Alternative Splicing Regulates the Subcellular Localization of A-kinase Anchoring Protein 18 Isoforms

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Abstract. The cAMP-dependent protein kinase (PKA) is localized to specific subcellular compartments by association with A-kinase anchoring proteins (AKAPs). AKAPs are a family of functionally related proteins that bind the regulatory (R) subunit of PKA with high affinity and target the kinase to specific subcellular organelles. Recently, AKAP18, a low molecular weight plasma membrane AKAP that facilitates PKA-mediated phosphorylation of the L-type Ca\textsuperscript{2+} channel, was cloned. We now report the cloning of two additional isoforms of AKAP18, which we have designated AKAP18\textsubscript{b} and AKAP18\textsubscript{g}, that arise from alternative mRNA splicing. The AKAP18 isoforms share a common R subunit binding site, but have distinct targeting domains. The original AKAP18 (renamed AKAP18\textsubscript{a}) and AKAP18\textsubscript{b} target the plasma membrane when expressed in HEK-293 cells, while AKAP18\textsubscript{g} is cytosolic. When expressed in epithelial cells, AKAP18\textsubscript{a} is targeted to lateral membranes, whereas AKAP18\textsubscript{b} is accumulated at the apical membrane. A 23-amino acid insert, following the plasma membrane targeting domain, facilitates the association of AKAP18\textsubscript{b} with the apical membrane. The data suggest that AKAP18 isoforms are differentially targeted to modulate distinct intracellular signaling events. Furthermore, the data suggest that plasma membrane AKAPs may be targeted to subdomains of the cell surface, adding additional specificity in intracellular signaling.

Key words: protein kinase A • AKAP • epithelia • targeting • green fluorescent protein

Horizontally induced changes in intracellular cAMP influence many cellular processes, including growth and differentiation, vesicular trafficking, cellular metabolism, and ion channel activity (Taylor et al., 1990; Francis and Corbin, 1994). These pleiotropic effects are predominantly due to activation of the cAMP-dependent protein kinase (PKA). The PKA holoenzyme is a tetramer containing two regulatory (R) and two catalytic (C) subunits; binding of cAMP causes dissociation of the R and C subunits, to reversibly activate the enzyme (Taylor et al., 1990). Although PKA has a broad substrate specificity in vitro, activation of cAMP-mediated processes by cell surface receptors results in phosphorylation of a specific and restricted set of protein substrates. Thus, compartmentalization of PKA in close proximity to specific targets may be crucial for controlling the specificity and efficiency of cAMP-mediated signaling in cells (Rubin, 1994; Dell’Aquila and Scott, 1997; Schillace and Scott, 1999b). Furthermore, compartmentalization of PKA, together with other protein kinases or protein phosphatases, may facilitate appropriate cross-talk between signaling pathways (Pawson and Scott, 1997).

The identification of a diverse family of A-kinase anchoring proteins (AKAPs) suggests that compartmentalization of PKA is a general mechanism for modulation of cAMP-mediated signaling. AKAPs bind with high affinity to the NH\textsubscript{2} terminus of the type II R subunit (RII) dimer, via an amphipathic helix in each AKAP (Carr et al., 1991; Newlon et al., 1999). Recent data suggests that a subset of AKAPs can also bind R I subunits (Huang et al., 1997; Ngelo and Rubin, 1998). Although the binding affinity is lower than for RII (Burton et al., 1997) and the physiological significance of the A K A P-R I interaction is yet to be established. In addition, each AKAP contains a unique targeting motif that directs the A K A P-mediated regula-
Materials and Methods

Cloning of AKAP18β and AKAP18γ

The pET11.RIIα plasmid was transformed into BL21(DE3) pLysS E. coli and grown at 37°C; protein expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h at 37°C, and the RIIα protein was dialyzed into 50 mM sodium bicarbonate, pH 8.5, and concentrated by centrifugation in a BioMax-10K centrifugal filter (Millipore Inc.). Purified RIIα (10 μM) was biotinylated by addition of 100 μM EZ-Link NHS-LC-Biotin (Pierce Chemical Co.). Excess biotin was removed by dialysis in 10 mM Tris-HCl, pH 7.4, + 0.15 M NaCl.

Biotin-RIIα was used as a probe to screen a λTriplEx human lung cDNA library (CLONTECH). Biotin-RIIα (10 nM) was prebound to 0.5 μg/ml streptavidin-alkaline phosphatase (SA-A P) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 for 4 h at 4°C, and the filters were incubated overnight at 4°C in TTBS containing the biotin-RIIα/SA-A P complex. A filter extensive washing in TTBS, bound RIIα/SA-A P complexes were visualized as described (Sparks et al., 1996). Plasmids were rescued from λTriplEx phage by in vivo excision, and inserts sequenced at the University of North Carolina Sequencing Facility.

An ~2-kb cDNA clone encoding a novel AKAP was isolated from the library screen; after sequencing, this clone was designated A K A P B 18b. To obtain upstream coding sequence, 5′ RACE was performed using M arthon-R-Ready human pancreas cDNA (CLONTECH); RACE products were cloned into pT-A dv (CLONTECH) and sequenced. A K A P B 18a was cloned using reverse transcriptase (RT)-PCR of human pancreas cDNA and KlenTaq DNA polymerase (CLONTECH), using oligonucleotides designed based on the previously reported human AKAP 18 sequence. PCR products were excised from the gel and subcloned into pT-A dv (CLONTECH).

