

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^{2+} channel, was cloned. We now report the cloning of two additional isoforms of AKAP18, which we have designated AKAP18 β and AKAP18 γ , 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 α) and AKAP18 β target the plasma membrane when ex-

pressed in HEK-293 cells, while AKAP18 γ is cytosolic. When expressed in epithelial cells, AKAP18 α is targeted to lateral membranes, whereas AKAP18 β is accumulated at the apical membrane. A 23-amino acid insert, following the plasma membrane targeting domain, facilitates the association of AKAP18 β 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

HORMONALLY 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)¹. 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'Acqua 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 NH₂ 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 some AKAPs can also bind RI subunits (Huang et al., 1997b; Angelo and Rubin, 1998), although the binding affinity is lower than for RII (Burton et al., 1997) and the physiological significance of the AKAP-RI interaction is yet to be established. In addition, each AKAP contains a unique targeting motif that directs the AKAP-mediated regula-

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1. *Abbreviations used in this paper:* AKAP, A-kinase anchoring protein; C, catalytic; nt, nucleotides; PKA, cAMP-dependent protein kinase; R, regulatory; RII, type II R subunit; RT, reverse transcriptase.

tory complex to the appropriate subcellular compartment (Glantz et al., 1993; Lester et al., 1996; Chen et al., 1997; Huang et al., 1999). Immunohistochemical and biochemical analyses have identified AKAPs that associate with cytoskeletal elements, mitochondria, ER, the Golgi complex, peroxisomes, and the centrosome (McCartney et al., 1995; Lester et al., 1996; Chen et al., 1997; Dransfield et al., 1997; Dong et al., 1998; Schmidt et al., 1999; Witczak et al., 1999).

AKAPs are implicated in the regulation of many plasma membrane-associated events, including modulation of ion channels and regulation of hormone secretion (Rosenmund et al., 1994; Gao et al., 1997; Lester et al., 1997; Ali et al., 1998; Fraser et al., 1998; Gray et al., 1998; Tibbs et al., 1998; Klusmann et al., 1999). Some AKAPs target the cell surface by association with submembranous cytoskeletal elements, and others by lipid modification or direct association with membrane phospholipids (Glantz et al., 1993; Dransfield et al., 1997; Dell'Acqua et al., 1998; Dong et al., 1998; Fraser et al., 1998; Gray et al., 1998).

AKAP18 (also named AKAP15) is a low molecular weight AKAP that associates with the cell membrane via NH₂-terminal lipid modifications (Fraser et al., 1998; Gray et al., 1998). In rat skeletal muscle, AKAP18 is concentrated in transverse tubules and overlaps the distribution of the L-type Ca²⁺ channel (Gray et al., 1997, 1998). Coexpression of AKAP18 and cardiac L-type Ca²⁺ channels in fibroblasts resulted in a significant increase in cAMP-responsive Ca²⁺ currents (Fraser et al., 1998), and the two proteins can be copurified after expression in fibroblasts (Gray et al., 1998). Together, these data indicate that AKAP18 may serve to functionally couple PKA and ion channels at the cell surface. We cloned two novel AKAP18-related cDNAs (named AKAP18 β and AKAP18 γ) using expression library screening and PCR-based approaches. Therefore, we compared the expression of these AKAP18-related mRNAs and proteins in cells and examined their subcellular distributions in HEK-293 and epithelial cell lines. Our data suggest that AKAP18-related proteins arise secondary to alternative mRNA splicing of a single gene, to generate a family of proteins that are differentially targeted and contain a common RII binding site.

Materials and Methods

Cloning of AKAP18 β and AKAP18 γ

The pET11.RII α plasmid was transformed into BL21(DE3) pLysS *Escherichia 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 RII α purified on cAMP agarose as described (Scott et al., 1990). The purified RII α protein was dialyzed into 50 mM sodium bicarbonate, pH 8.5, and concentrated by centrifugation in a Biomax-10K centrifugal filter (Millipore Inc.). Purified RII α (10 μ M) was biotinylated by addition of 100 μ M 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-RII α was used as a probe to screen a λ TriplEx human lung cDNA library (CLONTECH). Biotin-RII α (10 nM) was prebound to 0.5 μ g/ml streptavidin-alkaline phosphatase (SA-AP) in 50 ml TTBS (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-RII α /SA-AP complex. After extensive washing in TTBS, bound RII α /SA-AP complexes were visualized as described (Sparks et al., 1996). Plasmids

were rescued from λ TriplEx phage by in vivo excision, and inserts sequenced at the University of North Carolina Sequencing Facility.

An \sim 2-kb cDNA clone encoding a novel AKAP was isolated from the library screen; after sequencing, this clone was designated AKAP18 γ . To obtain upstream coding sequence, 5' RACE was performed using Marathon-Ready human pancreas cDNA (CLONTECH); RACE products were cloned into pT-Adv (CLONTECH) and sequenced. AKAP18 β 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 AKAP18 sequence. PCR products were excised from the gel and subcloned into pT-Adv (CLONTECH).

For RT-PCR analyses, total RNA from cultured cells was extracted using RNA STAT60 (Tel-test "B" Inc.) and treated with DNase (Promega). First strand cDNA was synthesized using Superscript II reverse transcriptase (GIBCO BRL) primed with random hexamers. PCR was performed using Taq DNA polymerase (GIBCO BRL) and AKAP18 α specific primers. PCR products were purified, ligated into pT-Adv 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 AKAP18 γ (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). After incubation, the blot was washed at room temperature for 30 min in 2 \times SSC + 0.05% SDS, followed by 0.1 \times SSC + 0.1% SDS for 40 min at 50°C. Blots were stripped and reprobed with 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, Hind III, or XbaI, was electrophoresed on 1% agarose gels. Gels were soaked in 0.5 M NaOH/1.5 M NaCl for 20 min at room temperature and washed in neutralization solution. The DNA was transferred to GeneScreen (New England Nuclear Life Sciences) by capillary diffusion in 20 \times SSC overnight at room temperature. Hybridizations were carried out at 42°C in ExpressHyb (CLONTECH) using a ³²P-labeled cDNA probe common to all known AKAP18 isoforms (nt 106–243 of AKAP18 α), and membranes were washed as described above.

