Molecular Characterization of a cDNA That Encodes Six Isoforms of a Novel Murine A Kinase Anchor Protein*

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We have cloned cDNA that encodes six novel A kinase anchor proteins (collectively named AKAP-KL). AKAP-KL diversity is generated by alternative mRNA splicing and utilization of two translation initiation codons. AKAP-KL polypeptides are evident in lung, kidney, and cerebellum, but are absent from many tissues. Different isoforms predominate in different tissues. Thus, AKAP-KL expression is differentially regulated in vivo. All AKAP-KL isoforms contain a 20-residue domain that avidly binds (k_d ~ 10 nM) regulatory subunits (RII) of protein kinase AII and is highly homologous with the RII tethering site in neuronal AKAP75. The distribution of AKAP-KL is strikingly asymmetric (polarized) in situ. Anchor protein accumulates near the inner, apical surface of highly polarized epithelium in tubules of nephrons. Both RII and AKAP-KL are enriched at an intracellular site that lies just below the plasma membrane of alveolar epithelial cells in lung. AKAP-KL interacts with and modulates the structure of the actin cytoskeleton in transfected cells. We also demonstrate that the tethering domain of AKAP-KL avidly ligates RII subunits in intact cells. AKAP-KL may be involved in (a) establishing polarity in signaling systems and (b) physically and functionally integrating PKAII isoforms with downstream effectors to capture, amplify, and precisely focus diffuse, trans-cellular signals carried by cAMP.

Protein kinase A (PKA) mediates actions of hormones and neurotransmitters that activate adenylyl cyclase (1–4). Signals carried by cAMP are often directed to effectors that accumulate at discrete intracellular sites (5–7). Targeting of signals to these sites can be achieved by generating a non-uniform distribution of PKA molecules. This occurs when PKAIIs and IIβ isoforms are attached to cytoskeleton or organelles by a kinase anchor proteins (AKAPs) (5, 6). Prototypic anchor proteins (AKAPs 75, 79, and 150) have a binding site for regulatory (RII) subunits of PKAII isoforms and distinct domains that mediate non-covalent coupling of AKAP-PKAII complexes to the microtubule-based dendritic cytoskeleton of neurons and the cortical actin cytoskeleton of non-neuronal cells (5–13). Both cytoskeletal locations are closely apposed to the plasma membrane. Thus, anchored PKAII is positioned near a signal generator (adenylate cyclase) and multiple PKA substrate/effector proteins (e.g. myosin light chain kinase, microtubule-associated protein-2, ion channels, serpentine receptors that couple with G_)

Anchored PKAII isoforms may be essential for dissemination of cAMP signals in highly polarized epithelium. Epithelial cells of lung and kidney provide examples of maximally polarized signal transduction, in which hormone-activated adenylyl cyclase and downstream target/effector can be located at opposite ends of the cell. Tight junctions between adjacent cells block communication by diffusible molecules and create two functionally distinct regions of plasma membrane. In epithelial layers of nephrons the basolateral surface is accessible to hormones in plasma and contain receptors and adenylyl cyclase; the apical surface is enriched in channel and transporter proteins that mediate absorption/elimination of ions, nutrients, metabolites, and water (17, 18). Activities of many channels and transporters that traverse the apical membrane are regulated by hormones that stimulate adenylyl cyclase, thereby promoting PKA activation (17–21). Several considerations suggest that C subunits derived from cytoplasmic PKAs may not mediate trans-epithelial signaling. Signals (cAMP) generated by modest, physiological levels of hormone at the basolateral surface can become weak, diffuse, and insufficient to activate target PKA molecules dispersed (at a relatively low concentration) in cytoplasm. This is due to the transient and intermittent nature of both hormone-release and hormone-mediated stimulation of adenylyl cyclase in situ, rapid desensitization of receptor-G protein interactions, dilution of cAMP into the large volume of the cytoplasm, and degradation mediated by cAMP phosphodiesterases. Moreover, studies on knock-out mice and cultured neurons indicate that the ability of PKA isoforms to respond to small changes in cAMP content is often crucial for regulation in vivo (14, 22, 23). Another constraint is that channels/transporters cluster into patches that constitute only a small portion of the apical membrane surface (24, 25). A mechanism that confers increased sensitivity in a specific microenvironment involves concentrating and colocalizing PKAII isoforms with substrate effectors via AKAPs. AKAP-PKAII complexes could serve as sensor/transducers that capture highly diffuse and relatively small trans-cellular signals (cAMP).
PhosphorImager analysis (Molecular Dynamics) (35). densitometry (Pharmacia-LKB Ultrascan XL laser densitometer) or visualized by autoradiography. Results were quantified by scanning assays. Residues 586–605 constitute the high-affinity RII site in AKAP-KL (see “Results”). RIIa was carried out at 0 °C for 90 min. Subsequently, 40 μl of buffer A containing 0.1 M NaCl. RIIa standards for the estimation of ovalbumin (45,000), and carbonic anhydrase (29,000) were used as