For RT-PCR analyses, total RNA from cultured cells was extracted using R N A STAT 60 (Tel-test Inc.) and treated with DNase I (Promega). First strand cDNA was synthesized using Superscript II reverse transcriptase (GIBCO BRL). PCR reactions were performed using Ta q DNA polymerase (GIBCO BRL) and A K A P 18a specific primers. PCR products were purified, ligated into pT-A dv cloning vector, and sequenced.

Northern Blot, Southern Blot, and Screening of Genomic Libraries

A human multiple tissue northern blot (CLONTECH) was probed with a 32P-labeled random-primed cDNA probe using the unique region of A K A P B 18y (nucleotides [nt] 357-689). The blot was prehybridized at 68°C for 30 min and incubated with the probe at 68°C for 1 h in ExpressHyb (CLONTECH). A fiber incubation, the blot was washed at room temperature for 30 min in 2× SSC + 0.1% SDS, followed by 0.1× SSC + 0.1% SDS for 40 min at 50°C. Blots were stripped and reprobed with a 32P-labeled β-actin probe (CLONTECH). All blots were analyzed using a STORM-840 PhosphorImager.

For Southern blot analysis, human genomic DNA (CLONTECH) digested with 100 units of BamHI, EcoRV, HindIII, or XbaI, was electrophoresed on 1% agarose gels. DNA was transferred to GeneScreen (New England Nuclear Life Sciences) by capillary diffusion in 20× SSC overnight at room temperature. Hybrids were washed at 42°C in ExpressHyb (CLONTECH) using a 32P-labeled DNA probe common to all known AKAP 18 isoforms (nt 106-243 of A K A P B 18b), and membranes were washed as described above.

A genomic DNA library was created for the mouse strain L EM1 in the Lambda Fix II vector (Stratagene). The library was screened with an α32PdCTP random-primed probe representing full-length A K A P B 18b (nt 205-450) or the sequence common to the three identified A K A P B 18 isoforms (nt 256-450).

Generation of AKAP18 Plasmids

The coding sequences of A K A P B 18a, A K A P B 18b, and A K A P B 18y were amplified by PCR using human pancreas cDNA as template. The sense primers incorporated an EcoRI site at the 5′ end and overlapped the initiator methionine of each AKAP 18 isoform. The antisense primer overlapped the COOH terminus and stop codon, and incorporated a BamH I site. The PCR fragments were digested with EcoRI and BamHI and subcloned into pC DNA 3.1 (−) (Invitrogen) digested with the same enzymes.

The cDNA encoding each A K A P B isoform was also fused in-frame at the 3′ end with the cDNA encoding GFP. The coding regions of A K A P B 18a, A K A P B 18b, and A K A P B 18y were amplified by PCR using the sense primers described above and a single antisense primer designed to remove the stop codon and incorporate a BamH I site at the 3′ end. Similarly, the 1-163b, 1-447, and 17-447 constructs were generated by PCR using a K A P B 18a DNA template. DNA sequencing confirmed the absence of mutations in all constructs generated by PCR.

Cell Culture and Transfection of Cells

CHO, human embryonic kidney (HEK-293), and M D C K type II were obtained from the American Type Culture Collection. The N T-I cell line, derived from mouse pancreatic β cells, was provided by Dr. L l o y d F r i c k e r (A l b e r t Einstein School of Medicine, Bronx, NY). Cultured cells were grown in the appropriate media as described previously (Chen et al., 1998; Short et al., 1998). CH O, H E K -293, and M D C K cells were grown to 30-50% confluency and transfected with the appropriate plasmid in Effec-
Western Blot and Immunoprecipitation

Rabbit antisera directed against A K A P 1 8 was generated in rabbits using recombinant A K A P 1 8 and affinity-purified as described (Fraser et al., 1998; Short et al., 1998) using rabbit anti-GFP (1:1,000; CLONTECH), affinity-purified rabbit anti-A K A P 1 8 (V O 5 7; 1:1,000 ohm cm; Transduction Laboratories). The AKAP18 antisera also recognize A K A P 1 8 a n d A K A P 1 8 β, Transfected cells were washed once with PBS and lysed in ice-cold buffer (20 mM H epes, pH 7.4, 20 mM NaCl, 5 mM EDTA, 2 μg/ml leupeptin, 1.6 μg/ml benzamidine, 0.3 μg/ml PM SF), with or without 1.0% Triton X-100. For some experiments, whole cell lysates were separated into soluble and particulate fractions by centrifugation at 40,000 g for 30 min at 4°C, and protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Co.). For immunoblotting, proteins were resolved on 12.5 or 15% SDS-P A GE gels and transferred to Immobilon-P (Millipore Inc.). Western blots were performed as described previously (Fraser et al., 1998; Short et al., 1998) using rabbit anti-GFP (1:1,000; CLONTECH), affinity-purified rabbit anti-A K A P 1 8 (V O 5 7; 1 μg/ml), or mouse anti-C subunit (1 μg/ml; Transduction Laboratories). Immunoprecipitations were carried out overnight at 4°C using 500 μg of protein (whole cell lysate or soluble and particulate fractions) and 1 μg affinity-purified A K A P 1 8 antisera (Fraser et al., 1998). Immune complexes were collected on protein A agarose and electrophoresed on 12.5 or 15% SDS-P A GE gels. R I I overlays were performed as described previously using [32P]-labeled mouse R I I α (Fraser et al., 1998).

