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

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Compartmentalization of protein kinases with substrates is a mechanism that may promote specificity of intracellular phosphorylation events. We have cloned a low-molecular weight A-kinase Anchoring Protein, called AKAP18, which targets the cAMP-dependent protein kinase (PKA) to the plasma membrane, and permits functional coupling to the L-type calcium channel. Membrane anchoring is mediated by the first 10 amino acids of AKAP18, and involves residues Gly1, Cys4 and Cys5 which are lipid-modified through myristoylation and dual palmitoylation, respectively. Transient transfection of AKAP18 into HEK-293 cells expressing the cardiac L-type Ca^{2+} channel promoted a 34 ± 9% increase in cAMP-responsive Ca^{2+} currents. In contrast, a targeting-deficient mutant of AKAP18 had no effect on Ca^{2+} currents in response to the application of a cAMP analog. Further studies demonstrate that AKAP18 facilitates GLP-1-mediated insulin secretion in a pancreatic β cell line (RINm5F), suggesting that membrane anchoring of the kinase participates in physiologically relevant cAMP-responsive events that may involve ion channel activation.

Keywords: AKAP18/insulin secretion/kinase anchoring/lipid modification/L-type Ca^{2+} channel

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 RII 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 RII–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 Ca^{2+} channels and Ca^{2+}-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 α_{1c} and β_{2a} subunits of the cardiac L-type Ca^{2+} channel enhances cAMP-responsive Ca^{2+} currents. Further studies show that localization of RII to the cell membrane by AKAP18 is also able to facilitate hormone-mediated insulin secretion in a pancreatic β cell line.

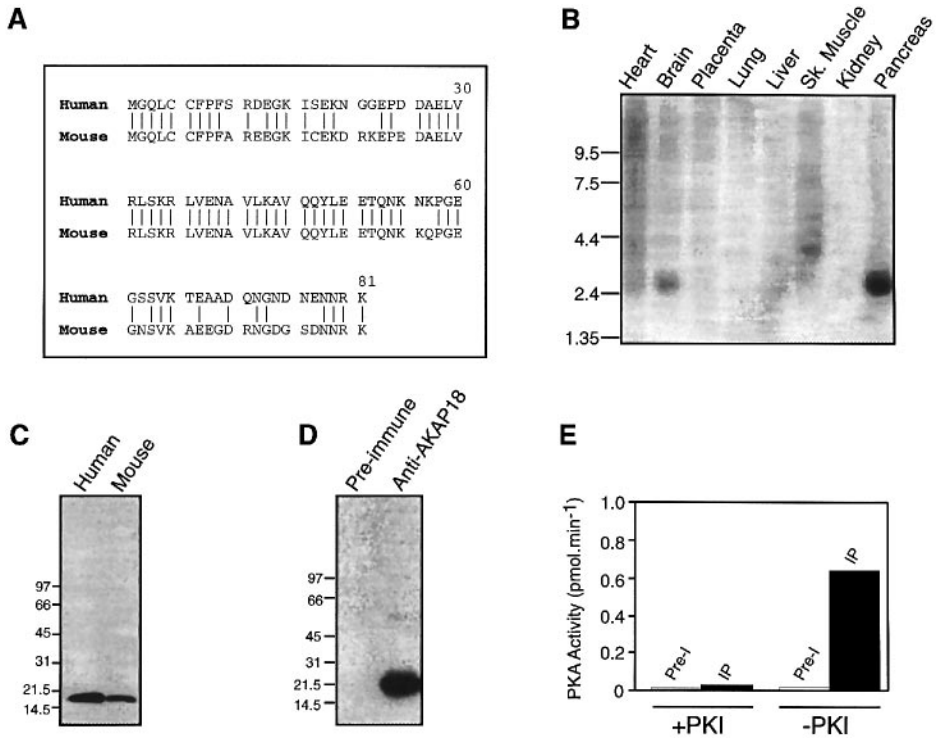

Fig. 1. Sequence of AKAP18. (**A**) Comparison of the human and mouse AKAP18 amino acid sequences. Common residues are indicated by a vertical line. (**B**) A human multiple tissue Northern blot probed with a ^{32}P-radiolabelled 187 bp fragment of the AKAP18 coding sequence. mRNA size markers are shown 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 ^{32}P-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 nucleotides upstream of the initiation codon showed no significant homology between mouse and human, while 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 ^{32}P-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

