Experiments were designed to test the idea that A kinase anchor proteins (AKAPs) tether regulatory subunits (RII) of protein kinase AII (PKAII) isoforms to surfaces of organelles that are bounded by phospholipid bilayers. S-AKAP84, one of three RII-binding proteins encoded by a single-copy murine gene, was studied as a prototypic organelle-associated AKAP. When S-AKAP84 was expressed in HEK293 cells, the anchor protein was targeted to mitochondria and excluded from other cell compartments. The RII tethering site is located in the cytoplasm adjacent to the mitochondrial surface. Endogenous RII subunits are not associated with mitochondria isolated from control cells. Expression of S-AKAP84 in transfected HEK293 cells triggered a redistribution of 15% of total RII to mitochondria. Thus, the tethering region of the organelle-inserted anchor protein is properly oriented and avidly binds RII (PKAII) isoforms in intact cells. Two critical domains in S-AKAP84 were mapped. Residues 1 to 30 govern insertion of the polypeptide into the outer mitochondrial membrane; amino acids 306–325 constitute the RII-binding site. Properties established for S-AKAP84 in vitro and in situ strongly suggest that a physiological function of this protein is to concentrate and immobilize RII (PKAII) isoforms at the cytoplasmic face of a phospholipid bilayer.

Type II isoforms of cAMP-dependent protein kinase (PKAIIa and PKAIIβ)1 are attached to cytoskeleton or organelles via binding of their regulatory subunits (RIIa, RIIβ) with protein kinase A anchor proteins (AKAPs) (1–3). Prototypic neuronal anchor proteins (bovine AKAP75 and its human (AKAP79) and rat (AKAP150) homologs) have a conserved binding site for RII subunits and domains that non-covalently link AKAP-PKAI complexes to the dendritic cytoskeleton of neurons and the cortical actin cytoskeleton of non-neuronal cells (4–9). Both cytoskeletal locations are closely apposed to the plasma membrane. Therefore, anchored PKAII is placed in proximity with a signal generator (hormone/neurotransmitter-activated adenylyl cyclase) and multiple PKA substrate/effector proteins (e.g. myosin light chain kinase, microtubule-associated protein-2, ion channels, serpentine receptors that couple with the GTP-binding protein Gs). This arrangement creates a target site for cAMP action (reviewed in Ref. 1).

Distinct RII-binding proteins mediate association of PKAII isoforms with peroxisomes, mitochondria, and other organelles in a variety of cell types (1, 2, 10, 11). These AKAPs have 20-residue RII-binding sites that are homologous with the RII-binding domain in AKAP75 (1, 2, 6, 10, 11). Otherwise, sequences of non-neuronal AKAPs diverge among themselves and differ from the sequence of AKAP75. Potential targeting domains for some non-neuronal AKAPs have been inferred from motifs in derived amino acid sequences and the distribution of the anchor proteins observed upon subcellular fractionation and immunostaining (1, 2, 10, 11). However, an experimental demonstration that an anchor protein governs accumulation of RII subunits at a specific location within intact cells has been accomplished only for neuronal AKAP75 (9, 12). AKAP75 targets RII to sites in cytoskeleton. We lack knowledge of the (a) abilities of non-neuronal AKAPs to sequester RII subunits from cytoplasmic (or other) pools in situ and (b) efficiency and selectivity with which AKAP-RII complexes are anchored at organelles (rather than cytoskeleton) in intact cells.

S-AKAP84 is a novel, organelle-associated RII-binding protein that is expressed in male germ cells (10). S-AKAP84 has an RII tethering site, a leucine-zipper segment, and a predicted, N-terminal targeting/anchoring domain (10). Biogenesis of S-AKAP84 is coordinated with the appearance of RIIa subunits (PKAIIa) during a late stage of spermiogenesis (10, 13, 14). Moreover, RII subunits are co-localized with S-AKAP84 as round spermatids differentiate into condensing spermatids, which contain a distinct flagellum. Newly-synthesized S-AKAP84 is incorporated into pre-existing mitochondria, which migrate to a location at which the flagellar cytoskeleton is assembled (15). A plausible model suggests that S-AKAP84 binds cytoplasmic PKAIa and anchors the kinase via insertion of its N-terminal targeting domain into the outer membrane of mitochondria (10). Anchored PKAIa is optimally positioned to phosphorylate proteins on the mitochondrial surface and components of microtubule motor systems, thereby regulating mitochondrial translocation to the site of cytoskeleton assembly. Insertion of S-AKAP84/RIIa into the outer mitochondrial membrane ensures delivery of PKAIa to the midpiece of the flagellum (16), where catalytic subunits will be juxtaposed with substrate/effector proteins in the cytoskeleton. Cyclic AMP-stimulated phosphorylation of target proteins presumably promotes sperm motility by eliciting sliding of an external cytoskeletal structure (fibrous sheath) over an internal...
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microtubular structure (16–19).