Confocal Microscopy

Rabbit antisera that specifically recognize A K A P 1 8 (NC 2 5 7) were generated using residues 15–43 of A K A P 1 8 coupled with keyhole limpet cytochrome c as immunogen. Complement proteins were removed from the whole serum by incubation with D E A E-Blue dextran (Pierce Chemical Co.). Transfected cells were grown on glass coverslips (HEK-293 and M D C K cells) or Transwell filters ( M D C K cells). M D C K cells were grown until confluent monolayers were observed and transepithelial resistances were >1,000 ohm-cm². Cells were washed with PBS and then fixed for 20 min in fresh 4.0% paraformaldehyde prepared in PBS. For immunochemistry, cells were permeabilized for 10 min in acetone/methanol (1:1), washed three times with PBS, and blocked at room temperature in 4% nonfat dry milk, 2 mg/ml BSA, and 0.1% Triton X-100 in PBS. Cells were rinsed in PBS and incubated for 1 h at room temperature with affinity-purified antisera as noted in the figure legends. A tier extensive washing, Texas red-conjugated secondary antibodies were applied for 1 h at room temperature. Cells were washed and mounted with VectaShield mounting medium (Vector Laboratories) and analyzed by confocal microscopy as described (Chen et al., 1998).

Results

Cloning of Two Additional A K A P 1 8 Isoforms

We screened a lung c D N A expression library using biotinylated R I I as probe and identified a novel c D N A that shares sequence homology with A K A P 1 8, a previously identified membrane-associated A K A P ( Fraser et al., 1998; Gray et al., 1998). The c D N A was identical to A K A P 1 8 at the 3’ end, but shared little homology with A K A P 1 8 at the 5’ end (data not shown). Therefore, we designated the c D N A and protein product A K A P 1 8γ, to indicate its relationship to other A K A P 1 8 family members, including the previously reported A K A P 1 8 (r e n a m e d A K A P 1 8α) and A K A P 1 8β (discussed below). The original A K A P 1 8γ c D N A contained a single open reading frame, but no consensus ribosome binding site or initiator methionine. Therefore, we used rapid amplification of c D N A ends ( R A C E ) with human pancreas c D N A as template to obtain the full-length A K A P 1 8γ sequence. The longest A K A P 1 8γ c D N A isolated was 2,917 nt in length with a single open reading frame from nt 107 to 1,086 (these sequence data have been submitted to the Genbank/EMBL/D D B J databases under the accession number A F 1 5 2 9 2 9). The upstream c D N A sequence contains three in-frame stop codons, and there are stop codons in the alternative reading frames (data not shown), suggesting that this is the correct open reading frame.

The A K A P 1 8γ c D N A encodes a protein of 326 amino acids with a calculated molecular mass of 37 kD and a pl of 5.8. The first 262 amino acids are unique and do not share significant homology with any known proteins in Genbank/EMBL/D D B J databases. However, the last 64 amino acids are identical to human A K A P 1 8α and include a conserved R I I binding site (Fig. 1 A). There are no consensus myristoylation or palmitoylation sites at the NH₂ terminus of A K A P 1 8γ, suggesting that the A K A P 1 8γ protein is not modified by lipid side chains. We used P S O R T (Nakai and Kanehisa, 1992) to predict potential subcellular targeting sequences, and found that amino acids 37 to 54 of A K A P 1 8γ fit the specifications for a consensus nuclear localization signal (Gerace, 1992; Gorlich, 1997).

In previous studies, m R N A s of ~2.4-, 3.6-, and 4.3-kb were observed in rat and human tissues using the A K A P 1 8α coding region as a probe (Fraser et al., 1998; Gray et al., 1998). We used Northern blot analysis to determine whether any of these m R N A s represented the A K A P 1 8γ m R N A and to determine the tissue distribution of the message (Fig. 1 B). Using a radiolabeled probe directed against the unique region of A K A P 1 8γ, we detected a dominant transcript of ~4.3-kb in heart, brain, placenta, lung, and pancreas, and a smaller transcript of 2.4-kb, which is abundantly expressed in pancreas.