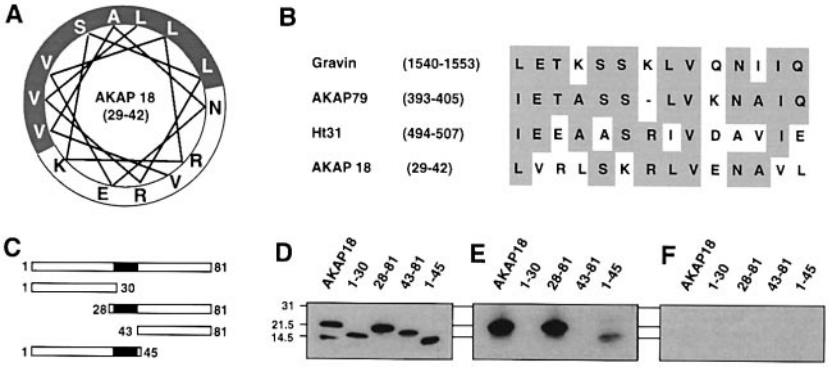

Fig. 2. Residues 29–42 constitute the RII-binding domain of AKAP18. (**A**) Arrangement of residues 29–42 of AKAP18 in a helical wheel configuration showing segregation of hydrophobic and hydrophilic amino acids. The α-helix contains 3.6 residues/turn and the hydrophobic face of the helix is shaded. (**B**) Alignment of the RII-binding domains of the A-kinase-anchoring proteins Gravin, AKAP79 and Ht31 with residues 29–42 of AKAP18. Homologous residues are shaded. (**C**) Recombinant fragments designed to map the RII-binding domain of AKAP18. The putative RII-binding site of AKAP18 is shown as a black box. (**D, E, F**) Purified recombinant protein (1 μg) for AKAP18 and the fragments 1–30, 28–81, 43–81 and 1–45 were separated by SDS–PAGE (4–15%) and electrotransferred to nitrocellulose. Proteins were detected by overlay with HRP-conjugated S-protein (D). The RII-binding capacity of the recombinant AKAP18 fragments was assessed by RII overlay using [^{32}P]RIIα in the absence (E) and presence (F) of 7.5 μM AKAP18 (25–48) peptide (DDAELVRLSKRLVENAVLKAVQQY). Data shown are representative of at least three separate experiments.

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 of amphipathic helix formation. This notion was further 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 pI 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 2E). More conclusive evidence that residues 29–42 were sufficient for binding was provided by experiments with a peptide encompassing residues 25–48 of AKAP18 (DDAELVRLSKRLVENAVLKAVQQY). 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