A genomic DNA library was created for the mouse strain ELM³ in the Lambda FIX II vector (Stratagene). The library was screened with an α [³²P]dCTP random-primed probe representing full-length AKAP18 α (nt 205–450) or the sequence common to the three identified AKAP18 isoforms (nt 256–450).

Generation of AKAP18 Plasmids

The coding sequences of AKAP18 α , AKAP18 β , and AKAP18 γ 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 AKAP18 isoform. The antisense primer overlapped the COOH terminus and stop codon, and incorporated a BamHI site. The PCR fragments were digested with EcoRI and BamHI, and subcloned into pcDNA3.1(–) (Invitrogen) digested with the same enzymes.

The cDNA encoding each AKAP18 isoform was also fused in-frame at the 3' end with the cDNA encoding GFP. The coding regions of AKAP18 α , AKAP18 β , and AKAP18 γ were amplified by PCR using the sense primers described above and a single antisense primer designed to remove the stop codon and incorporate a BamHI site at the 3' end. Similarly, the 1-16 α , 1-44 β , and 17-44 β constructs were generated by PCR using AKAP18 β DNA as 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 MDCK type II were obtained from the American Type Culture Collection. The NIT-1 cell line, derived from mouse pancreatic β cells, was provided by Dr. Lloyd Fricker (Albert 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). CHO, HEK-293, or MDCK cells were grown to 30–50% confluency and transfected with the appropriate plasmid in Effec-

tene reagent (Qiagen) for 15 h in complete media. Stable cell lines were selected for 3 wk in media containing 0.5 mg/ml G418, and cloned by serial dilution.

Western Blot and Immunoprecipitation

Rabbit antisera directed against AKAP18 α was generated in rabbits using recombinant AKAP18 α and affinity-purified as described (Fraser et al., 1998); these antisera also recognize AKAP18 β and AKAP18 γ . Transfected cells were washed once with PBS and lysed in ice-cold buffer (20 mM Hepes, pH 7.4, 20 mM NaCl, 5 mM EDTA, 2 μ g/ml leupeptin, 1.6 μ g/ml benzamidine, 0.3 μ g/ml PMSF), 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-PAGE 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-AKAP18 (VO57; 1 μ g/ml), or mouse anti-C subunit (1 μ g/ml; Transduction Laboratories). Immunoprecipitations were carried out overnight at 4°C using 500 μ g of protein (whole cell lysate or soluble and particulate fractions) and 1 μ g affinity-purified AKAP18 antiserum (Fraser et al., 1998). Immune complexes were collected on protein A agarose and electrophoresed on 12.5 or 15% SDS-PAGE gels. RII overlays were performed as described previously using ³²P-labeled mouse RII α (Fraser et al., 1998).

Confocal Microscopy

Rabbit antisera that specifically recognize AKAP18 β (NC 257) were generated using residues 15–43 of AKAP18 β coupled with keyhole limpet cyanin as immunogen. Complement proteins were removed from the whole serum by incubation with DEAE-Blue dextran (Pierce Chemical Co.). Transfected cells were grown on glass coverslips (HEK-293 and MDCK cells) or Transwell filters (MDCK cells). MDCK cells were grown until confluent monolayers were observed and transepithelial resistances were >1,000 ohm-cm². Cells were washed once with PBS and then fixed for 20 min in fresh 4.0% paraformaldehyde prepared in PBS. For immunocytochemistry, 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. After 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 AKAP18 Isoforms

We screened a lung cDNA expression library using biotinylated RII as probe and identified a novel cDNA that shares sequence homology with AKAP18, a previously identified membrane-associated AKAP (Fraser et al., 1998; Gray et al., 1998). The cDNA was identical to AKAP18 at the 3' end, but shared little homology with AKAP18 at the 5' end (data not shown). Therefore, we designated the cDNA and protein product AKAP18 γ , to indicate its relationship to other AKAP18 family members, including the previously reported AKAP18 (renamed AKAP18 α) and AKAP18 β (discussed below). The original AKAP18 γ cDNA contained a single open reading frame, but no consensus ribosome binding site or initiator methionine. Therefore, we used rapid amplification of cDNA ends (RACE) with human pancreas cDNA as template to obtain the full-length AKAP18 γ sequence. The longest AKAP18 γ cDNA isolated was 2,917 nt in length with a single open reading frame from nt 107 to

1086 (these sequence data have been submitted to the GenBank/EMBL/DDBJ databases under the accession number AF152929). The upstream cDNA 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 AKAP18 γ cDNA encodes a protein of 326 amino acids with a calculated molecular mass of 37 kD and a pI of 5.8. The first 262 amino acids are unique and do not share significant homology with any known proteins in GenBank/EMBL/DDBJ databases. However, the last 64 amino acids are identical to human AKAP18 α and include a conserved RII binding site (Fig. 1 A). There are no consensus myristoylation or palmitoylation sites at the NH₂ terminus of AKAP18 γ , suggesting that the AKAP18 γ protein is not modified by lipid side chains. We used PSORT (Nakai and Kanehisa, 1992) to predict potential subcellular targeting sequences, and found that amino acids 37 to 54 of AKAP18 γ fit the specifications for a consensus nuclear localization signal (Gerace, 1992; Gorlich, 1997; Nigg, 1997).

In previous studies, mRNAs of ~2.4-, 3.6-, and 4.3-kb were observed in rat and human tissues using the AKAP18 α coding region as a probe (Fraser et al., 1998; Gray et al., 1998). We used Northern blot analysis to determine whether any of these mRNAs represented the AKAP18 γ mRNA and to determine the tissue distribution of the message (Fig. 1 B). Using a radiolabeled probe directed against the unique region of AKAP18 γ , 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 AKAP18 α and AKAP18 γ , we used unique sense primers paired with a common antisense primer in RT-PCR reactions. Using a sense primer specific for the AKAP18 γ cDNA, we obtained a 979-nt product whose sequence exactly matched that obtained from the cDNA library screen and 5' RACE reactions (Fig. 2 A). Surprisingly, primers designed to specifically amplify AKAP18 α consistently amplified two bands of 246 and 315 nt (Fig. 2 A). The 246-nt fragment was the expected size of the AKAP18 α product, which was confirmed by DNA sequencing. The sequence of the 315-nt fragment matched AKAP18 α at the 5' and 3' ends, but contained a 69-bp insert (these sequence data have been submitted to the GenBank/EMBL/DDBJ databases under the accession number AF161075); we named this cDNA AKAP18 β . We used RT-PCR to determine whether AKAP18 α and - β are differentially expressed in cell lines and tissues. Both cDNAs were detected in fibroblast, endocrine, and epithelial cell lines, indicating that the two mRNAs are broadly expressed (Fig. 2 B). Although we were unable to reliably amplify the AKAP18 β cDNA from human brain, a weak signal was observed in some reactions (data not shown).