The preceding model remains tentative because predicted properties of S-AKAP84 have not been established in the context of intact cells. Critical questions are: does S-AKAP84 alone contain sufficient information to undergo selective targeting to mitochondria? Alternatively, are additional spermatid proteins required to promote association of S-AKAP84 with mitochondria? Is S-AKAP84 inserted via its predicted N-terminal targeting domain, leaving the remainder of the protein (including the RII tethering site) accessible to cytoplasm? Does S-AKAP84 bind RII subunits with sufficient affinity in situ to effect a redistribution of PKAII isoforms to mitochondria? Is S-AKAP84 targeted to mitochondria or dispersed in multiple cell compartments? Does the putative leucine-zipper domain of S-AKAP84 facilitate anchoring of PIIA isoforms? Finally, detection of a discrete RII-binding protein that is antigenically related to S-AKAP84 in spermatocytes (10) raises the following queries: Are there multiple isoforms of S-AKAP84 proteins? If so, what is the molecular basis for their origin?

We now describe experiments that address and resolve questions posed above. The results define structure-function relationships for S-AKAP84 in situ and support the S-AKAP84 functional model. In addition, insights were gained regarding the ability of a non-neuronal AKAP to selectively bind and target PIIA isoforms to an organelle in the context of the internal milieu of intact cells.

EXPERIMENTAL PROCEDURES

Isolation of cDNAs Encoding Mouse S-AKAP84 Isoforms—Complementary DNA encoding human S-AKAP84 (10) was digested with NdeI and the resulting 1.8-kilobase pair fragment was used as a template to generate a random-primed, 32P-labeled probe. This probe was used to screen a mouse testis cDNA library in bacteriophage gt11 (CLONTECH) as indicated previously (20, 21). Positive recombinant phage clones were plaque purified and cDNAs (0.9–2.9 kilobase pair) were sequenced by a dideoxynucleotide chain termination procedure (22) using T7, SP6, and custom oligonucleotide primers. T7 Dye Deoxy Terminator Cycle Sequencing Kits (Applied Biosystems) were used according to the manufacturer’s instructions. DNA products were separated and analyzed in a model 377 automated DNA Sequencer (Applied Biosystems) at the Albert Einstein College of Medicine DNA Analysis Facility.

Computer Analysis—cDNAs were sequenced by a dideoxynucleotide chain termination procedure (22) using T7, SP6, and custom oligonucleotide primers. T7 Dye Deoxy Terminator Cycle Sequencing Kits (Applied Biosystems) were used according to the manufacturer’s instructions. DNA products were separated and analyzed in a model 377 automated DNA Sequencer (Applied Biosystems) at the Albert Einstein College of Medicine DNA Analysis Facility.

Characterization of the Extreme 5′ End of S-AKAP84 and AKAP121 cDNAs—Complementary DNAs corresponding to 5′-terminal regions of mouse S-AKAP84 and AKAP121 mRNAs were synthesized, amplified, cloned, and sequenced as described in Land et al. (20, 25). Two rounds of amplification, via the polymerase chain reaction (PCR), were used to obtain cDNAs. The 5′ primer (5′-ATTAGCGGCGGCCTCTGAC-3′) contained a NotI restriction site and a poly(T) tail that hybridizes with dA residues appended to the antisense cDNA strand via terminal transferase (20). The initial 3′ primer (5′-GCTGCTCTTCTTCTCCAGGAGCTGTTG-3′) corresponds to the inverse complement of nucleotides 406 to 432 in S-AKAP84 cDNA (Fig. 1A). The second 3′ primer (5′-GTACGAGTCCTG- GGGGCCCCACAGAGA-3′) contained the inverse complement of nucleotides 381 to 399 in S-AKAP84 cDNA. Nucleotides 5–10 of the second 3′ primer correspond to the Snc1 recognition sequence. After digestion with NotI and Snc1, amplified cDNAs were cloned into plasmid pGEMTZ and sequenced.