**PROTEIN SEQUENCES**

**Isolation of cDNAs Encoding AKAP-KL**—A full-length cDNA clone encoding murine RIIa was obtained as described previously for RIIβ (28). RIIa cDNA was subcloned into the yeast-based plasmid pAS1 (CLONTECH) to generate a fusion gene. The gene encodes a hybrid protein in which the yeast GAL4 DNA-binding domain is appended at the N terminus of RIIa. A rat brain cDNA library in the yeast expression plasmid (library vector) pGAD10 was obtained from CLONTECH and screened via the two-hybrid interaction assay as described by Fields (27) and Durfee et al. (28). Growth of auxotrophic yeast on medium lacking Trp, His, and Leu and β-galactosidase assays were used to isolate and characterize cDNAs encoding candidate AKAPs. A fragment of cDNA (1.4 kilobase pairs) encoding a portion of a novel RII-binding protein was excised from recombinant pGAD10 (by digestion with EcoRI) and used as a template to generate a random-primed, [32P]labeled probe. This probe was used to screen a 5-stretched mouse brain cDNA library in bacteriophage agt11 (CLONTECH) as indicated in previous plagues (29, 30). Six positive recombinant phage clones were plaque purified and the cDNAs (0.9–3.8 kilobase pairs) were subcloned in plasmids pGEMTZ (Promega) and pBluescript (Stratagene) and sequenced.

**DNA Sequence Analysis**—cDNA inserts were sequenced by a dideoxynucleotide chain termination procedure (31) using T7, T1, and custom synthetic oligonucleotide primers as described previously (30).

**Computer Analysis**—Analysis of sequence data, sequence comparisons, and data base searches were performed using PCGENE-Intelli
genetics software (IntelliGenetics, Mountainview, CA) and BLAST programs (32, 33) provided by the NCBI server at the National Institutes of Health.

**Electrophoresis of Proteins**—Proteins were denatured in gel loading buffer and subjected to electrophoresis in 9 or 10% polyacrylamide gels containing 0.1% SDS as described previously (8). Myosin (M, = 210,000) phosphorylase b (97,000), transferrin (77,000), albumin (68,000), ovalbumin (45,000), and carbonic anhydrase (29,000) were used as standards for the estimation of M, values. Western Immunoblot Assays—Size-fractionated proteins were transferred from denaturing polyacrylamide gels to an Immobilon-P membrane (Millipore Corp.) (34). Blots were blocked, incubated with anti-serum directed against AKAP-KL (1:2000), and washed as described previously (34, 35). Antigen-IgG complexes were visualized by an indirect chemiluminescence procedure (34, 35). Signals were recorded on Kodak XAR-5 x-ray film.

**Overlay Assay for RII Binding**—Overlay binding assays have been described previously (8, 9). In brief, a Western blot is probed with [32P]labeled RIIα (using a subunit concentration of 0.3 nM and 1 × 10⁶ cpm of [32P]radioactivity/ml). Complexes of [32P]-RIIα and AKAPs are visualized by autoradiography. Results were quantified by scanning densitometry (Pharmacia-LKB Ultroscan XL laser densitometer) or PhosphorImager analysis (Molecular Dynamics) (35).

**DNA Sequencing**—Aliquots (80 μl) of highly purified partial AKAP-KL protein (residues 534–741, Fig. 2A) were used to screen binding assays. Residues 586–605 constitute the high-affinity RIIα-binding site in AKAP-KL (see “Results”). RIIα was expressed, purified, and labeled with [32P] as described previously (8, 26). Assays were performed in 250 μl of buffer A (10 mM Tris-HCl, 50 mM sodium phosphate, pH 8.0, 0.1 mM NaCl). [32P]Labeled RIIα was varied from 0.1 to 75 μM. Incubations were performed at 0 °C for 90 min. Subsequently, 40 μl of a 50% suspension of Ni²⁺-chelate Sepharose 4B beads (Talon resin, CLONTECH), which was pre-equilibrated with Buffer A, was added and the incubation continued for 30 min. Next, samples were diluted to 1 ml with buffer A and the beads were pelleted by centrifugation at 2,000 × g for 5 min at 4 °C. The beads were washed an additional 5 times with suspension in Buffer A (1 ml of buffer A). Bound [32P]-labeled RIIα was eluted from the beads in 0.2 ml of 1% SDS and radioactivity was determined in a scintillation counter. 32P radioactivity in an aliquot of the first supernatant solution was determined to measure the amount of free RIIα.