The first 16 amino acids of human AKAP18 β are identical to AKAP18 α and are followed by an insert of 23 unique amino acids (Fig. 2 C). Distal to this 23-amino acid insert, AKAP18 α and - β are identical to each other (Fig. 2, C and D). Thus, we have identified three AKAP18 isoforms (named α , β , and γ) which share a common RII

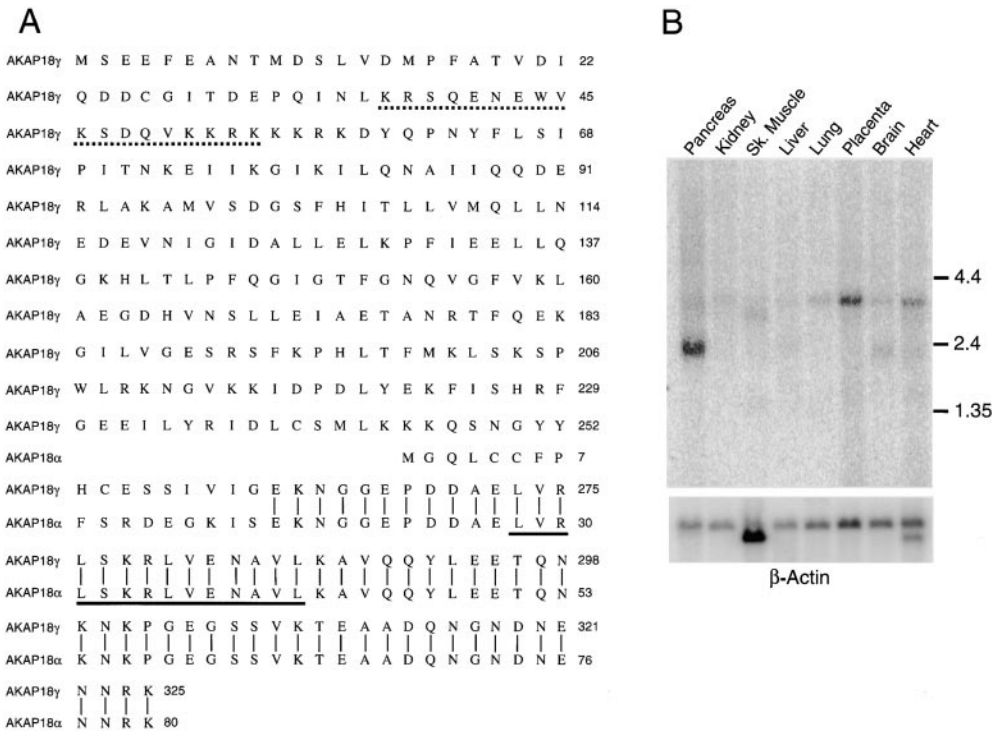


Figure 1. Cloning of human AKAP18 γ . A, Comparison of human AKAP18 γ and AKAP18 α amino acid sequences. Common residues are indicated by a vertical line, a dashed line marks the putative nuclear localization signal, and the RII binding site is underlined. B, A human multiple tissue Northern blot was hybridized with a random primed 32 P-labeled probe generated against the unique region of AKAP18 γ (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.

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. Ge-

nomeric 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 AKAP18 α , - β , and - γ mRNAs arise as alternate products