RNase Protection Analysis—A cDNA fragment (nucleotides 1744–2225, Fig. 1A) was generated by digestion with Snc1 and Ncol and was cloned in pGEM5Z. Recombinant plasmid was linearized by digestion with Ncol and 32P-labeled antisense RNA was synthesized by bacteriophage SP6 RNA polymerase as described previously (25). The antisense RNA contains overlapping sequences of 397, 345, and 260 nucleotides, that are complementary to corresponding regions in S-AKAP84, AKAP100, and AKAP121 mRNAs, respectively. RNase protection analysis was performed as described previously (20, 25) using 20 μg of total RNA from mouse germ cells (10, 12).

Expression and Purification of S-AKAP84 Fusion Protein—A 741-bp fragment of S-AKAP84 cDNA (nucleotides 810–1550, Fig. 1A) was amplified by PCR, using primers that appened a 5′ Ndel site and a 3′ BamHI site. Amplified cDNA was digested with Ndel and BamHI and cloned into the pET14b expression plasmid (Novagen), which was cleaved by the same enzymes. This placed cDNA encoding amino acids 205–451 of S-AKAP84 downstream from the T7 RNA polymerase promoter, thereby directing synthesis of an N-terminal fusion peptide. The peptide contains a stretch of six consecutive His residues, which form a nickel-binding domain. Escherichia coli BL21(DE3) was transformed with the expression plasmid and induced with 0.4 mm isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C. The bacterium contains a chromosomal copy of the phage T7 RNA polymerase gene under the control of the lac promoter. Bacteria were harvested, disrupted, and separated into soluble and particulate fractions as described previously (20). Soluble S-AKAP84 fusion protein was purified to near-homogeneity by nickel-chelate chromatography (27). Approximately 2 mg of purified S-AKAP84 fusion protein was obtained from a 500-ml culture of E. coli.

Production of Antibodies That Bind Marine S-AKAP84—S-AKAP84 fusion protein was injected (0.33 mg per injection, 2 mg for each of three booster injections) into the New Zealand White rabbit. Serum was collected at 3-week intervals.

Expression of S-AKAP84 and AKAP121 in HEK293 Cells—A 2.8-kb cDNA containing an uninterrupted coding region for AKAP121 was excised from pGEM7Z by digestion with HindIII and XhoI. The insert was cloned into the expression plasmid pCEP4 (Invitrogen), which was cleaved with the same restriction enzymes. This placed the cDNA downstream from a constitutive cytomegalovirus promoter and upstream from a polyadenylation signal. pCEP4 also contains a bacterial hygromycin B phosphotransferase gene under the regulation of a strong, viral thymidine kinase promoter. The AKAP121 coding region (2571 bp, 857 amino acids) in the cDNA insert was flanked by a 145-bp 5′-untranslated region and the 3′-untranslated region shown in Fig. 1A. A 1.9-kb cDNA that directs the synthesis of S-AKAP84 was also cloned in pCEP4. S-AKAP84 cDNA contains an internal, alternative exon (nucleotides 1774–1880, Fig. 1A) that introduces a proximal stop codon. It encodes a protein composed of 547 residues.

Human embryonic kidney cells (HEK293 cells) were grown and transfected as described previously (9, 12, 28). Stably transfected cells were selected by growth in 200 μg/ml hygromycin B as described previously (12).

Preparation of Cell Extracts and Isolation of Purified Mitochondria—Cytosolic and particulate fractions of HEK293 cells were isolated as described previously (28), with one modification. Triton X-100 was omitted from cell lysis buffer. Highly purified mitochondria were isolated by differential centrifugation and sedimentation through a Percoll gradient as described by Hovias et al. (29). Cytochrome c oxidase activity was determined spectrophotometrically by following the decrease in cytochrome c absorbance at 550 nm (30).