To further compare the expressions of A K A P 1 8α and A K A P 1 8γ, we used unique sense primers paired with a common antisense primer in RT-PCR reactions. Using a sense primer specific for the A K A P 1 8γ c D N A, we obtained a 979-nt product whose sequence exactly matched that obtained from the c D N A library screen and 5’ R A C E reactions (Fig. 2 A). Surprisingly, primers designed to specifically amplify A K A P 1 8α consistently amplified two bands of 246 and 315 nt (Fig. 2 A). The 246-nt fragment was the expected size of the A K A P 1 8α product, which was confirmed by D N A sequencing. The sequence of the 315-nt fragment matched A K A P 1 8α at the 5’ and 3’ ends, but contained a 69-nt insert (these sequence data have been submitted to the Genbank/EMBL/D D B J databases under the accession number A F 1 6 1 0 7 5); we named this c D N A A K A P 1 8β. We used RT-PCR to determine whether A K A P 1 8α and -γ are differentially expressed in cell lines and tissues. Both c D N A s were detected in fibroblast, endocrine, and epithelial cell lines, indicating that the two m R N A s are broadly expressed (Fig. 2 B). Although we were unable to reliably amplify the A K A P 1 8γ c D N A from human brain, a weak signal was observed in some reactions (data not shown).

The first 16 amino acids of human A K A P 1 8α are identical to A K A P 1 8α and are followed by an insert of 23 unique amino acids (Fig. 2 C. D) to this 23-amino-acid insert, A K A P 1 8α and -γ are identical to each other (Fig. 2, C and D). Thus, we have identified three A K A P 1 8 isoforms (named α, β, and γ) which share a common R I I sequence.
binding site, but have unique NH₂-terminal sequences (Fig. 2 D).

We used Southern blot analysis to determine whether these AKAP18 isoforms arise from a single gene. Genomic DNA was digested and hybridized with a probe common to all three AKAP18-related cDNAs. A single fragment was visualized on Southern blots, suggesting that AKAP18α, β, and γ mRNAs arise as alternate products.

**Figure 1. Cloning of human AKAP18γ.** A, Comparison of human AKAP18γ and AKAP18α amino acid sequences. Common residues are indicated by a vertical line, a dashed line marks the putative nuclear localization signal, and the RII binding site is underlined. B, A human multiple tissue Northern blot was hybridized with a random primed 32P-labeled probe generated against the unique region of AKAP18γ (nt 357-689). The blot was stripped and rehybridized with a β-actin probe. mRNA size markers are shown in kb. Similar results were obtained in two separate blots.

**Figure 2. Cloning of AKAP18β.** A, Primers used to amplify human pancreas cDNA are shown schematically; PCR was performed using human pancreas cDNA and the primer pairs indicated. + indicates addition of 1 ng cDNA and – indicates no cDNA. Samples were electrophoresed on 1% agarose gels and visualized with ethidium bromide. mRNA size markers are shown in kb. No PCR product was observed using primers A + D, indicating that cDNAs containing both AKAP18γ and α-specific sequence does not exist in this tissue. The data are representative of three PCR reactions using human pancreas or lung cDNA as template. B, RT-PCR with AKAP18α-specific primers (C + B) was performed using cDNA from cultured cell lines, human pancreas, and human brain. cDNA quality was verified by amplifying each sample with human cyclophilin primers. + indicates AKAP18α or cyclophilin plasmid control and – indicates no addition of template. Samples were electrophoresed on 1% agarose gels and visualized with ethidium bromide. mRNA size markers are shown in kb. Data are representative of two separate experiments. C, Comparison of AKAP18α and AKAP18β amino acid sequences. Common residues are indicated by a vertical line and the RII binding site is underlined. D, Schematic of three AKAP18 isoforms. The proteins are drawn to scale and unique regions marked by different shadings.
of one gene (Fig. 3 A). This is consistent with the fact that the 3’ untranslated regions of AKAP18α, -β, and -γ are identical (data not shown).

Preliminary analysis of mouse AKAP18 genomic sequence (Fig. 3 B) indicates that residues 1–16 of AKAP18α (also contained in AKAP18β) are encoded by a single exon; this exon contains the determinants for lipid modification. A shorter short exon encodes the 23-amino acid residues specific to AKAP18β. In addition, the COOH-terminal RII binding domain found in all AKAP18 isoforms is contained within a single exon. Taken together, the data indicate that alternative splicing of a single AKAP18 gene gives rise to (at least) three distinct AKAP18 mRNAs encoding different protein products.

Protein Analysis of AKAP18 Isoforms

To determine whether each AKAP18 isoform is capable of binding PKA, we transiently transfected HEK-293 cells with cDNA encoding each isoform, and immunoprecipitated the expressed proteins with AKAP18-specific antisera. As expected, each of the immunoprecipitated AKAP18 isoforms was able to bind the RII subunit in overlay assays (Fig. 4 A). However, a new classification for AKAPs has been proposed, whereby the anchoring proteins must be able to interact with the PKA holoenzyme inside cells (Colledge and Scott, 1999). Therefore, we also probed AKAP18-specific immunoprecipitates with antisera directed against the C subunit of PKA. The C subunit was detected in immunoprecipitates for each isoform, but

Figure 3. Alternative splicing gives rise to three distinct AKAP18 mRNAs. A, Human genomic DNA digested with the enzymes indicated, or positive control CDNA was hybridized with a radiolabeled probe overlapping the common region of all three AKAP18 isoforms. DNA size standards are shown in kb. B, Schematic of the mouse AKAP18 gene and mRNAs encoding each AKAP18 isoform. Exons encoding the lipid modification determinant (Exon L), the AKAP18β-specific sequence (Exon B), and the RII binding region (Exon R) have been identified.