**Production of IgGs Directed against AKAP-KL**—Samples of AKAP-KL fusion protein were injected into rabbits (0.4 mg initial injection; 0.2 mg for each of four booster injections) at Covance Laboratories (Vienna, VA) at 3-week intervals. Serum was collected at 3-week intervals after the first injection.

**Deletion and Site-directed Mutagenesis of AKAP-KL**—Deletion mutagenesis was performed via polymerase chain reaction as described for AKAP75 and S-AKAP84 (11, 15). Amino acid substitutions were introduced into the RII-binding domain of AKAP-KL via site-directed mutagenesis, as described previously (11).

**Determination of AKAP-KL Localization by Immunofluorescence Analysis and Immunoperoxidase Histochemistry**—HEK293 cells that were stably transfected with an AKAP-KL-transgene were fixed and incubated with anti-AKAP-KL antibodies using procedures described by Li et al. (13). AKAP-KL-IgG complexes were visualized by incubation with fluorescein isothiocyanate-coupled secondary antibodies and the utilization of a laser scanning confocal microscope system as described previously (13). F-Actin was visualized by its interaction with rhodamine-tagged phalloidin, as previously reported (13). Mice were sacrificed by in situ fixation (12). Sections of kidney and lung were prepared, probed with antibodies directed against AKAP-KL, and stained via an indirect peroxidase procedure as previously reported (12). A black precipitate is formed at sites containing AKAP-KL-IgG complexes.

**RESULTS AND DISCUSSION**

**Discovery of a Novel Anchor Protein, AKAP-KL**—A cDNA encoding full-length RIIα was cloned in the yeast plasmid pAS1-CYH2. This recombinant “bait” vector was used to screen a rat brain cDNA library inserted into the yeast “library” vector pGAD10. Interaction of the GAL4 DNA-binding domain-RIIα fusion protein encoded by the bait vector with RII-binding proteins fused to the activation domain of GAL4 (encoded by library vector) reconstitutes a transcription factor that stimulates GAL4-dependent promoters to transcribe HIS3 and lacZ-reporter genes. The combination of chimeric genes in plasmids and interaction-dependent HIS3 gene expression enable selection of candidate AKAPs by growth of auxotrophic yeast colonies on Trp-leu His medium. Yeast carrying four different
cDNAs were obtained from 10⁶ transformants. Each yeast clone produced positive results in β-galactosidase assays. Library plasmids were recovered from yeast and amplified in E. coli. Reconstitution assays showed that plasmids encoding candidate AKAPs did not activate HIS3 or lacZ alone, or in combination with irrelevant bait plasmids. In contrast, co-transformation of yeast with selected library plasmids and the original bait plasmid enabled growth on “triple minus” medium and β-galactosidase expression. RII-binding assays were performed on samples of total protein from yeast carrying positive, recombinant plasmids. One fusion protein bound ~10-fold more 32P-labeled RIα than the others (Fig. 1, lane 8). When the corresponding cDNA was sequenced it yielded a derived protein comprising 415 residues. A translation termination codon was 415 residues. A translation termination codon was sponding cDNA was sequenced it yielded a derived protein

Total protein (35

(AATAAA) are present (nucleotides 3192–3197 and 3367–3372) to determine the binding activities of the fusion proteins. Lanes 1, 2, and 8 received fusion proteins from yeast transformed with recombinant pGAD10 plasmids that encode three distinct RII-binding proteins, which were identified in the 2-hybrid interaction screen. Lanes 4 and 5 contained proteins from non-transformed yeast and yeast containing the RIα cDNA in the bait vector, respectively. Lanes 6 and 7 received proteins from yeast transformed with pGAD10 plasmids encoding proteins that do not interact with RIα. The sample in lane 8 corresponds to a fragment AKAP-KL. An autoradiogram is shown. Only the relevant part of the blot is presented.

Fig. 1. Expression of RIIα-binding proteins in yeast. Samples of total protein (35 μg) from colonies of transformed yeast were subjected to electrophoresis in a denaturing polyacrylamide gel (10%). Subsequently, size-fractionated proteins were transferred to an Immobilon P (Millipore) membrane and probed with 32P-labeled RIα (overlay assay, see “Experimental Procedures”) to determine the binding activities of the fusion proteins. Lanes 1, 2, and 8 received fusion proteins from yeast transformed with recombinant pGAD10 plasmids that encode three distinct RII-binding proteins, which were identified in the 2-hybrid interaction screen. Lanes 4 and 5 contained proteins from non-transformed yeast and yeast containing the RIα cDNA in the bait vector, respectively. Lanes 6 and 7 received proteins from yeast transformed with pGAD10 plasmids encoding proteins that do not interact with RIα. The sample in lane 8 corresponds to a fragment AKAP-KL. An autoradiogram is shown. Only the relevant part of the blot is presented.