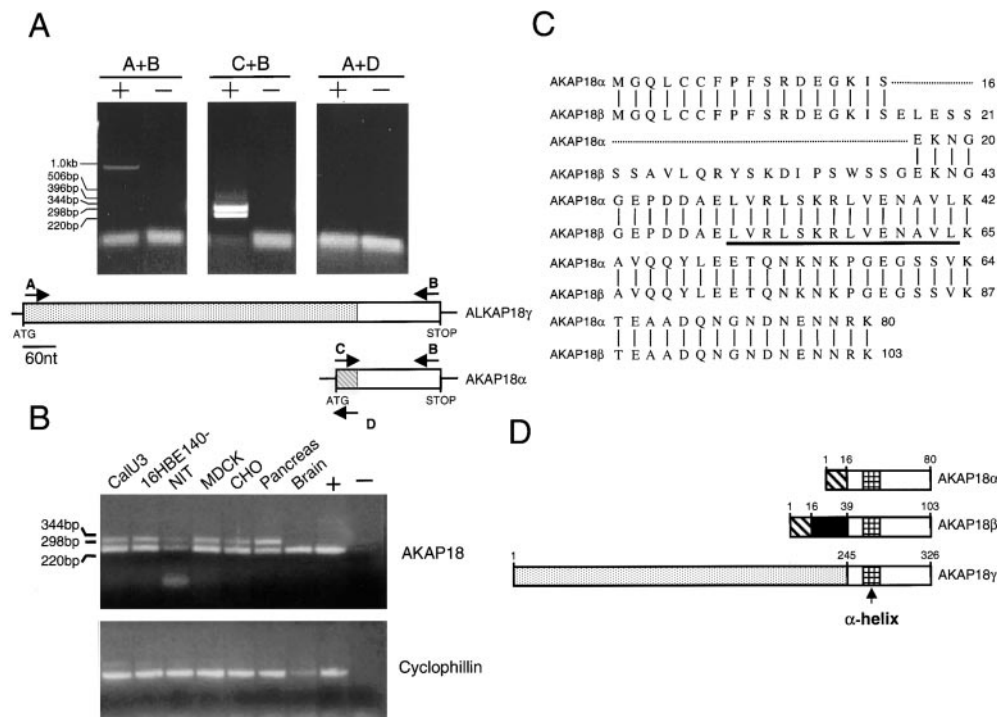


Figure 2. Cloning of AKAP18 β . A, Primers used to amplify human pancreas cDNA are shown schematically; PCR was performed using human pancreas cDNA and the primer pairs indicated. + indicates addition of 1 ng cDNA and - indicates no cDNA. Samples were electrophoresed on 1% agarose gels and visualized with ethidium bromide. mRNA size markers are shown in kb. No PCR product was observed using primers A + D, indicating that cDNAs containing both AKAP18 γ - 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 AKAP18 α -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 AKAP18 α or cyclophilin plasmid control and - indicates no addition of template. Samples were electrophoresed on 1.0% agarose gels and visualized with ethidium bromide. DNA size markers are shown in kb. Data are representative of two separate experiments. C, Comparison of AKAP18 α and AKAP18 β 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.

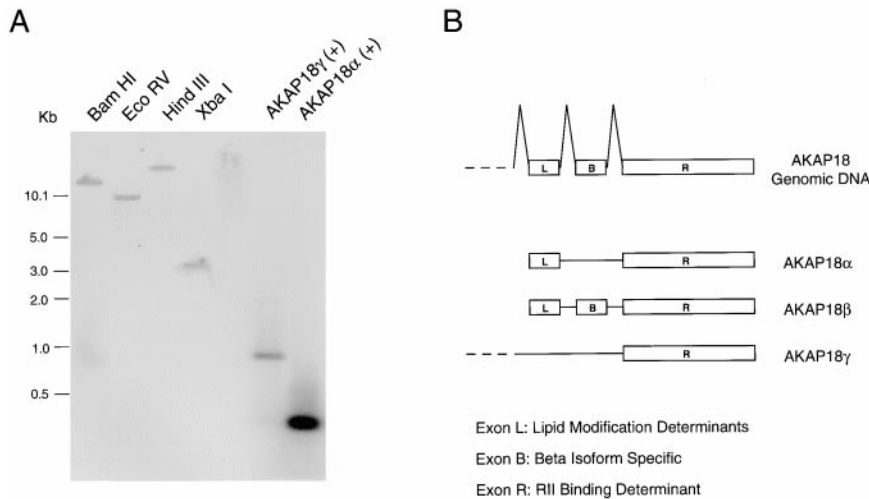


Figure 3. Alternative splicing gives rise to three distinct AKAP18 mRNAs. A, Human genomic DNA digested with the enzymes indicated, or positive control cDNAs were hybridized with a radiolabeled probe overlapping the common region of all three AKAP18 isoforms. DNA size standards are shown in kb. B, Schematic 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.

of one gene (Fig. 3 A). This is consistent with the fact that the 3' untranslated regions of AKAP18 α , - β , and - γ are identical (data not shown).

Preliminary analysis of mouse AKAP18 genomic sequence (Fig. 3 B) indicates that residues 1–16 of AKAP18 α (also contained in AKAP18 β) are encoded by a single exon; this exon contains the determinants for lipid modification. Another short exon encodes the 23-amino acid residues specific to AKAP18 β . 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

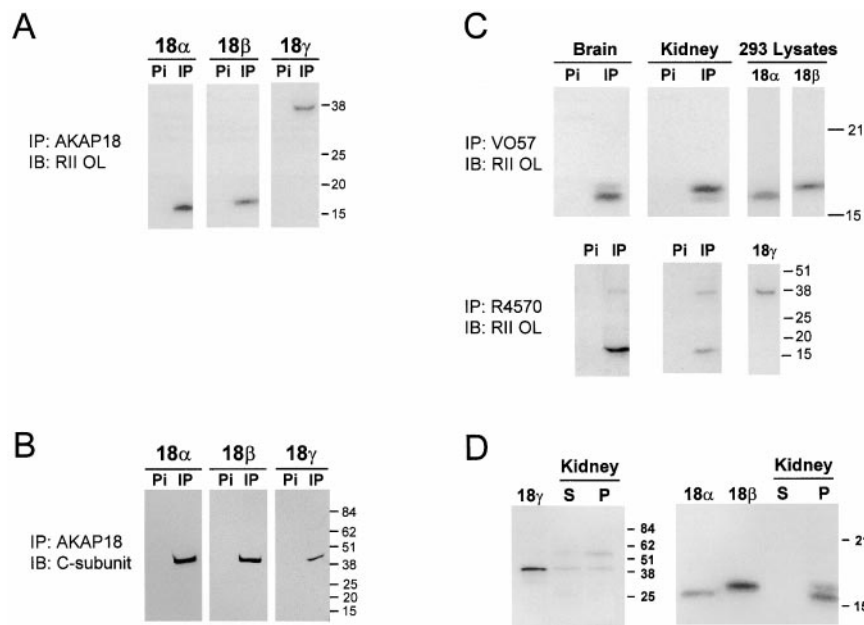


Figure 4. Analysis of AKAP18-related proteins. A, Lysates prepared from HEK-293 cells transiently expressing individual AKAP18 isoforms were immunoprecipitated with antisera directed against AKAP18. Samples were separated by SDS-PAGE and proteins visualized by RII overlay. B, Lysates prepared from HEK-293 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 bonafide 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 AKAP18 β 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 AKAP18 β protein in brain, two bands immunoprecipitated from both brain and kidney with VO57 antiserum corresponding to AKAP18 α and β . Both of these proteins were preferentially accumulated in the particulate fraction of rat kidney (Fig. 4 D). Bands corresponding to AKAP18 α and γ were immunoprecipitated from both tissues using R4570 antisera (Fig. 4 C). We also examined the distribution of AKAP18 γ 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 AKAP18 α is dependent upon lipid modification through myristylation of Gly¹ and palmitoylation of Cys⁴ and Cys⁵ (Fraser et al., 1998). Accordingly, the first ten amino acids of AKAP18 α 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 cDNAs encoding each construct into HEK-293 cells and compared the intracellular distribution of the proteins by differential fractionation and immunofluorescent microscopy. AKAP18 α 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 AKAP18 α and β with the particulate fraction of rat kidney (Fig. 4 D). In contrast, a significant fraction of the overexpressed AKAP18 γ partitioned with the soluble fraction, although ~20% was found in the particulate fraction (Fig. 5 A). The expressed AKAP18 γ 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 AKAP18 γ in the soluble and particulate fractions of rat kidney suggests that overexpression of AKAP18 γ in HEK-293 cells saturates a protein-protein interaction required to maintain a particulate pool of this isoform.