Figure 4. Analysis of AKAP18-related proteins. A, Lysates prepared from HEK-293 cells transiently expressing individual AKAP18 isoforms were immunoprecipitated with antisera directed against AKAP18. Samples were separated by SDS-PAGE and proteins visualized by RII overlay. B, Lysates prepared from HEK-293 cells transiently expressing individual AKAP18 isoforms were immunoprecipitated with rabbit antisera directed against AKAP18. Samples were separated by SDS-PAGE and blots were probed with mouse anti-PKA C subunit. C, Whole rat brain or kidney lysates were immunoprecipitated with rabbit antisera directed against AKAP18 as indicated. Samples were electrophoresed on SDS-PAGE and visualized by RII overlay. D, Whole rat kidney lysates were fractionated into soluble (S) and particulate (P) fractions in hypotonic buffers lacking detergent. Equal ratios of the soluble and particulate fractions were electrophoresed on SDS-PAGE and visualized by RII overlay. For C and D, lysates were also prepared from HEK-293 cells transiently transfected with AKAP18 isoforms, and samples were electrophoresed and analyzed in parallel with the tissue samples. For all panels, protein size standards are shown in kD and the data are representative of at least four similar experiments. IP, immunoprecipitation; IB, immunoblotting; Pi, preimmune sera.
was absent from control experiments with preimmune sera (Fig. 4 B). Thus, each of the AKAP18 isoforms functions as a bona fide AKAP in cells.

We next determined whether each of the novel AKAP18 isoforms was expressed in rat tissues. We chose kidney as a tissue where mRNA was detected for all three AKAP18 isoforms, and brain as a tissue source where AKAP18β mRNA levels were low (Figs. 1 B and 2 B). Detergent soluble extracts were prepared from brain and kidney, immunoprecipitations were carried out with AKAP18 specific antisera (VO57 or R4570), and AKAPs were detected by RII overlay (Fig. 4 C). Although there was less AKAP18β protein in brain, two bands immunoprecipitated from both brain and kidney with VO57 antiserum corresponding to AKAP18α and -β. Both of these proteins were preferentially accumulated in the particulate fraction of rat kidney (Fig. 4 D). Bands corresponding to AKAP18α and -γ were immunoprecipitated from both tissues using R4570 antisera (Fig. 4 C). We also examined the distribution of AKAP18γ in rat kidney, and it was equally distributed in the soluble and particulate fractions (Fig. 4 D). Collectively, these results suggest that all three cloned AKAP18 isoforms are expressed as proteins in cells.

Localization of AKAP18 Isoforms in Cells

Accumulating evidence suggests that AKAPs compartmentalize PKA at discrete subcellular compartments to facilitate cAMP-responsive events and control the specificity of intracellular signaling (Colledge and Scott, 1999). Therefore, each AKAP contains a targeting domain responsible for localizing PKA to specific organelles or subcellular compartments (Schillace and Scott, 1999a). The targeting of AKAP18α is dependent upon lipid modification through myristylation of Gly1 and palmitoylation of Cys5 and Cys7 (Fraser et al., 1998). Accordingly, the first ten amino acids of AKAP18α encompass the minimal sequence necessary to target a reporter protein to the plasma membrane (Fraser et al., 1998). To determine whether AKAP18 isoforms are differentially targeted, we transiently transfected cDNA's encoding each construct into HEK-293 cells and compared the intracellular distribution of the proteins by differential fractionation and immunofluorescent microscopy. AKAP18α and -β fractionated exclusively with the cell membranes in buffers lacking detergent (Fig. 5 A) and both proteins were distributed at the cell surface (Fig. 5 B). This is consistent with the segregation of endogenous AKAP18α and -β with the particulate fraction of rat kidney (Fig. 4 D). In contrast, a significant fraction of the overexpressed AKAP18γ partitioned with the soluble fraction, although ~20% was found in the particulate fraction (Fig. 5 A). The expressed AKAP18γ protein was visualized throughout the cytoplasm of cells, but did not significantly accumulate in the nucleus (Fig. 5 B). The even distribution of the endogenous AKAP18γ in the soluble and particulate fractions of rat kidney suggests that overexpression of AKAP18γ in HEK-293 cells saturates a protein–protein interaction required to maintain a particulate pool of this isoform.

Localization of AKAP18α and AKAP18β in Epithelial Cells

Although both AKAP18α and -β are targeted to membranes in HEK-293 cells, recent data indicate that the formation of specialized plasma membrane microdomains is crucial for efficient intracellular signaling in many cell...
types (Shaul and Anderson, 1998; Fanning and Anderson, 1999; Ostrom and Insel, 1999). Therefore, we con-
sidered whether the unique 23-amino acid insert present in A K A P18β directs this isoform to specific targets or micro-
domains of the plasma membrane. M D C K cells, a well-
characterized kidney epithelial cell line, form a tight
monolayer with distinct apical, basolateral, and junctional
surfaces when grown on permeable filter supports. We sta-
bly expressed cD N A s encoding GFP-tagged A K A P18α
and -β in M D C K cells to compare their distributions in po-
larized cells. Previous experiments established that the fu-
sion of GFP to the COOH terminus of A K A P18α does
not disrupt membrane targeting of the chimeric protein
(Fraser et al., 1998).