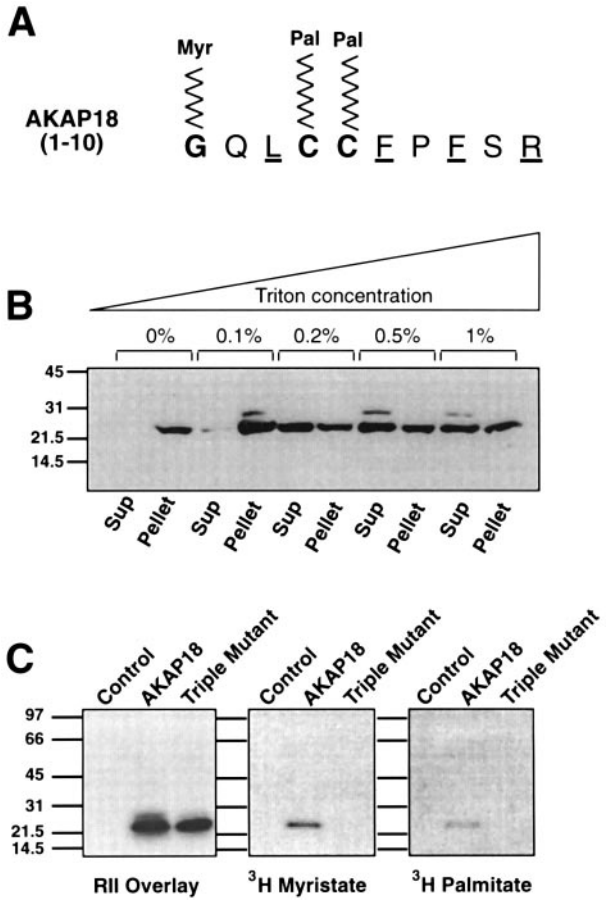

Fig. 3. AKAP18 is subject to N-terminal lipid modification. (**A**) The first 10 residues of AKAP18 are shown with the schematic addition of a myristate group on Gly1 and palmitate groups on Cys4 and Cys5. Hydrophobic residues are underlined. (**B**) Subcellular fractionation of AKAP18-transfected HEK-293 cells in the presence of increasing concentrations of Triton X-100 (0–1%). Soluble (sup) and particulate (pellet) fractions at each Triton concentration were separated by SDS–PAGE (total protein content per lane = 50 μg). AKAP18 protein was detected by immunoblot using a polyclonal antibody raised against recombinant AKAP18. The data shown are representative of three independent experiments. (**C**) AKAP18 incorporates [^{3}H]myristate and [^{3}H]palmitate in cell culture. HEK-293 cells were 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 [^{3}H]myristate or [^{3}H]palmitate. The presence of AKAP18 protein in immunoprecipitates was confirmed by RII overlay and ^{3}H incorporation was detected by fluorography. Data shown are representative of four independent experiments.

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 [^{3}H]myristic acid or [^{3}H]palmitic acid, subjected to immunoprecipitation with anti-AKAP18 antibodies and the incorporation of ^{3}H in cell extracts was analyzed by SDS–PAGE and fluorography. Figure 3C shows incorporation of both [^{3}H]myristate and [^{3}H]palmitate by a protein migrating at the correct molecular weight for the wild-type AKAP18 expression construct, while there was no significant incorporation of label by the AKAP18 triple mutant. An RII overlay of the same protein samples confirmed the presence of an RII-binding protein in both extracts, demonstrating effective expression of both AKAP18 constructs.

Further mutants were generated to test the hypothesis 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 (C4,5S) (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 C4,5S 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 immunochemical 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 (C4,5S) 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 single cysteine residue (G1A/C4S and G1A/C5S). GFP fusions of these mutants were transiently expressed in HEK-293 cells and are shown in Figure 4D. Removal of either single cysteine did not significantly affect the peripheral localization of AKAP18, whereas the mutation of a single cysteine in combination with substitution of the glycine residue was sufficient to delocalize the G1A/C4S and G1A/C5S mutants (Figure 4D). Taken together with the results shown in Figure 4B and C, this mutational analysis demonstrates that alteration of any two of the three amino acid residues which are targets for lipid modification results in a loss of AKAP18 targeting.