(Fig. 2A). The sequence of the acidic polypeptide (pI ~ 5.0) is not homologous with sequences of previously characterized proteins. However, several domains that could potentially contribute to functional roles for AKAP-KL are evident. The sequence between residues 586 and 605 (Fig. 2A) of AKAP-KL aligns with the RII-binding site of AKAP75 (11) to yield 45% overall identity (Fig. 3A). A central core of 7 amino acids (Leu⁵⁹³ to Gln⁵⁹⁸ in AKAP-KL) is nearly invariant (86% identity) in the two proteins. Furthermore, five amino acids with large aliphatic side chains, which coordinately regulate RII binding affinity in AKAP75 (11), are conserved in AKAP-KL (Leu⁵⁹⁶, Leu⁵⁹⁷, Val⁵⁹⁸, Ile⁶⁰⁸, and Ile⁶⁰⁹). A partial AKAP-KL protein (residues 354–714) that includes the putative tethering site binds both RIIα (Fig. 3B, lane 1) and RIIβ (not shown). Two approaches verified the functionality of the potential RII-binding site in AKAP-KL. First, a hydrophobic residue (Ile⁶⁰⁸) predicted to be essential for binding RII (by analogy with AKAP75) was mutated to Ala. This substitution diminished RII binding activity of partial AKAP-KL by >95% (Fig. 3B, lane 2). A similar effect was observed when the corresponding Ile in AKAP75 was replaced with Ala (11). Mutation of an amino acid predicted to be non-essential (Ala⁵⁹⁰ to Ser) had little effect on the tethering of RII (Fig. 3B, lane 2). Partial AKAP-KL proteins that lack residues 586–605 have no RII binding activity. ²

RII-binding regions in AKAP75 and AKAP-KL are predicted to fold as an amphipathic α-helix with one predominantly hydrophobic surface (10). Interactions between this hydrophobic surface and a complementary apolar region near the N terminus of RII subunits stabilize AKAP75-RII (PKAII) complexes (26, 40). The binding of PKAII isoforms by AKAP-KL. Replacement of a critical Ile residue with Ala is a conservative substitution that is unlikely to alter secondary structure or the apolar nature of the RII tethering site. Rather, it appears that a reduction in the size of the hydrophobic binding surface compromises tethering activity (see Ref. 11 for details).

If AKAP-KL mediates accumulation of PKAII in a discrete microenvironment within cells, then anchor protein-RII complexes should have a low dissociation constant, indicative of stable protein-protein interactions. Purified His-tagged AKAP-KL fusion protein (residues 354–714) was incubated to equilibrium with various concentrations of 32P-labeled RIα. Subsequently, AKAP-KL-RIα complexes were recovered on Ni²⁺-chelate Sepharose 4B beads and amounts of bound and free radiolabeled ligand were measured in a scintillation counter. Analysis of typical results (Fig. 3C) yielded a Kd value of 9.5 nM, indicating that AKAP-KL avidly binds RII (PKAII). In many tissues the total RII concentration is ~150 nM (41). If the concentration of AKAP-KL is assumed to be 10–100 nM, then the Kd and mass law considerations (42) indicate that >85% of the tethering sites will be occupied by RII (PKA) isoforms.

Two segments of AKAP-KL (residues 246–316 and 729–766, Fig. 2A) are predicted to be coiled-coil domains (43). Such structures mediate homomeric and heteromeric protein-protein interactions. A segment of AKAP-KL (residues 274–312) is composed of Gln (79%) interspersed with occasional Leu residues. The sequence LQQQQ appears 5 times within this region. Studies on proteins and model peptides indicate that Gln-rich domains serve as “zippers” that interlock polypeptides into oligomeric structures (44). Although the LQ₄ motif has not been previously described as a specific entity, it is established that Leu side chains are crucial components of zippers (actually coiled-coil regions) that facilitate protein-protein interactions (45). Thus, it is possible that the LQ₄ repeat region and coiled-coil domains mediate oligomerization of AKAP-KL and/or targeting and anchoring of AKAP-KL-PKAII complexes. Possible
functions of coiled-coil regions in AKAP-KL can be rigorously evaluated in future studies by employing a combination of mutagenesis, transfection/expression, biochemical analysis, and immunolocalization techniques.