Confocal microscopy performed on well-polarized M D C K
cell cultures indicated that the distributions of GFP-tagged
A K A P18α and -β differed. A K A P18α/GFP was accumu-
lated predominantly along the lateral margins of the trans-
fected cells. In contrast, A K A P18β/GFP was present at
the apical membrane (Fig. 6 A), and overlapped the dis-
tribution of the apical membrane glycoprotein gp135
(Ojakian and Schwimmer, 1988; Fig. 6 B). The A K A P18α/
GFP and A K A P18β/GFP proteins were expressed at rela-
tively equal levels, and both proteins were present in the
particulate fraction when cells were lysed in hypotonic
buffers lacking detergent (data not shown). The lateral
membranes of polarized epithelial cells are comprised of
two membrane specializations, the tight and adherens
junctions. The distribution of A K A P18α significantly
overlapped the distribution of β-catenin, a protein that ac-
cumulates at the adherens junctions (Nathke et al., 1994;
Fig. 6 B). When the distribution of A K A P18α and -β was
compared with the distribution of Z O -1, a marker for tight
junctions (Willott et al., 1992; Itoh et al., 1993), neither
protein was found to significantly overlap (Fig. 6 B).

Lateral and apical distributions of A K A P18α/GFP and

Figure 6. A K A P18α and -β are differentially targeted in M D C K
cells. A , M D C K cells stably expressing A K A P18α/GFP, A K A P18β/
GFP, or GFP alone were grown on Transwell filters. Confluent monolayers were fixed in 4% paraformaldehyde and analyzed by confo-
cal microscopy in X Y and X Z planes. A t least three individual cell lines expressing each construct were analyzed and similar results
were obtained. B ar, 10 μm. B , The distributions of A K A P18α/GFP and A K A P18β/GFP were compared to the distribution of markers
for tight junctions (Z O -1), adherens junctions (β-catenin), and apical membranes (gp135). Stably transfected cells were fixed and
stained with rat anti Z O -1 (1:400), rabbit anti-β-catenin (1:400), or mouse anti-gp135 (1:50), followed by the appropriate Texas red-con-
jugated secondary antibody. Images are shown as X Z scans and are representative of images collected in two independent experiments.
AKAP18β/GFP, respectively, were observed in several independent clonal cell lines and in transient transfection assays (data not shown). AKAP18β was observed at lateral surfaces of well-polarized MDCK cells in clones that expressed high levels of the transfected protein (data not shown), suggesting that a saturable protein–protein or protein–lipid interaction mediates the selective association of AKAP18β/GFP with apical membranes.

The differential targeting of AKAP18α and -β was not due to overexpression of the proteins in the MDCK cells, since we found the endogenous proteins were also differentially distributed (Fig. 7). To compare the distributions

Figure 7. Localization of endogenous AKAP18 in MDCK cells. A, MDCK cells stably expressing AKAP18β/GFP or AKAP18α/GFP were grown on Transwell filters and confluent monolayers were fixed in 4% paraformaldehyde. Cells were permeabilized, blocked, and stained with NC257 (1:1000 dilution) followed by Texas red-conjugated secondary antibody. Antibody staining was compared with the distribution of the GFP fusion proteins by confocal microscopy. B, MDCK cells stably expressing AKAP18β/GFP or AKAP18α/GFP were grown on Transwell filters and confluent monolayers were fixed and stained with VO64 (1 μg/ml) as described in A. For experiments in A and B, scanning in one channel was performed with the other laser off to assure that there was no bleed-through. C, Wild-type MDCK cells were grown on Transwell filters and confluent monolayers were fixed and stained with VO64 or NC257 as described above. Preimmune sera and normal rabbit IgG at the same concentrations failed to stain any structures in wild-type or transfected MDCK cells. Bar, 10 μm.
of AKAP18 and -β in MDCK cells, we generated an antibody directed against residues 15–43 of AKAP18β (NC 257); this antibody specifically recognizes the overexpressed AKAP18β/GFP stably expressed in MDCK cells (Fig. 7 A). Furthermore, the antibody does not detect AKAP18α/GFP on the lateral borders of stably transfected MDCK cells, although we did observe apical membrane labeling of these cells, as well as some punctate staining towards the apical pole (Fig. 7 A). In contrast, antisera VO64 generated against recombinant AKAP18α, which detects multiple AKAP18 isoforms on Western blots (data not shown), recognized both AKAP18β/GFP and AKAP18α/GFP in stably transfected MDCK cells (Fig. 7 B). Having established that NC257 selectively recognized AKAP18β while VO64 recognized both AKAP18 isoforms, we stained wild-type MDCK cells with each antisera and compared the distribution of endogenous AKAP18 in these cells. In cells stained with VO64, AKAP18 proteins were found distributed along the lateral cell membranes (Fig. 7 C) with a staining pattern resembling the distribution of the AKAP18α/GFP. Punctate staining was also observed at the apical cell surface in confocal sections (data not shown), which is consistent with the staining (presumably of endogenous AKAP18β) observed in AKAP18α/GFP cells (Fig. 7 B). In contrast, no staining of the lateral cell surface was observed when cells were stained with NC257 directed against the β-specific exon, although robust staining of subapical and apical vesicles was observed (Fig. 7 C). Therefore, we conclude that in polarized MDCK cells, endogenous AKAP18α and -β are differentially targeted to the lateral and apical cell surfaces, respectively. The formation of detergent-resistant membranes or lipid rafts is implicated in signal transduction and in the sorting of proteins to the apical cell surface (Brown and London, 1998). Therefore, we tested whether the differential targeting of AKAP18α and -β correlated with the selective accumulation of AKAP18β in detergent-insoluble lipid rafts. To do this, we performed subcellular fractionation experiments in the presence of different concentrations of Triton X-100 and compared the solubilities of AKAP18α and -β. Since both proteins were easily extracted in buffers containing 0.2% Triton X-100 (data not shown), we conclude that AKAP18β is not associated with detergent insoluble complexes at the apical surface of MDCK cells.