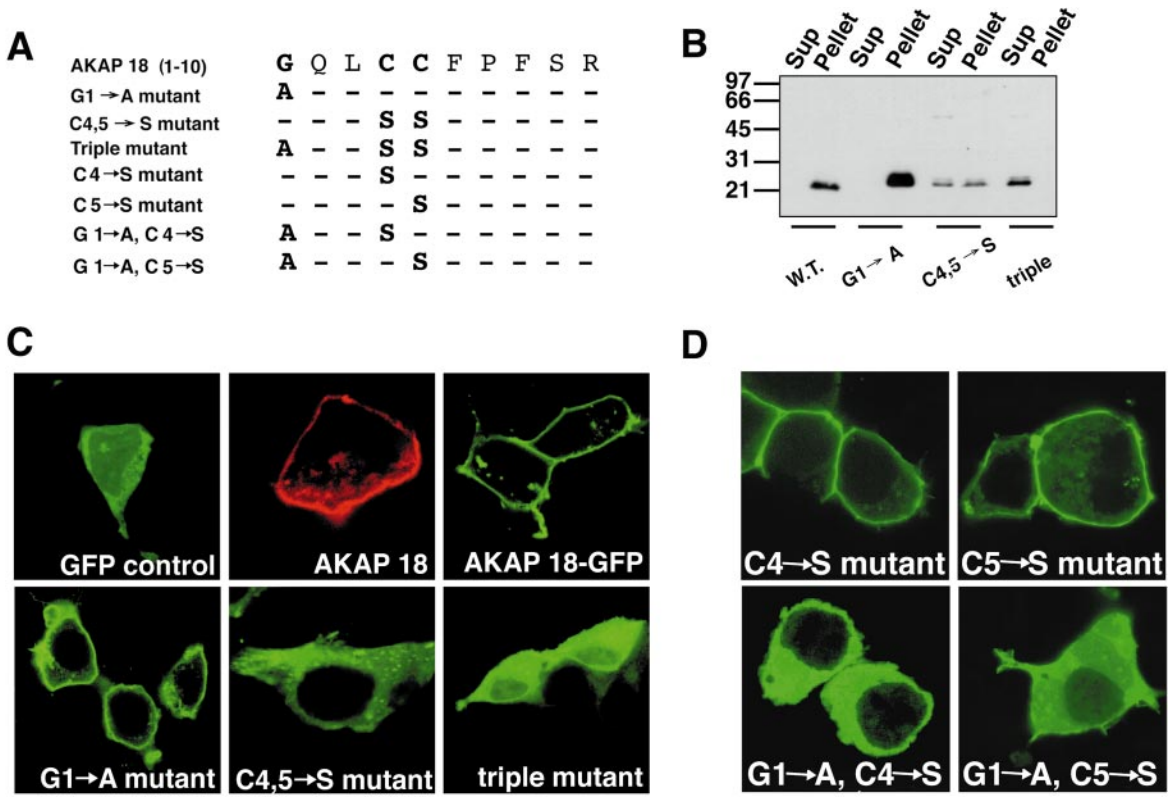

Fig. 4. Mutations in the first five N-terminal amino acids of AKAP18 disrupt targeting function. (**A**) Residues 1–10 of AKAP18 showing amino acid changes incorporated for the N-terminal mutants. (**B**) Subcellular fractionation of HEK-293 cells transfected with AKAP18 and the G1A, C4,5S and triple mutants in the pcDNA3.1/Myc.His expression vector. Soluble (sup) and particulate (pellet) fractions from each construct were separated by SDS–PAGE (total protein content per lane = 50 μg). AKAP18 proteins were detected by immunoblot using a monoclonal antibody to the c-Myc epitope tag. (**C, D**) Confocal microscopy of HEK-293 cells transfected with a GFP control plasmid, wild-type AKAP18 and GFP fusions of AKAP18 and the G1A, C4,5S, triple, C4S, C5S, G1A/C4S and G1A/C5S mutants. The subcellular localization of wild-type AKAP18 was detected by immunochemical staining whereas the GFP fusions were visualized directly by fluorescent excitation at 490 nm. The data shown in (B), (C) and (D) represent at least three separate transfections for each construct.

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 α_{1c} and β_{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