Six Isoforms of AKAP-KL Are Produced in Mammalian Cells—Sequencing of 3' ends of AKAP-KL cDNAs revealed two alternative modes of AKAP-KL mRNA splicing. In the first instance, a splice donor sequence at nucleotide 2878 (Fig. 2A) is joined to an acceptor site at position 3097, thereby deleting 218 nucleotides in cDNA. This deletion shifts the reading frame after codon 789 so that the succeeding 21 nucleotides encode a novel, C-terminal hexapeptide (Fig. 2B) and a translation termination signal. As a result, the C terminus of the anchor protein is truncated by 90 residues. AKAP-KL proteins that lack 90 C-terminal residues are designated AKAP-KL3; AKAP-KL proteins that retain this region are designated AKAP-KL1. A second mode of RNA splicing joins a donor sequence at nucleotide 3058 (Fig. 2A) with the acceptor site at nucleotide 3097. This splicing reaction excises 13 codons (for amino acids 849–861) and does not alter the reading frame (Fig. 2B). Anchor proteins that lack residues 849–861 are named AKAP-KL2.

Further diversity in AKAP-KL structure is introduced by utilization of either of two translation start codons. Like codon 1 in Fig. 2A, Met codon 125 is included within the context of a consensus translation initiation sequence (ANNATG, nucleotides 883–889). Both start sites are used in cultured cells and in vivo (see Fig. 4, below). Thus, AKAP-KL proteins that lack 90 C-terminal residues are designated AKAP-KL3; AKAP-KL proteins that retain this region are designated AKAP-KL1. A second mode of RNA splicing joins a donor sequence at nucleotide 3058 (Fig. 2A) with the acceptor site at nucleotide 3097. This splicing reaction excises 13 codons (for amino acids 849–861) and does not alter the reading frame (Fig. 2B). Anchor proteins that lack residues 849–861 are named AKAP-KL2.

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The physiological significance of various AKAP-KL isoforms remains to be determined. However, the ability of individual cells to produce up to 6 related, but distinct AKAPs from a single gene suggests mechanisms for the diversification and specialized adaptation of PKAII-mediated signal transduction. For example, different AKAP-KL isoforms may be targeted to distinct intracellular locations. (All forms of the anchor protein contain the RII tethering site.) Differences in stability (t1/2) among anchor protein variants could alter the concentration of immobilized (versus cytoplasmic) PKAII, thereby shifting the focus of PKAII-mediated signaling from one compartment to another.
Properties, Expression, and Polarized Targeting of AKAP-KL

The smaller anchor protein (S-AKAP84) is uniquely produced from a single gene transcript by alternative splicing (37, 46). S-AKAP84 and AKAP121 (D-AKAP-1) mRNAs are derived from reverse transcripts of AKAP-KL mRNAs from this library does not imply that brain is the principal site of accumulation of the AKAP-KL isoforms. As described under “Experimental Procedures” and “Results and Discussion,” a sample of total protein from induced E. coli (30 μg, lane 2), proteins in the column flow-through (30 μg, lane 1), and protein from the pooled peak fractions eluted with 1 M imidazole (1 μg, lane 3) were size-fractionated by denaturing electrophoresis. A 10% polyacrylamide gel stained with Coomassie Blue is shown. The partial AKAP-KL protein has an apparent Mr of 50,000. B, cytosol (100,000 × g supernatant solution) and two particulate fractions (P1, 10,000 × g pellet; P2, 100,000 × g pellet) were prepared from mouse kidney as described previously (8, 12). Samples (35 μg) of cytosolic, P1 and P2 proteins were size-fractionated in a denaturing polyacrylamide gel (9%) and transferred to an Immobilon P membrane. Lanes 1 and 5 received cytosolic proteins; lanes 2 and 6 contained P2 proteins; lanes 3, 4, and 7–9 were loaded with proteins from the P1 pellet. Lanes 1–4 from the Western blot were incubated with preimmune serum and antisera. Lanes 5 and 6 were probed with preimmune serum and antiserum. The immunoblots were developed by an enhanced chemiluminescence procedure and signals were recorded on x-ray film (see “Experimental Procedures”). RI-binding proteins in the P1 pellet were identified and characterized by the overlay binding assay (lane 9). An autoradiogram is shown.