Electrophoresis of Proteins and Western Immunoblot Assays—Samples of proteins (20–50 μg) from cell fractions were denatured in gel loading buffer and subjected to electrophoresis in a 5% polyacrylamide gel containing 0.1% SDS as described previously (31). β-Galactosidase (Mw = 116,000), phosphorylase (97,000), transferrin (77,000), albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (29,000) were used as standards for estimation of Mw values. Western blots of the size-fractionated proteins were blocked, incubated with antiserum directed against epitopes included in both S-AKAP84 and AKAP121 (1:2000), and washed as described previously (12, 28). Anchor proteins were visualized and quantified by an indirect chemiluminescence procedure as previously reported (12, 28).

Immunofluorescence Analysis—HEK293 cells were fixed, washed, and incubated sequentially with antibodies against S-AKAP84, and then fluorescein isothiocyanate-tagged goat IgGs directed against rabbit immunoglobulins, as described by Li et al. (9). Prior to fixation, cells were incubated for 30 min with 0.2 μm Mitotracker Red (Molecular Probes, Eugene OR), a rosamine dye that is selectively incorporated into mitochondria. A chloromethyl group in the dye covalently couples to mitochondrial proteins, thereby ensuring its retention in the organelle upon subsequent fixation and washing. Covalently-coupled Mitotracker Red emits intense red fluorescence with λmax = 599 nm (absorption maximum is at 578 nm), which makes it suitable for co-
staining with complexes of antigen, antibodies, and fluorescein-tagged secondary antibodies ($\lambda_{max}$, emission = 520 nm). Fluorescence signals were collected with a Bio-Rad MRC 600 laser scanning confocal microscope (Image Analysis Facility, Albert Einstein College of Medicine) as described in previous publications (9, 20).

Mutagenesis and Expression of Partial S-AKAP84 Proteins in E. coli—Deletion mutagenesis was performed via PCR as described for human S-AKAP84 (10). Amplified cDNAs were cloned in pET14b and truncated proteins were expressed in E. coli as described previously (27). Table I gives the nomenclature for the truncated proteins and indicates the segment of mouse S-AKAP84 that was expressed in bacteria. One deletion mutant, which encodes amino acids 31–547 of S-AKAP84, was generated as described by Glanza et al. (7) and expressed in transiently transfected HEK293 cells.

**Assay for RII Binding Activity**—The methodology and application of the overlay binding assay have been described in several published papers (4, 5, 7–10). Results were quantified as described previously (12).

**RESULTS AND DISCUSSION**

Transcripts of the Murine S-AKAP84 Gene Encode Three Predicted A Kinase Anchor Proteins—Complementary DNAs encoding proteins homologous with human S-AKAP84 were retrieved from a mouse testis cDNA library in bacteriophage Agt11 and characterized. Three related, but distinct mRNAs encode S-AKAP84 homologs in mouse testis. The longest cDNA (Fig. 1A) encodes the shortest polypeptide, which is named S-AKAP84 (apparent $M_r$ = 84,000 in denaturing electrophoresis). Inserts obtained from the testis cDNA library contained nucleotides 53–2990 in Fig. 1A. Extreme 5′ regions of mouse anchor protein cDNAs were characterized by a coupled reverse transcriptase-anchored PCR procedure known as RACE (rapid amplification of cDNA ends, see “Experimental Procedures”). Male germ cell mRNA served as a template for cDNA synthesis. Sequences of six independent cDNAs created in this manner were identical (Fig. 1B). Each had 347 bp of previously established downstream sequence (nucleotides 53–399 in Fig. 1A) linked to a novel upstream segment. This 5′ terminal sequence (nucleotides 1–52) was appended to the downstream sequence described above (nucleotides 53–2990) to yield a composite full-length S-AKAP84 cDNA (Fig. 1A).

A predicted initiator Met codon (nucleotides 198–200, Fig. 1A) lies within the context of a consensus translation start site (ANNNTGG) (31). An open reading frame of 546 codons follows the initiator ATG and precedes a translation termination signal at nucleotides 1839–1841 (Fig. 1A). The upstream cDNA sequence (nucleotides 1–197) lacks an in-frame Met codon, and includes a translation stop signal (nucleotides 33–35). Thus, nucleotides 1–197 evidently constitute a unique 5′ untranslated sequence for S-AKAP84 mRNAs synthesized in male germ cells. The preceding observations also define the transcription start site (G, +1) and indicate that a single promoter governs initiation of S-AKAP84 gene transcription in spermatic cells.