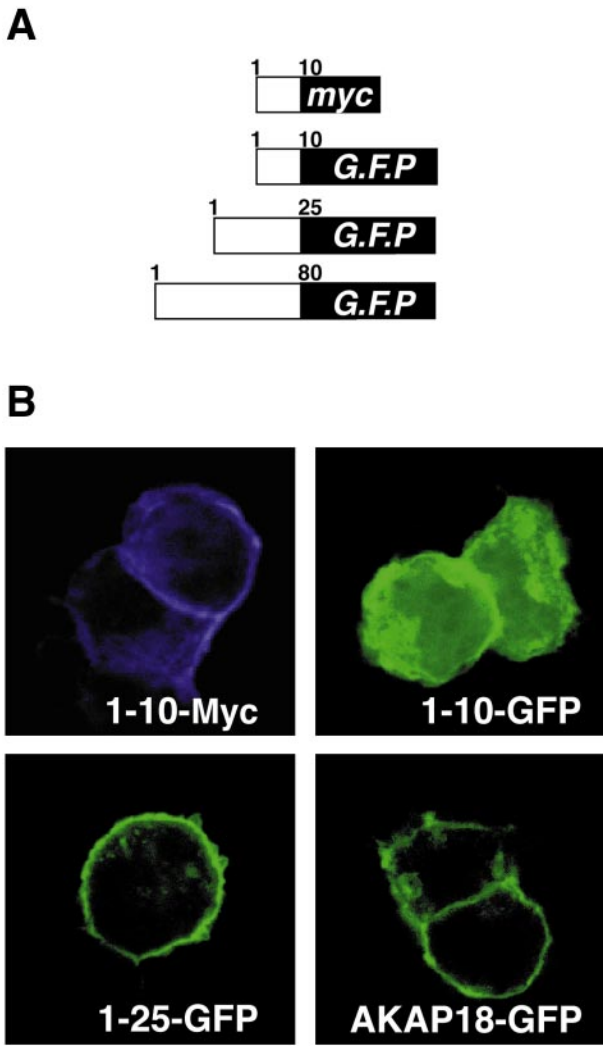

Fig. 5. Residues 1–10 of AKAP18 are sufficient for membrane targeting. (**A**) Schematic representation of constructs prepared to determine the minimal AKAP18 targeting sequence. (**B**) Confocal microscopy of HEK-293 cells transfected with constructs detailed in (A). The 1–10/Myc protein was detected by immunochemical staining using a monoclonal antibody to the c-Myc epitope tag and an FITC-conjugated secondary antibody. This construct is pseudo-colored blue to distinguish it from the 1–10, 1–25 and full-length AKAP18/GFP fusions. Cells imaged are representative of at least two independent transfection studies with each construct.

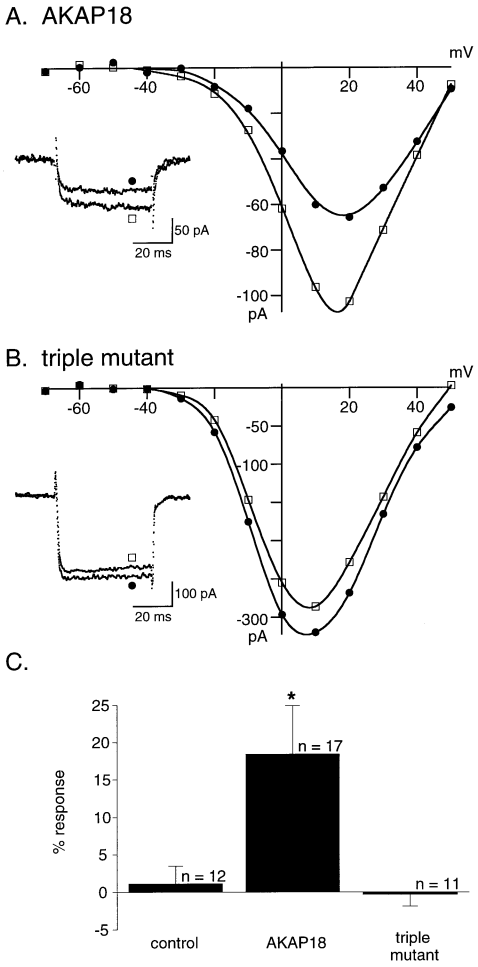

Fig. 6. Membrane targeting of AKAP18 is required for cAMP-dependent modulation of L-type Ca^{2+} channel currents. (**A**) Whole-cell current with 10 mM barium as charge carrier was evoked by depolarization from a holding potential of –80 mV. Current–voltage relationship is shown for HEK-293 cells transfected with the α_{1c} and β_{2a} cardiac Ca^{2+} channel subunits and wild-type AKAP18. Currents recorded following 2–3 min bath application of the cAMP analog 8-CPTcAMP (1 mM) (□) were augmented compared with before treatment (●). Currents were monitored during drug application by test pulses to 0 mV. Inset shows traces evoked by a voltage step to +10 mV. (**B**) Modulation of the L-type Ca^{2+} channel was not seen following co-expression of α_{1c} and β_{2a} Ca^{2+} channel subunits with the untargeted AKAP18 triple mutant. Current–voltage relation is shown in the absence (●) and presence (□) of 8-CPTcAMP (1 mM). Inset shows currents evoked by a voltage step to +10 mV from a holding potential of –80 mV. (**C**) Percentage response to 8-CPTcAMP for each condition tested. Percentage response was calculated as: [(8-CPTcAMP – control)/control]×100%. Response was measured from currents evoked by a voltage step to 0 mV from a holding potential of –80 mV. Barium current through L-type Ca^{2+} channels was significantly (*P <0.05) augmented by 8-CPTcAMP in AKAP18-transfected cells, while cells transfected with the untargeted triple mutant were not significantly different from controls.