Names and sizes of AKAP-KL isoforms

| Name        | Number of amino acid residues | Calculated Mr |
|-------------|------------------------------|---------------|
| AKAP-KL1A   | 885                          | 98,000        |
| AKAP-KL1B   | 761                          | 85,000        |
| AKAP-KL2A   | 872                          | 96,000        |
| AKAP-KL2B   | 748                          | 83,000        |
| AKAP-KL3A   | 795                          | 87,000        |
| AKAP-KL3B   | 671                          | 74,000        |

Preparation and Specificity of Antibodies Directed against AKAP-KL—AKAP-KL cDNAs were cloned from a brain cDNA library. Since a high proportion of the complete constellation of mammalian genes is expressed in the central nervous system, the efficiency of screening is maximized. However, isolation of reverse transcripts of AKAP-KL mRNAs from this library does not imply that brain is the principal site of accumulation of the anchor protein. To determine the tissue and cellular distributions, as well as functions of AKAP-KL it is essential to generate highly specific anti-AKAP-KL IgGs.
A fragment (1164 base pairs) of AKAP-KL cDNA encoding amino acids 354–741 (Fig. 2A) was amplified by the polymerase chain reaction using 5′ and 3′ primers that introduced NdeI and BamHI restriction sites, respectively. This enabled cloning of the cDNA fragment into the pET14b expression plasmid. The inserted cDNA lies downstream from a bacteriophage T7 promoter and DNA encoding a fusion peptide composed of 20 amino acids. Included in the fusion peptide are six consecutive His residues, which constitute a Ni²⁺-binding site. E. coli BL21 (DE3) was transformed with recombinant pET14b plasmid and induced to synthesize fusion protein with 1 mM isopropyl-1-thio-β-D-galactopyranoside. After lysis of bacteria in a French press, the soluble, partial AKAP-KL fusion protein was purified to near homogeneity by affinity chromatography on a metal chelate-Sepharose 4B resin (Fig. 4A).

Antibodies directed against the partial AKAP-KL protein were produced in rabbits. The antibodies will bind with all AKAP-KL isoforms (Table I) because the cDNA fragment encoding the antigen begins downstream from the second translation initiation codon and terminates upstream from donor sites for alternative mRNA splicing. Western immunoblot analysis revealed that two major AKAP-KL polypeptides (with apparent Mr values of 105,000 and 120,000) accumulate in the 10,000 × g pellet fraction (P1) of mouse kidney homogenates (Fig. 4B, lane 7). Two minor AKAP-KL proteins (apparent Mr values of 115,000 and 130,000) are also detected. Lower levels of the same proteins are evident in the 100,000 × g particulate fraction (P2), whereas cytosol lacks the RII-binding protein (Fig. 4B, lanes 5 and 6). Thus, AKAP-KL is tightly associated with cytoskeleton and/or organelles. Polypeptides with the same Mr values avidly bind 32P-labeled RIIα (Fig. 4B, lane 9). AKAP-KL isoforms (Table I), like other AKAPs (11, 15), are acidic proteins that exhibit aberrantly large Mr values in denaturing gels.

Expression of AKAP-KL Is Tissue-specific and Isoform-selective—Analysis of P1 fractions from various tissues disclosed that AKAP-KL is abundantly expressed in lung (Fig. 5A, lane 1). In contrast, the anchor protein is either absent or produced at very low levels in multiple tissues, including liver, heart, and cerebral cortex (Fig. 5A, lanes 3–5). Moderate levels of AKAP-KL were detected in thymus and cerebellum. AKAP-KL isoforms observed in cerebellum (Fig. 5A, lane 6) do not co-migrate with anchor proteins that accumulate in lung. Thus, expression of the AKAP-KL gene is tightly regulated. Accumulation of AKAP-KL polypeptides is restricted to certain cell/tissue types and anchor protein isoforms that predominate in a given tissue are evidently determined by a combination of regulated AKAP-KL mRNA splicing and utilization of two translation initiation codons.

It is possible to identify AKAP-KL isoforms expressed in vivo by comparison with AKAPs encoded by transgenes in transfected cells. Hamster AV-12 cells lack endogenous AKAP-KL. AV-12 cells transfected with an AKAP-KL2 transgene accumulate anchor proteins with apparent Mr values of 115,000 and 130,000 (Fig. 5B, lane 3). In contrast, an AKAP-KL3 transgene programs the synthesis of 105- and 120-kDa RII-binding proteins (Fig. 5B, lane 2). Deletion of 120 codons at the 5′ end of AKAP-KL3 cDNA forces the exclusive utilization of the second initiator Met codon and results in the production of a single anchor protein with an apparent Mr of 105,000 (Fig. 5B, lane 1). Comparison of anchor proteins synthesized in lung tissue (Fig. 5B, lane 4) with those expressed in transfected cells disclosed that AKAP-KL3A and AKAP-KL3B (Table I) are prominent mediators of PKAII immobilization in pulmonary tissue. Longer exposures of the x-ray film revealed low levels of immunoactive proteins with Mr values of 115,000, 117,000, 130,000, and 133,000 (data not shown), indicating that all AKAP-KL isoforms (Table I) are synthesized in lung. The principal anchor proteins in cerebellum (see Fig. 5A) were identified as AKAP-KL2A and AKAP-R2B by applying the same methodology.