A lengthy 3′-untranslated sequence (nucleotides 1842–2990) follows the translation stop codon in S-AKAP84 cDNA (Fig. 1A). A classical poly(A) addition signal (AATAAA) is not present in 10–30 nucleotides upstream from the polyadenylate tail. However, the demonstration (via sequencing) that 30–40 A residues are attached to nucleotide 2990 in several cloned cDNAs indicates that atypical, alternative poly(A) addition signals (e.g. candidates are AGGACA, at nucleotides 2962–2967; AGAACG, nucleotides 2975–2980) interact with 3′-end processing enzymes. The size of the cDNA sequence (2990 nucleotides) matches the expected attachment of a typical segment of polyadenylate (~150–200 nucleotides, Ref. 32) in vivo to predict that mature S-AKAP84 mRNA will include ~3140–3190 bases. This is in agreement with the size (3.2 kb) of mouse S-AKAP84 mRNA detected on Northern blots of spermatid RNA (10).

The cDNA in Fig. 1A encodes a novel mouse protein (calculated $M_r$ = 58,000) that is homologous with human S-AKAP84 (Fig. 2A), but is unrelated to other polypeptides in standard data bases. Mouse S-AKAP84 is enriched in Ser, Pro, and Glu, which account for 31% of its 547 amino acid residues. Like other AKAPs (1, 28), the mouse spermatid anchor protein is highly acidic (predicted $pI$ = 4.6) and exhibits an apparent $M_r$ (84,000) in denaturing gel electrophoresis that exceeds its calculated $M_r$ by ~40%.

The amino acid sequence of mouse S-AKAP84 is only 55% identical with its human counterpart (Fig. 2A). Thus, inspection of the aligned sequences enables identification of conserved protein segments that are probable mediators of critical functions. For example, the putative mitochondrial membrane targeting region (residues 1–28) is 96% identical in mouse and human S-AKAP84 polypeptides. As a result, this N-terminal domain was selected for functional analysis in intact cells (see Fig. 10 and text below). Regions of marked divergence between the sequence of S-AKAP84 and its human homolog are also informative. A potential leucine zipper region in human S-AKAP84 (residues 310–343, Fig. 2A) is not present in the mouse homolog. Thus, this domain does not play an essential role in targeting and anchoring PKAII isoforms.

Alignment of a partially characterized (10), extended RII-binding region from human S-AKAP84 (residues 331–389, Fig. 2A) with the corresponding region of mouse S-AKAP84 revealed only one highly-conserved cluster of amino acids: residues 306–325 in mouse S-AKAP84 are 75% identical (85% similar) with amino acids 344–363 in the human anchor protein (Fig. 2A). Alignment of this cluster of amino acids from S-AKAP84 with the thoroughly characterized 20-residue RII-binding site from AKAP75 (7) was also illuminating (Fig. 3A). The S-AKAP84 sequence contains amino acids with large hydrophobic side chains that align in perfect register with six Leu, Ile, and Val residues that are critical for high-affinity binding of RII and PKA isoforms by AKAP75 (7). Potential boundaries for the S-AKAP84 tethering site were identified from divergent features in the human and mouse anchor protein sequences that surround the conserved 20-residue cluster.

The C-terminal sequence contiguous with Leu325 (mouse S-AKAP84) is not highly conserved and contains Pro327. Proline is excluded from RII-binding domains in previously characterized AKAPs; moreover mutant AKAPs that contain Pro substitutions in the tethering domain are unable to form stable complexes with RII (6, 33). The dispensable leucine zipper motif is adjacent to the N terminus of the conserved candidate RII-binding region in human S-AKAP84. These observations suggest that the RII-binding site of S-AKAP84 is generated from amino acid residues included between Ile306 and Leu325 (Ile314 and Val316) in human S-AKAP84. Analysis of deletion mutations in the predicted RII tethering region experimentally verified that residues 306–325 mediate formation of S-AKAP84-RII complexes (see below).