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-CPTcAMP (1 mM) significantly increased the barium current of cells co-transfected with AKAP18 compared with controls (18.4 ± 6.5%; n = 17 versus 1.1 ± 2.4%; 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-CPTcAMP (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

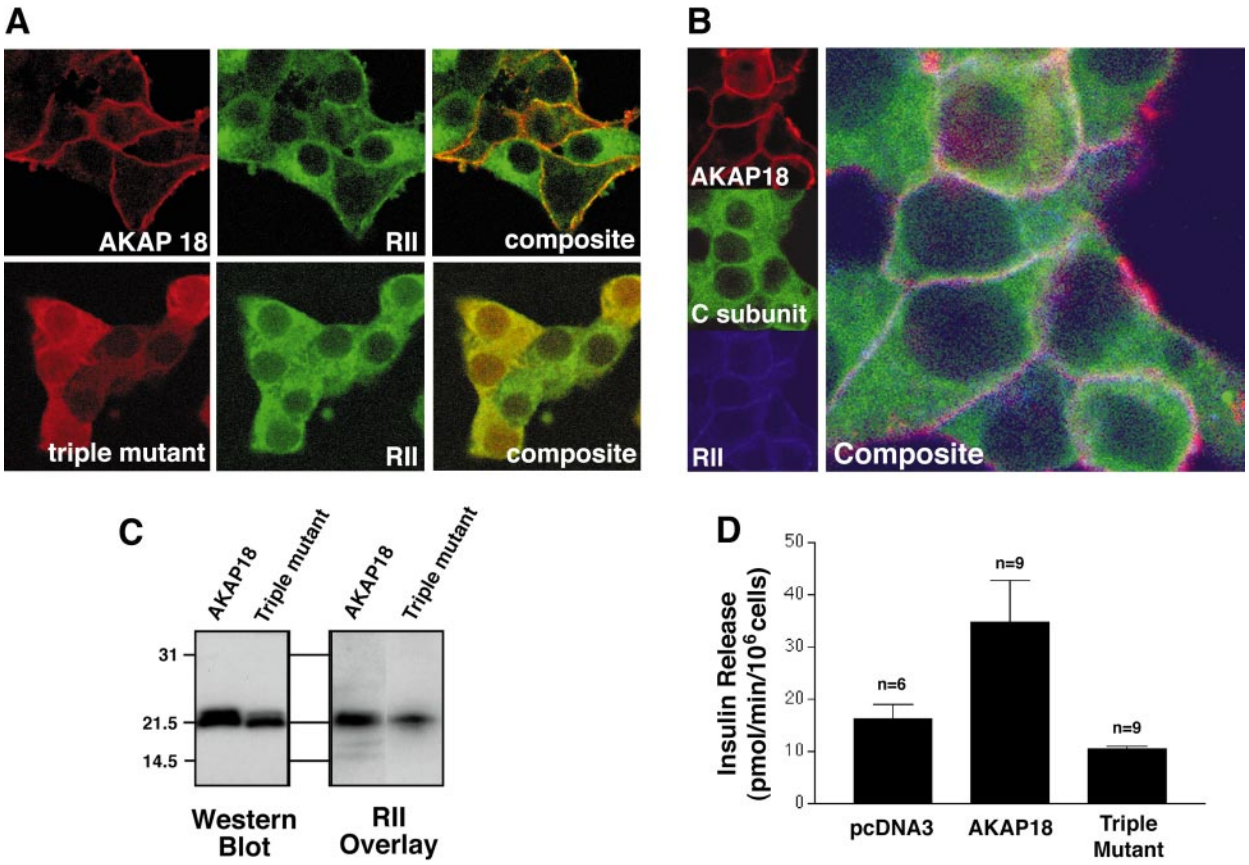

Fig. 7. AKAP18 mediates a redistribution of RII and influences GLP-1-mediated insulin secretion in RINm5F cells. (**A**) Confocal microscopy of RINm5F cells transfected with constructs encoding wild-type AKAP18 and the untargeted triple mutant. Immunocytochemical analysis was carried out using polyclonal antibodies raised against recombinant AKAP18 (red) and rat RIIα (green) in rabbit and goat, respectively. Texas Red-conjugated anti-rabbit and FITC-conjugated anti-goat secondary antibodies were used to permit the visualization of both proteins. In the panels showing immunochemical staining of AKAP18 and the triple mutant alone, several untransfected cells are evident due to the non-clonal nature of the AKAP18-transfected RINm5F cell lines. (**B**) Co-staining of AKAP18 (red), RII (blue) and C subunit (green) in transfected RINm5F cells using secondary antibodies conjugated to Texas Red, Cy5 and FITC, respectively. (**C**) Expression of AKAP18 and triple mutant proteins in transfected RINm5F cells. Whole-cell lysates (~50 μg total protein per lane) were separated by SDS–PAGE and electrotransferred to PVDF membrane. AKAP proteins were identified by both immunoblotting with the polyclonal antibody to AKAP18 and RII overlay as described in Materials and methods. (**D**) RINm5F cells transfected with wild-type AKAP18, the triple mutant and a pcDNA3 control plasmid were monitored for GLP-1-mediated insulin secretion. Secretion levels are represented as the increase in pmol insulin released/min/10^6 cells relative to basal levels. Statistical differences were determined by analysis of variance ($P < 0.05$).