Properties of AKAP-KL in Vitro and in Cells—Extraction with buffers containing 0.5% Triton X-100 failed to solubilize the anchor protein, indicating that it is not embedded in a lipid bilayer (Fig. 6A). Buffers containing 0.5% sodium deoxycholate (which disrupts association of proteins in cytoskeleton) efficiently solubilize AKAP-KL. Confocal immunofluorescence microscopy of HEK293 cells that were stably transfected with cDNA encoding full-length AKAPs KL2A and 2B revealed the intracellular distribution of the anchor protein. AKAP-KL accumulates in regions of cortical cytoskeleton that appear as projections or large clusters of antigen (Fig. 6B). These structures are not present in control HEK293 cells or cells transfected with unrelated transgenes (13). In contrast, AKAP75 is dispersed throughout the cortical cytoskeleton in the same cells (13). Thus, AKAP-KL appears to be targeted to specific microenvironments in cytoskeleton. It is possible that AKAP-KL actively promotes assembly/organization of specialized structures via interactions with other proteins in cytoskeleton. The differential clustering of AKAP-KL in subdomains of cytoskeleton in HEK293 cells may also reflect properties involved in targeting of the anchor protein to the vicinity of the apical surface of polarized epithelial cells (see below).

Cytochalasin D disrupts the F-actin network, causes marked cell rounding and elicits a redistribution of AKAP-KL to two sites (Fig. 6C). In part, the anchor protein is dispersed along the cell periphery; the remainder of AKAP-KL is aggregated in large internal structures. Double immunostaining disclosed that both peripheral and internal AKAP-KL are associated with F-actin in cytochalasin-treated cells (Fig. 6D). F-actin is distributed evenly in the cortex of
FIG. 6. AKAP-KL associates with actin cytoskeleton. Proteins were solubilized from the P1 particulate fraction of kidney homogenates as described previously (29). Supernatant fractions (40 μg of protein) derived from the P1 pellet were collected and assayed for AKAP-KL polypeptides by a Western immunoblot assay. Lane 2 received proteins solubilized with 0.5% sodium deoxycholate; lane 3 contained proteins solubilized with 1% Triton X-100; lane 4 was loaded with proteins extracted with 1 M NaCl; lane 1 contained 30 μg of total P1 particulate proteins. B, confocal immunofluorescence microscopy of HEK293 cells stably transfected with an AKAP-KL transgene. Signals were obtained with anti-AKAP-KL antibodies and secondary antibodies tagged with fluorescein isothiocyanate. No signals were seen after blocking with excess antigen or with preimmune serum. AKAP-KL accumulates in clusters and projections. C, cells shown in B were incubated with 5 μM cytochalasin D for 1 h. Immunofluorescence microscopy shows that AKAP-KL re-distributes uniformly at the periphery and in large internal aggregates. D, double immunostaining for AKAP-KL and F-actin is shown after treating cells described in B with cytochalasin D. The distribution of AKAP-KL (shown on the left) was determined by incubating with anti-AKAP-KL serum and fluorescein isothiocyanate-coupled secondary antibodies. The location of F-actin (right) was established by probing with rhodamine-phalloidin.

FIG. 7. Distribution of AKAP-KL in kidney and lung. A, immunoperoxidase staining of kidney sections that contain proximal tubules. The stain for AKAP-KL (heavy black precipitate) is located at the lumenal (apical) surface of the tubules. No staining was detected with preimmune serum or with immune serum preincubated with excess AKAP-KL antigen. Magnification × 400. B, examination of proximal tubule staining at lower magnification confirms that AKAP-KL is selectively targeted to the apical surface (as) of polarized epithelial cells. The lumen is marked as lu; the (unstained) glomerulus as g. Magnification × 200. Panels C and D show the immunoperoxidase staining of alveolar epithelial cells for AKAP-KL and total RII, respectively. The anchor protein accumulates in long strand-like structures (e.g. see arrowhead in C) that lie just under the plasma membranes of highly flattened and polarized epithelial cells that mediate gas exchange between blood and air. A substantial portion of RII in alveolar epithelial cells (arrowhead in D) appears to be co-localized in the same structures as AKAP-KL. Counterstained nuclei appear as dark spheroids in the lung sections. Preincubation of anti-AKAP-KL serum with excess purified partial AKAP-KL antigen (panel E) eliminates the anchor protein signal; likewise, panel F shows that excess purified antigens (3 μg of RIIα and RIIβ) abrogate signals evident in panel D.
untreated, transfected cells (e.g. see Ref. 13). Thus, AKAP-KL binds with actin or actin-associated proteins in cytoskeleton. However, cytochalasin D-sensitive interactions with other (cytoskeletal?) proteins apparently restrict and enrich AKAP-KL in discrete microenvironments. A speculation is that such mechanisms might contribute to assembly of polarized distal signaling modules that include anchored PKAII.