An alternatively processed transcript of the murine S-AKAP84 gene is diminished in size by deletion of nucleotides 1774–1880 and 2142–2166 (Fig. 1A). The deletions eliminate the translation termination codon at nucleotides 1839–1841 and shift the reading frame after codon 525. These changes create a contiguous coding region for an additional 332 C-terminal amino acids (Fig. 3B). The resulting “long” form of the anchor protein (named AKAP121 for its apparent $M_r$ in denaturing electrophoresis) is composed of 857 residues and has a calculated $M_r$ of 84,000 in denaturing gel electrophoresis that exceeds its calculated $M_r$ by ~40%.

The amino acid sequence of mouse AKAP121 is not highly conserved with its human counterpart (Fig. 2B). The resulting C-terminal segment of AKAP121 is not homologous with previ-
ously characterized polypeptides. Nucleotide sequences at the 5' and 3' boundaries of the first deleted DNA segment (nucleotides 1774–1880) constitute consensus splice donor and acceptor sites. Thus, accumulation of S-AKAP84 in germ cells appears to be controlled (in part) by suppression of splicing at this exon. In contrast, excision of the exon is essential for the biogenesis of AKAP121. Deletion of nucleotides 2142–2166 is addressed below.

Fig. 1. Sequence of murine S-AKAP84 cDNA. Panel A presents the nucleotide sequence for S-AKAP84. The derived amino acid sequence is given below the corresponding codons. Panel B shows the unique sequence determined for the extreme 5' end of S-AKAP84 and AKAP121 cDNAs (uppercase letters) by application of an anchored PCR procedure (see "Experimental Procedures"). The sequence of adjoining 3' nucleotides (lowercase letters) is identical with a portion of the sequence obtained from cDNA inserts isolated from the mouse testis library in bacteriophage λgt11. The upstream flanking DNA (lowercase letters) was appended via a 5' PCR primer. Nucleotides 1–8 constitute a NotI restriction site. A stretch of dT residues was introduced to promote hybridization with a poly(dA) sequence that is added to the antisense cDNA strand by incubation with terminal transferase and dATP (20).
The mechanism that generates mouse AKAP121 is also operative in man. We previously isolated a human testis cDNA (10) that encodes an anchor protein composed of 903 amino acids (AKAP149, Fig. 2 B). AKAP149 mRNA (3.1 kb) is derived from a 3.2-kb transcript of the human S-AKAP84 gene via excision of a 107-bp alternative exon, as described above for AKAP121. (Full-length human S-AKAP84 cDNA is composed of 2380 nucleotides reported in Fig. 1 A (Ref. 10), plus 640 nucleotides of contiguous 3'-terminal sequence2,3 and polyadenylate.) The recent cloning of human AKAP149 cDNA from an intestinal carcinoma library (34) revealed that two discrete mRNAs encode a single anchor protein. The intestinal cDNA is derived from a 4.3-kb mRNA that accumulates in a variety of somatic cells (10, 34). The 4.3-kb mRNA shares an identical coding region with human germ cell AKAP149 mRNA, but contains a much longer 3'-untranslated region.

Alignment of the mouse AKAP121 and human AKAP149 homologs disclosed that the two anchor proteins are 71% identical overall (Fig. 2, A and B). Moreover, C-terminal segments of mouse AKAP121 and its human counterpart are 96% identical over 308 residues, indicating that this portion of the protein mediates an essential function in certain somatic cells. The absence of this sequence in the principal spermatid anchor protein (S-AKAP84) and the demonstrated ability of S-AKAP84 to bind and anchor RII subunits in situ (see below) suggest that the C-terminal region of AKAP121 does not participate in the docking of PKAII isoforms. Instead, one segment of the C-terminal region may sequester RNA at the mitochondrial surface. A fragment of AKAP121 bounded by amino acids 565 and 613 contains 13 key residues that are hallmarks of a consensus sequence known as a KH domain (Fig. 3 B and Refs. 34–37). KH domains are invariably involved in the creation and/or stabilization of RNA-binding sites in multiple proteins (35–37). Physiological functions of these proteins have not been defined with precision, but they appear to play roles in mRNA processing, translocation, and translation (35–37). The function of the KH domain in AKAP121 has not been established. The remainder of the C-terminal portion of AKAP121 (residues 614–857) is highly conserved and may contain additional functional domains.