observations), suggesting that the larger current amplitudes were not due to transfection with AKAP18 or the triple mutant. These results suggest that AKAP18 contributes to the cAMP-dependent augmentation of L-type Ca^{2+} current and that the membrane-targeting domain of AKAP18 appears to be required for this modulation.

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 α_{1c} and β_{2a} 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). Although we cannot rule out the contribution of secondary protein–protein interactions which could sustain a channel/AKAP complex, our data support a model for AKAP targeting that is essentially independent of the channel. Hence, AKAP18 may be more generally distributed across the lipid bilayer and could participate in placing the kinase close to several transmembrane substrates.

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 myristate and palmitate (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 Alanine-Rich C-Kinase Substrate protein (MARCKS), is membrane targeted through an N-terminal myristate 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 Ca^{2+} channels (Johnson *et al.*, 1994, 1997; Gao *et al.*, 1997), and Ca^{2+}-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 Ca^{2+} channels (Gao *et al.*, 1997). While AKAP79 and AKAP18 are both capable of targeting PKA to the Ca^{2+} channel in a heterologous expression system, there are several reasons why AKAP18 is more likely to be the physiological partner to the Ca^{2+} channel. The tissue distribution of AKAP18 is more consistent with the expression pattern of the L-type Ca^{2+} 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.*, 1994; Sjoholm *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 Ca^{2+} 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 Ca^{2+} release sites such as the IP_3 receptor and the ryanodine receptor may have their own pools of anchored PKA (Gromada *et al.*, 1995; Widmann *et al.*, 1995, 1996; Thorens *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 (Ammala *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 Ca^{2+} 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 [^{32}P]RIIα 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 *Nco*I–*Eco*RI 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 *Spe*I restriction enzyme site 85 bp downstream from the *Nco*I site, the 1–30 recombinant fragment was subcloned *Nco*I–*Spe*I. The 1–45, 43–81 and 28–81 fragments were all amplified by PCR using synthetic oligonucleotide primers to introduce restriction enzyme sites at the appropriate positions in the AKAP18 sequence. 1–45 was subcloned *Nco*I–*Eco*RI after introduction of a termination codon and an *Eco*RI site, while 43–81 and 28–81 were also subcloned *Nco*I–*Eco*RI using *Nco*I sites incorporated by PCR and the same downstream *Eco*RI 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).

Construction of AKAP18 mammalian expression plasmids

A synthetic oligonucleotide primer was designed to remove the AKAP18 termination codon while introducing a *Bam*HI site at the 3′ end of the coding sequence. Following PCR amplification, wild-type AKAP18 was subcloned *Eco*RI–*Bam*HI (using an *Eco*RI 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 *Bam*HI site immediately downstream of the termination codon. Upon *Eco*RI–*Bam*HI 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 *Kpn*I 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 *Kpn*I–*Bam*HI 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 *Kpn*I and *Bam*HI 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 *Bam*HI site immediately after the codon for amino acid 25. This fragment was PCR-amplified and subcloned *Eco*RI–*Bam*HI 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 [^{32}P]RIIα 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 bacterially 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 c-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 cDNAs by Ca^{2+} phosphate precipitation. Cells were incubated with the DNA for 16 h at 37°C under 5% CO_2, washed with phosphate-buffered saline (PBS), then incubated for a further 24–48 h in fresh growth medium [DMEM, 10% fetal calf serum (FCS), 1% penicillin/streptomycin]. ^{3}H cell-labeling experiments involved addition of [^{3}H]myristic acid or [^{3}H]palmitic acid (NEN-Du Pont) 48 h post-transfection to a final concentration of 25 μCi/ml and incubation for a further 3 h at 37°C under 5% CO_2. After immunoprecipitation and SDS–PAGE, ^{3}H incorporation was detected by fluorography using EN^{3}HANCE (NEN-Du Pont).