Localization of AKAP18 α and AKAP18 β in Epithelial Cells

Although both AKAP18 α 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

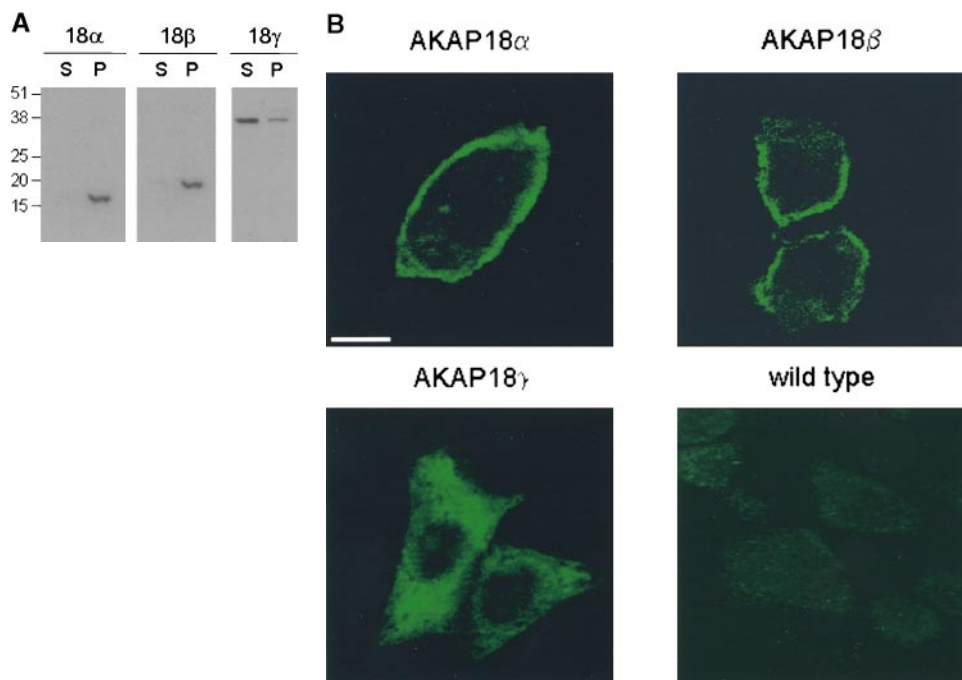


Figure 5. Targeting of AKAP18 isoforms in HEK-293 cells. A, HEK-293 cells were transiently transfected with cDNAs encoding each AKAP18 isoform. Cells were separated into soluble (S) and particulate (P) fractions and 25 μ g of each sample was resolved by SDS-PAGE. The distribution of AKAP18 isoforms was determined by immunoblot analysis using rabbit antisera directed against AKAP18 α . Protein size standards are shown in kD. The data are representative of three similar experiments. B, HEK-293 cells were transiently transfected with AKAP18 cDNAs and plated on glass coverslips. Eight hours after transfection, wild-type and transfected cells were stained with affinity-purified rabbit anti-AKAP18 IgG (VO57; 1 μ g/ml) and FITC-conjugated secondary antibody. The cells imaged are representative of at least four independent transfection studies with each plasmid. Bar, 10 μ m.

types (Shaul and Anderson, 1998; Fanning and Anderson, 1999; Ostrom and Insel, 1999). Therefore, we considered whether the unique 23-amino acid insert present in AKAP18 β directs this isoform to specific targets or microdomains of the plasma membrane. MDCK 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 stably expressed cDNAs encoding GFP-tagged AKAP18 α and - β in MDCK cells to compare their distributions in polarized cells. Previous experiments established that the fusion of GFP to the COOH terminus of AKAP18 α does not disrupt membrane targeting of the chimeric protein (Fraser et al., 1998).

Confocal microscopy performed on well-polarized MDCK cell cultures indicated that the distributions of GFP-tagged AKAP18 α and - β differed. AKAP18 α /GFP was accumulated predominantly along the lateral margins of the trans-

ected cells. In contrast, AKAP18 β /GFP was present at the apical membrane (Fig. 6 A), and overlapped the distribution of the apical membrane glycoprotein gp135 (Ojakian and Schwimmer, 1988; Fig. 6 B). The AKAP18 α /GFP and AKAP18 β /GFP proteins were expressed at relatively 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 AKAP18 α significantly overlapped the distribution of β -catenin, a protein that accumulates at the adherens junctions (Nathke et al., 1994; Fig. 6 B). When the distribution of AKAP18 α and - β was compared with the distribution of ZO-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 AKAP18 α /GFP and