The Unique Sequence of AKAP18β Contains Apical Targeting Information

Our expression studies show that AKAP18α and -β are differentially targeted in polarized epithelial cells, yet they only differ by the presence of an alternative exon encoding 23 amino acids (Figs. 2 and 3). To further explore the function of this AKAP18β-specific sequence, we generated three GFP fusion proteins corresponding to exons in the AKAP18 gene: 1-16αβ/GFP, which encompasses the common membrane targeting domain; 17-44β/GFP, which encompasses the AKAP18β specific sequence; and 1-44β/GFP, which includes both exons (Fig. 8 A). We first transiently expressed each of the GFP chimeras in HEK-293 cells to compare the efficiency with which the expressed proteins were targeted to the cell surface. Both 1-16αβ/GFP and 1-44β/GFP were detected at the cell surface, whereas the 17-44β/GFP protein was uniformly distributed throughout the cell (Fig. 8 B). Similarly, when the 17-44β/GFP chimera was stably expressed in MDCK cells, the protein was distributed throughout the cytoplasm and nucleus (Fig. 8 C). These results indicate that the 23-amino acid insert unique to AKAP18β is not sufficient to mediate membrane targeting. However, the localization of the 1-16αβ/GFP and 1-44β/GFP proteins clearly differed when stably expressed in MDCK cells. The 1-16αβ/GFP protein targeted the plasma membrane, and most of the expressed protein was distributed along the lateral borders of the cells (Fig. 8 C). The 1-44β/GFP protein was also targeted to the plasma membrane in MDCK cells. However, a significant fraction of the 1-44β/GFP protein was present at the apical cell surface, although protein was detected along the lateral borders. Collectively, these data indicate that the 23-amino insert unique to AKAP18β facilitates targeting of AKAP18β to the apical membrane.

Discussion

In this report, we describe the identification and characterization of two additional isoforms of AKAP18, a plasma membrane-associated AKAP suggested to play a role in modulation of L-type Ca²⁺ channels (Fraser et al., 1998; Gray et al., 1998). We propose to call the original AKAP18 cDNA AKAP18α, and the newly described cDNA AKAP18β and -γ. Taken together, Southern blot analyses and partial sequencing of the mouse AKAP18 gene indicate that these cDNAs arise secondary to alternative splicing of exons in a single gene (Fig. 3). Although the sequencing of the mouse AKAP18 gene is not complete, we have already identified exons encoding the lipid modification domain found in AKAP18α and -β, the 23-amino acid insert found in AKAP18β, and the RII binding site in all three AKAP18 isoforms (Fig. 3 B).

Several other AKAPs are known to exist in multiple forms (Lin et al., 1995; Dong et al., 1998; Schmidt et al., 1999), and the generation of AKAP diversity by differential mRNA splicing may be a common occurrence. In theory, differential splicing of AKAP genes may result in the expression of proteins that are targeted to different subcellular compartments, or proteins that target the same compartment, but recruit additional binding partners. Some AKAPs bind other kinases or phosphatases (Kauck et al., 1996; Schillace and Scott, 1999a), or contain additional putative interaction domains (Dong et al., 1998; Schmidt et al., 1999). An alternative splicing could, therefore, generate AKAP-mediated multiprotein complexes with different compositions. Although splicing of several AKAP genes is well documented, there are few examples where the function of the splicing is well established. For example, there are six known isoforms of AKAP-KL which share a common RII binding site, but the function of the unique sequence in each isoform is not known (Dong et al., 1998). An alternative splicing generates S-AKAP84, AKA8121, and D-AKAP1-related proteins (Lin et al., 1995; Chen et al., 1997; Huang et al., 1997a, 1997b). Each of these proteins share a common RII binding site; however, while S-AKAP84 and AKA8121 are targeted to the outer mitochondrial membrane, D-AKAP1 splice var-
The unique 23 amino acids in AKAP18β facilitate apical targeting. A, Schematic diagram of the three GFP fusion proteins expressed in HEK-293 and MDCK cells. GFP is not drawn to scale. The unique region of AKAP18β is filled in black. B, Each construct was expressed transiently in HEK-293 cells and the distributions of the expressed GFP-tagged proteins analyzed by confocal microscopy. Bar, 10 μm. The images are representative of three individual experiments. C, MDCK cells stably expressing the constructs shown in A were grown to confluence on glass coverslips and the distribution of the GFP chimeras was determined by confocal microscopy. Images were collected in the XY and XZ planes. Similar results were obtained in transient transfection assays and in at least three clonal cell lines for each construct. Bar, 10 μm.
alternative splicing of the AKAP18 gene generates proteins that may target distinct subcellular compartments, but contain a common R11 binding determinant (Fig. 2 D). Furthermore, our data indicate that alternative splicing may dictate the targeting of AKAP18 isoforms to micro-domains of the cell surface in some cell types (Figs. 6–8). However, it is likely that another consequence of this splicing is to regulate differential association with other cellular proteins.