Subcellular fractionation and immunoprecipitation

Transfected HEK-293 cells at 100% confluency were resuspended in 500 μl of ice-cold hypotonic buffer (20 mM HEPES pH7.4, 20 mM NaCl, 5 mM EDTA, 2 μg/ml leupeptin/pepstatin, 1 mM benzamidine, 1 mM AEBSF, with or without Triton X-100) and homogenized on ice with 30 strokes of a Dounce homogenizer. Unlysed cells, nuclei and cell debris were pelleted by centrifugation at 325 *g* for 2 min (this step was omitted in experiments where cell lysis was carried out in the presence of Triton X-100). Soluble (supernatant) and particulate (pellet) fractions were generated by centrifugation at 40 000 *g* for 30 min. Immunoprecipitation was carried out from 500 μg of cell lysate by incubation overnight with 10 μg of affinity-purified AKAP18 antiserum or preimmune IgG. Immunecomplexes were recovered using protein A–Sepharose as previously described (Nauert *et al.*, 1997), analyzed by SDS–PAGE and assayed for PKA catalytic activity using Kemptide as substrate (Corbin and Reimann, 1974).

Confocal microscopy

Transfected cells at 50–80% confluency were seeded onto glass coverslips and incubated at 37°C under 5% CO_2 for 24–48 h in fresh growth medium. Cells were washed twice with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and then fixed for 10 min in PBS/3.7% formaldehyde. Cells destined for detection of GFP fusions were washed with PBS, then mounted directly for confocal microscopy. For immunocytochemistry, cells were permeabilized for 1 min in ice-cold acetone, washed with PBS, then blocked for 30 min in PBS/0.1% BSA. Cells were then incubated for 1 h with the appropriate dilution of primary antibody and washed three times with PBS/0.1% BSA. After incubation for 1 h in Texas Red, Cy5 or FITC-conjugated secondary antibody (Molecular Probes) and further washing, the cells were mounted using the Prolong system (Molecular Probes). Immunofluorescent staining or intrinsic GFP fluorescence was visualized on a laser-scanning confocal microscope (Zeiss).

Ca^{2+} channel measurements

HEK-293 cells were plated at low density (1:80 from confluent parent plate) on 35 mm dishes and transiently transfected with Ca^{2+} phosphate-precipitated cDNAs encoding the α_{1c} and β_{2a} cardiac Ca^{2+} channel subunits (0.1 μg) (obtained from M.Hosey, Northwestern University), and the CD4 cell surface marker (0.25 μg) at 24 h after plating. Cells were co-transfected with cDNAs (0.1 μg) encoding AKAP18 and the untargeted AKAP18 triple mutant as indicated. Recordings were made 48 h post-transfection. Whole-cell barium currents (Hamill *et al.*, 1981) were recorded using an Axopatch 200A amplifier (Axon Instruments, Forest City, CA). Whole-cell capacitance and series resistance compensation were employed. Whole-cell capacitance was 23.4 ± 1.4 pF (mean ± SEM; $n = 20$), while series resistance was 2–5 MΩ at ~95% compensation. Cells were perfused in an external recording solution consisting of 125 mM NaCl, 5 mM KCl, 20 mM TEA-Cl, 10 mM $BaCl_2$, 1 mM $MgCl_2$, 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 120 mM Cs methanesulfonate, 20 mM TEA-Cl, 5 mM $MgCl_2$, 5 mM ATP(Na), 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-CPTcAMP, currents were leak- and capacity-subtracted using a P/4 pulse protocol. P/4 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-CPTcAMP 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-CPTcAMP 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-CPTcAMP were measured 2–3 min following its application. Only currents in which a 10% enhancement of amplitude occurred were considered as positive responses. Data are expressed as mean ± SEM. 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 lipofectamine method as previously described (Lester *et al.*, 1997). Cell lines were plated at 1×10^5 cells per 35 mm well. After 24–48 h in culture, the cells were rinsed in low-glucose KRBH and incubated for 30 min with high-glucose KRBH. Insulin secretion was measured by incubating the transfected β cells with KRBH containing 16.7 mM glucose, 10 nM GLP-1 (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).

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