**AKAP-KL Is Targeted to the Apical Surface of Polarized Epithelial Cells**—Anti-AKAP-KL IgGs were used in an indirect immunoperoxidase staining procedure to determine the location of AKAP-KL in sections of rat kidney. The anchor protein is expressed in epithelial cells and accumulates exclusively at the apical surface, which abuts the lumen (e.g. see epithelial cells in proximal tubules, Figs. 7, A and B). RIIa is also concentrated at the apical surface of epithelial cells in renal proximal tubules (21). In lung, AKAP-KL accumulates in long, linear structures just below the luminal surface of alveolar epithelial cells (Fig. 7C). In parallel, a substantial amount of RII is also concentrated in the elongated structures near the apical surface of alveolar epithelial cells (Fig. 7D). The preceding results suggest that AKAP-KL and RII are co-localized and co-enriched in two types of polarized epithelium. Confirmation of this working hypothesis will ultimately require high-resolution, immunoelectron microscopy and manipulation of the system by molecular genetics (e.g. disrupting AKAP-KL-RII complexes by expressing AKAP-KL mutants that bind RII, but are mis-targeted, or by ablating AKAP-KL gene expression) in future studies. However, the present observations support the idea that AKAP-KL incorporates PKAII into distal signaling modules at sites near junctions of cytoskeleton and apical plasma membrane. Such modules might include PKA substrate/effector proteins (e.g. transporters) which span the apical membrane. This mode of organization can greatly facilitate cAMP-mediated trans-epithelial signal transduction.

**AKAP-KL Binds RII with High Affinity in Intact Cells**—The pEBG expression vector (36) was used to assess the ability of the tethering domain AKAP-KL to sequester RII in intact cells. pEBG contains a powerful EF1α promoter that lies upstream from the glutathione S-transferase (GST) gene and a 3′ multiple cloning region. AKAP-KL cDNA was inserted, in-frame with the GST gene, and the vector was co-transfected with Rγ/dCMV (an expression plasmid which contains the neo3 gene under control of an SV40 promoter) into hamster AV-12 cells. Stable cell lines expressing GST-AKAP-KL were isolated by selection with G418. Typically, ~80% of GST-AKAP-KL appeared to be anchored, whereas 20% was present in cytosol. Cytosolic GST-AKAP-KL complexes were purified to near homogeneity by affinity chromatography on Glutathione Sepharose 4B. Western blot analysis revealed that a large proportion of available cytosolic RII was complexed with the anchor protein (Fig. 8). Two conditions were varied to exclude the possibility that AKAP-KL-RII complexes assembled after cell homogenization. Since AKAP-KL-RII complexes dissociate very slowly at 4 °C, cells were disrupted: (a) in a volume of lysis buffer that was increased 20-fold to reduce concentrations of free RII (PKAII) and anchor protein or (b) in a standard volume of buffer that contained a vast excess (15 μg/ml) of His-tagged AKAP-KL partial protein (see Fig. 4A). The partial anchor protein avidly binds RII (Fig. 3C), but is not sequestered by Glutathione-Sepharose 4B resin. Neither dilution nor post-lysis competition with an RII-binding protein altered the results obtained in Fig. 8 (data not shown). Thus, stable AKAP-KL-RII complexes are efficiently produced in the environment of the internal milieu of intact cells.

**Conclusions and Implications**—AKAP-KL isoforms are differentially expressed and selectively targeted to apical regions of polarized lung and kidney epithelial cells. This family of novel anchor proteins efficiently sequesters RII (PKAII) in vitro and in situ. AKAP-KL proteins may be involved in (a) establishing polarity in trans-epithelial signaling systems and (b) creating/organizing distal PKEffectector complexes that receive, amplify, and focus trans-cellular signals carried by cAMP. Generation of six AKAP-KL isoforms by post-transcriptional processes creates substantial anchor protein diversity and suggests possible mechanisms by which intracellular localization of PKAII isoforms may be modulated in different cell types and in response to environmental stimuli.

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