The NH2-terminal targeting domain of AKAP18a and -b clearly directs the expressed proteins to the plasma membrane (Figs. 5, 6, and 8). Although computer-based resources for identifying organelle targeting signals predicted that AKAP18y would be found in the nucleus (Fig. 1 a), we observed that AKAP18y was distributed in cytoplasm of transiently transfected fibroblasts (Fig. 5 b) and stably transfected MDCK cells (data not shown). A approximately 50% of the native protein in rat kidney and 20% of the exogenously expressed AKAP18y in HEK-293 cells was found in the particulate fraction (Figs. 4 D and 5 A), suggesting association with cellular membranes or cytoskeletal structures. Due to the increased proportion of soluble AKAP18y in overexpression studies, we speculate that the targeting of the protein may rely strictly on association with an endogenous protein expressed at low levels in HEK-293 cells. However, we cannot rule out the possibility that AKAP18y functions to bind PKA in the cytoplasm of cells. There is some precedence for cytosolic AKAPs, such as AKAP9, which treatment of ovarian granulosa cells with forskolin stimulating hormone induced the expression of an ~80-kD AKAP that was found predominantly in the cytosol of fractionated cells (Carr et al., 1993). The generation of AKAP18-specific antisera for immunohistochemical studies and the identification of proteins that associate with the unique region of AKAP18y will hopefully resolve these questions.

AKAP18a and -b are well situated to modulate AMP-mediated signaling at the plasma membrane, since both proteins accumulate at the cell surface when expressed in cells (Figs. 5 B and 6). This is not surprising since residues 1–16, present in both isoforms, contain membrane targeting information (Fraser et al., 1998; Gray et al., 1998). A ction alone, the 23-amino acid insert unique to AKAP18 does not function to redistribute a GFP reporter protein to the plasma membrane (Fig. 8). Therefore, these amino acids do not contain plasma membrane targeting information. However, AKAP18a is restricted to the lateral surfaces of polarized MDCK cells, whereas AKAP18b is preferentially localized apically (Fig. 6). Indeed, our data demonstrate that acting in tandem with residues 1–16, the unique sequence (residues 17–39) facilitates apical targeting (Fig. 8 C). Although we considered the possibility that association with apical membrane lipid rafts explained the preferential apical distribution of AKAP18b, the protein showed no difference in its solubility with AKAP18a. Therefore, we speculate that the selective targeting of AKAP18b to the apical membrane is due to specific protein–protein interactions involving residues 17–39.

In epithelial cells, AKAP18b is restricted to the lateral cell membranes overlapping the distribution of b-catenin (Fig. 6 B). Endogenous AKAP18 was also observed along the lateral membranes of cells, but only when cells were stained with V064 antiserum, which recognizes multiple AKAP18 isoforms, including AKAP18a (Fig. 7). Although these data strongly support our AKAP18a/GFP studies (Figs. 6 and 8), additional immunohistochemical analyses of intact human tissues will help further characterize the subcellular localization of AKAP18. Nonetheless, our data suggest that AKAP18a may localize PKA to sites of cell–cell contact, where PKA is known to play a role (together with other protein kinases) in regulation of functional stability (Citi, 1992; Nilsson et al., 1996; Collores-Buzato et al., 1998; K ovbasnjuk et al., 1998). In contrast, AKAP18b is predicted to serve a different function in polarized cells. Many ion channels and transporters at the apical cell surface are regulated by PKA-mediated phosphorylation, and recent data implicate AKAPs in several events restricted to the apical cell surface. For example, AKAPs facilitate vasopressin-induced translocation of aquaporin-2 water channels in kidney (Klussmann et al., 1999) and modulation of the cystic fibrosis transmembrane conductance regulator CI- channels in airway epithelia (Huang et al., 1999). In MDCK cells, endogenous AKAP18 is distributed at the apical cell surface, although some staining was also observed on intracellular vesicles (Fig. 7). Thus, AKAP18b may be required for PKA-mediated regulation of apical ion or water transport, and may also be involved in vesicular trafficking to this membrane. It will be important to compare the distributions of AKAP18b and -b in different epithelial tissues and in other tissues containing specialized plasma membrane domains, including neurons and skeletal or cardiac muscle. In addition, it will be important to determine whether a specific AKAP18 isoform targets L-type Ca2+ channels. The identification of proteins that associate specifically with AKAP18b, and the generation of reagents to selectively disrupt a single isoform, will hopefully elucidate the function of each AKAP18 isoform.
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