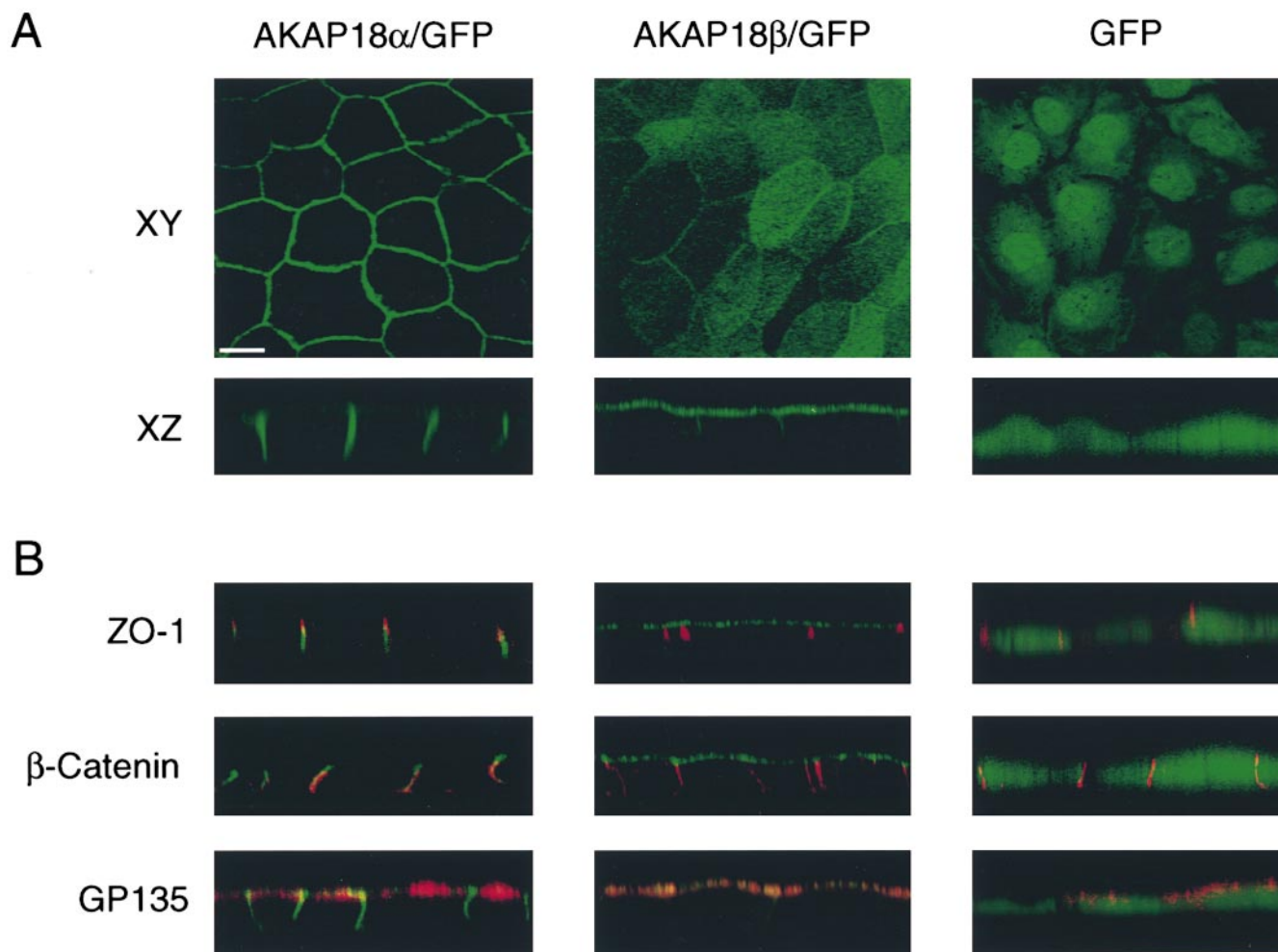


Figure 6. AKAP18 α and - β are differentially targeted in MDCK cells. **A**, MDCK cells stably expressing AKAP18 α /GFP, AKAP18 β /GFP, or GFP alone were grown on Transwell filters. Confluent monolayers were fixed in 4% paraformaldehyde and analyzed by confocal microscopy in XY and XZ planes. At least three individual cell lines expressing each construct were analyzed and similar results were obtained. Bar, 10 μ m. **B**, The distributions of AKAP18 α /GFP and AKAP18 β /GFP were compared to the distribution of markers for tight junctions (ZO-1), adherens junctions (β -catenin), and apical membranes (gp135). Stably transfected cells were fixed and stained with rat anti ZO-1 (1:400), rabbit anti- β -catenin (1:400), or mouse anti-gp135 (1:50), followed by the appropriate Texas red-conjugated secondary antibody. Images are shown as XZ scans and are representative of images collected in two independent experiments.

AKAP18 β /GFP, respectively, were observed in several independent clonal cell lines and in transient transfection assays (data not shown). AKAP18 β 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 AKAP18 β /GFP with apical membranes.

The differential targeting of AKAP18 α 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

