health grants N01-DH-20020 (to S.J.T.) and training grant NS07381 (to S.J.T.), DK02353 (to L.B.L.), NS29806 (to N.V.M.), GM48331 (to J.D.S.) and a grant from the Cystic Fibrosis Foundation to J.D.S. (SCOTT95GO).

References


Received December 23, 1997; revised February 17, 1998; accepted February 24, 1998