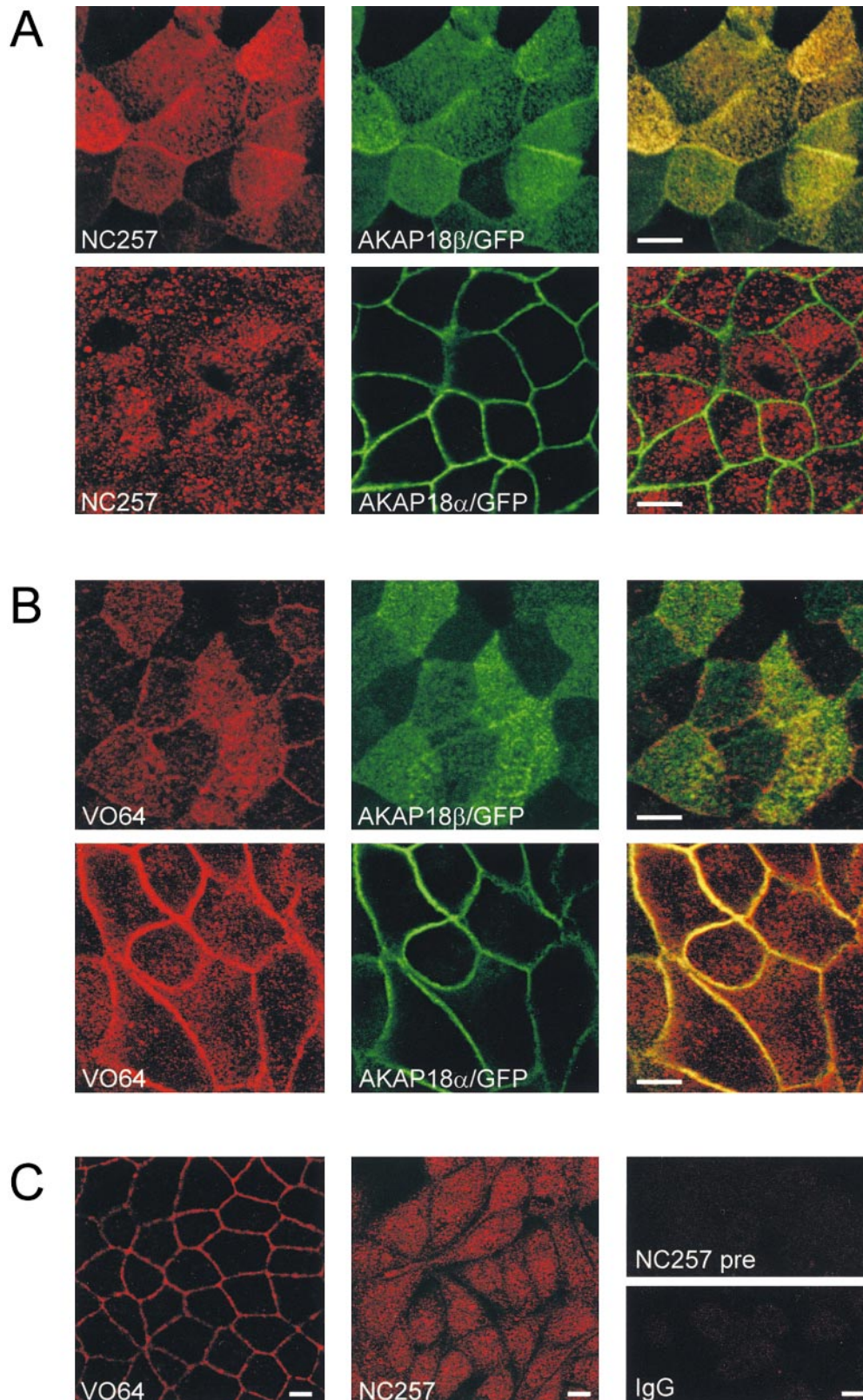


Figure 7. Localization of endogenous AKAP18 in MDCK cells. **A**, MDCK cells stably expressing AKAP18 β /GFP or AKAP18 α /GFP were grown on Transwell filters and confluent monolayers were fixed in 4% paraformaldehyde. Cells were permeabilized, blocked, and stained with NC257 (1:1,000 dilution) followed 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 AKAP18 β /GFP or AKAP18 α /GFP were grown on Transwell filters and confluent monolayers were fixed and stained with VO64 (1 μ g/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 μ m.

of AKAP18 α and - β in MDCK cells, we generated an antibody directed against residues 15–43 of AKAP18 β (NC 257); this antibody specifically recognizes the overexpressed AKAP18 β /GFP stably expressed in MDCK cells (Fig. 7 A). Furthermore, the antibody does not detect AKAP18 α /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, antiserum VO64 generated against recombinant AKAP18 α , which detects multiple AKAP18 isoforms on Western blots (data not shown), recognized both AKAP18 β /GFP and AKAP18 α /GFP in stably transfected MDCK cells (Fig. 7 B). Having established that NC257 selectively recognized AKAP18 β while VO64 recognized both AKAP18 isoforms, we stained wild-type MDCK cells with each antiserum 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 AKAP18 α /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 AKAP18 β) observed in AKAP18 α /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 AKAP18 α 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 AKAP18 α and - β correlated with the selective accumulation of AKAP18 β 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 AKAP18 α and - β . Since both proteins were easily extracted in buffers containing 0.2% Triton X-100 (data not shown), we conclude that AKAP18 β is not associated with detergent insoluble complexes at the apical surface of MDCK cells.

The Unique Sequence of AKAP18 β Contains Apical Targeting Information

Our expression studies show that AKAP18 α 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 AKAP18 β -specific sequence, we generated three GFP fusion proteins corresponding to exons in the AKAP18 gene: 1-16 α β /GFP, which encompasses the common membrane targeting domain; 17-44 β /GFP, which encompasses the AKAP18 β specific sequence; and 1-44 β /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 1-16 α β /

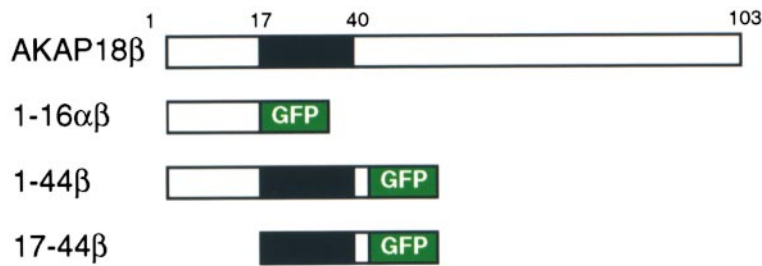
GFP and 1-44 β /GFP were detected at the cell surface, whereas the 17-44 β /GFP protein was uniformly distributed throughout the cell (Fig. 8 B). Similarly, when the 17-44 β /GFP chimera was stably expressed in MDCK cells, the protein was distributed throughout the cytoplasm and nucleus (Fig. 8 C). These results indicate that the 23-amino acid insert unique to AKAP18 β is not sufficient to mediate membrane targeting. However, the localization of the 1-16 α β /GFP and 1-44 β /GFP proteins clearly differed when stably expressed in MDCK cells. The 1-16 α β /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 1-44 β /GFP protein was also targeted to the plasma membrane in MDCK cells. However, a significant fraction of the 1-44 β /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 acid insert unique to AKAP18 β facilitates targeting of AKAP18 β 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 AKAP18 α , and the newly described cDNAs AKAP18 β 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 AKAP18 α and - β , the 23-amino acid insert found in AKAP18 β , and the RII binding site in all three AKAP18 isoforms (Fig. 3 B).

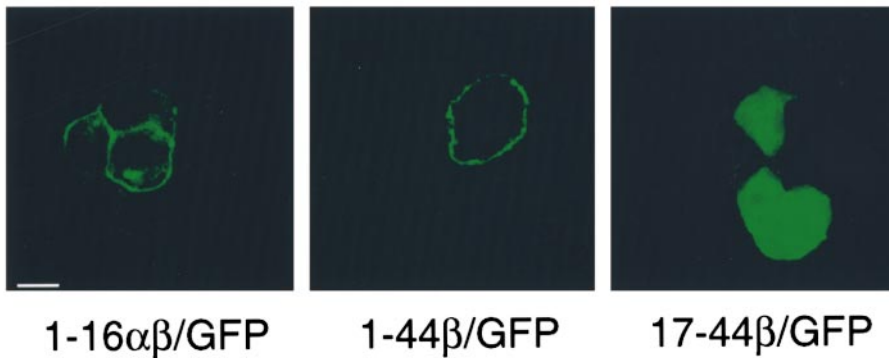
Several other AKAPs are known to exist in multiple forms (Lin et al., 1995; Dong et al., 1998; Schmidt et al., 1999), and the generation of AKAP diversity by differential mRNA splicing may be a common occurrence. In theory, differential splicing of AKAP genes may result in the expression of proteins 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 (Klauck et al., 1996; Schillace and Scott, 1999a), or contain additional putative interaction domains (Dong et al., 1998; Schmidt et al., 1999). 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). Alternative splicing generates S-AKAP84, AKAP121, and D-AKAP1-related proteins (Lin et al., 1995; Chen et al., 1997; Huang et al., 1997a, 1999). Each of these proteins share a common RII binding site; however, while S-AKAP84 and AKAP121 are targeted to the outer mitochondrial membrane, D-AKAP1 splice vari-

A



B

HEK293



C

MDCK

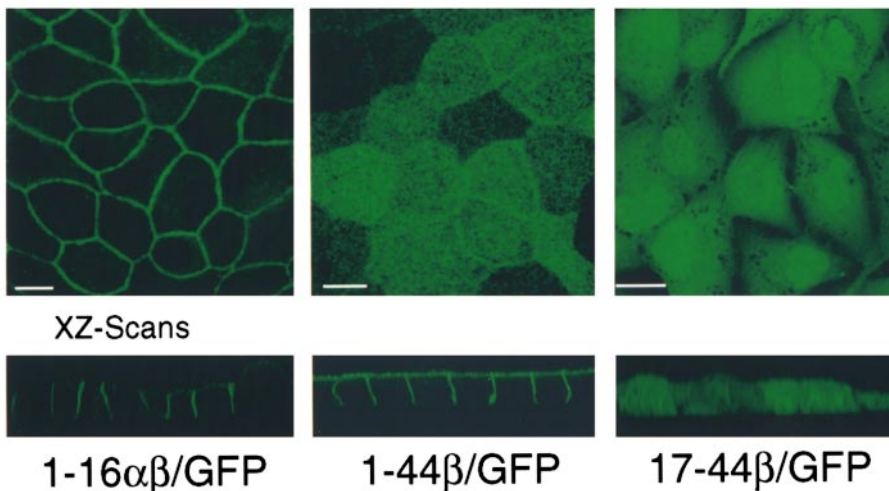


Figure 8. The unique 23 amino acids in AKAP18 β facilitate apical targeting. A, Schematic diagram of the three GFP fusion proteins expressed in HEK-293 and MDCK cells. GFP is not drawn to scale. The unique region of AKAP18 β is filled in black. B, Each construct was expressed transiently in HEK-293 cells and the distributions of the expressed GFP-tagged proteins analyzed by confocal microscopy. Bar, 10 μ m. 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 μ m.

ants may target mitochondria or the ER (Lin et al., 1995; Chen et al., 1997; Huang et al., 1997a, 1999). However, not all splicing generates AKAP proteins with the same RII binding domain. Splicing of a single gene on chromosome 7q21 generates three AKAP350-related proteins (with the

same RII binding domain) and yotiao, which contains a different RII binding site (Lin et al., 1998; Schmidt et al., 1999; Witczak et al., 1999). Furthermore, AKAP350 targets PKA to the centrosome (Schmidt et al., 1999; Witczak et al., 1999), whereas yotiao is localized at neuronal syn-

apses (Lin et al., 1998). Our data clearly demonstrate that alternative splicing of the AKAP18 gene generates proteins that may target distinct subcellular compartments, but contain a common RII binding determinant (Fig. 2 D). Furthermore, our data indicate that alternative splicing may dictate the targeting of AKAP18 isoforms to microdomains 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 AKAP18 α and - β 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 AKAP18 γ would be found in the nucleus (Fig. 1 A), we observed that AKAP18 γ was distributed in cytoplasm of transiently transfected fibroblasts (Fig. 5 B) and stably transfected MDCK cells (data not shown). Approximately 50% of the native protein in rat kidney and 20% of the exogenously expressed AKAP18 γ 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 AKAP18 γ 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 AKAP18 γ functions to bind PKA in the cytoplasm of cells. There is some precedence for cytosolic AKAPs, since treatment of ovarian granulosa cells with follicle 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 γ -specific antisera for immunohistochemical studies and the identification of proteins that associate with the unique region of AKAP18 γ will hopefully resolve these questions.

AKAP18 α and - β are well situated to modulate cAMP-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). Acting alone, the 23-amino acid insert unique to AKAP18 β 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, AKAP18 α is restricted to the lateral surfaces of polarized MDCK cells, whereas AKAP18 β 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 AKAP18 β , the protein showed no difference in its solubility compared with AKAP18 α . Therefore, we speculate that the selective targeting of AKAP18 β to the apical membrane is due to specific protein–protein interactions involving residues 17–39.

In epithelial cells, AKAP18 α is restricted to the lateral cell membranes overlapping the distribution of β -catenin (Fig. 6 B). Endogenous AKAP18 was also observed along

the lateral membranes of cells, but only when cells were stained with VO64 antiserum, which recognizes multiple AKAP18 isoforms, including AKAP18 α (Fig. 7). Although these data strongly support our AKAP18 α /GFP studies (Figs. 6 and 8), additional immunohistochemical analyses of intact human tissues will help further characterize the subcellular localization of AKAP18 α . Nonetheless, our data suggest that AKAP18 α 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 junctional stability (Citi, 1992; Nilsson et al., 1996; Colares-Buzato et al., 1998; Kovbasnjuk et al., 1998). In contrast, AKAP18 β 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 Cl⁻ channels in airway epithelia (Huang et al., 1999). In MDCK cells, endogenous AKAP18 β is distributed at the apical cell surface, although some staining was also observed on intracellular vesicles (Fig. 7). Thus, AKAP18 β 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 AKAP18 α and - β 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 AKAP18 β , and the generation of reagents to selectively disrupt a single isoform, will hopefully elucidate the function of each AKAP18 isoform.

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