A third type of S-AKAP84 cDNA encodes a mouse anchor protein (AKAP100) of intermediate size (637 residues). Amino acids 1–613 of AKAP100 are identical with the sequence of AKAP121 (Fig. 2). Thus, the alternative exon that lies between nucleotides 1774 and 1880 (Fig. 1 A) is excised in both AKAPs 121 and 100. However, nucleotides 2142–2166 (Fig. 1 A) are retained in AKAP100 cDNA. As a result, the reading frame-shifting at this point (yielding C614VSVLTRRLSAPCRQ-SSELDWEEV637) and a translation termination signal (TAA, nucleotides 2216–2218) is introduced after codon 637. Inspection of the cDNA sequence (Fig. 1) suggests that two closely spaced, alternative splice acceptor sites are probably present in the primary S-AKAP84 transcript. When splicing occurs at the distal AGGC consensus site (nucleotides 2165–2168) the mRNA encodes AKAP121; when exons are joined at the proximal GC dinucleotide (positions 2142, 2143) AKAP100 mRNA is generated. Differential utilization of several splice sites in S-AKAP84 pre-mRNA could provide a mechanism for controlling types and levels of anchor protein isoforms in spermatids and various somatic cells.

Messenger RNAs Encoding both S-AKAP84 and AKAP121 Accumulate in Developing Male Germ Cells—Northern gel analysis previously documented the appearance of 3.2-kb S-AKAP84 mRNA during late stages of spermatid development (10). Since this method is not sufficiently sensitive to distinguish between 3.2-kb S-AKAP84 and 3.1-kb AKAP121 mRNAs, we characterized germ cell transcripts by RNase protection analysis (Fig. 4 A). A cDNA insert, corresponding to nucleotides 1744–2225 (Fig. 1 A) was cloned in pGEM-7Z to generate a template for in vitro synthesis of 32P-labeled antisense RNA. Possible protected radiolabeled fragments that could be derived from the 481-nucleotide probe are shown schematically in Fig. 4 B. After hybridization of radiolabeled probe with germ cell RNA and digestion with RNases three protected fragments...
were detected (Fig. 4A, lanes 1 and 2). Fragments composed of 397 and 260 nucleotides correspond to cRNA sequences protected by S-AKAP84 and AKAP121 mRNAs, respectively (Fig. 4B). Densitometry revealed that similar amounts of these two mRNAs accumulate in vivo in germ cells. The strong signal produced by a 60-nucleotide cRNA fragment (Fig. 4A) is consistent with the predicted hybridization of the 5′ end of antisense RNA with both S-AKAP84 and AKAP121 mRNAs (Fig. 2).
The absence of protected antisense RNA that contains either 481 or 345 nucleotides indicates that (a) the distal splice acceptor site at nucleotide position 2166 (see above) is preferentially utilized for processing S-AKAP84 gene transcripts in spermatids and (b) AKAP100 mRNA is either a very minor species in germ cells or is produced only in somatic cells of testis.

**Preparation and Specificity of Antibodies Directed against Mouse S-AKAP84 and AKAP121 Polypeptides—When Western blots of murine male germ cell proteins were probed with antibodies directed against human S-AKAP84, the IgGs bound murine S-AKAP84 and also detected a low level of a 121-kDa polypeptide (10). The difference in apparent Mr values between the two immunoreactive proteins (equivalent to ~320 amino acids) suggested that the larger polypeptide could be encoded by the 3.1-kb AKAP121 mRNA. A caveat regarding this interpretation is that antibodies to human S-AKAP84 were directed against a portion of the protein (residues 193–353, Fig. 2A) that is only 41% identical with the mouse homolog. To rigorously investigate the relationship between the 121-kDa protein and the 3.1-kb mRNA, and also generate a tool for elucidating properties of mouse S-AKAP84 in situ, we produced an antiserum directed against the murine anchor proteins.

A CDNA insert (nucleotides 810–1550, Fig. 1A) which encodes a partial polypeptide shared by S-AKAP84 and AKAP121 (amino acids 205–451, Fig. 2) was cloned into the expression plasmid pET14b. This enabled high-level synthesis (in *E. coli*) of a fusion protein in which a 247-residue partial S-AKAP84/AKAP121 protein is preceded by a 20-residue N-terminal fusion peptide. The fusion peptide contains a stretch of six consecutive His residues that facilitate purification of recombinant protein to near-homogeneity via affinity chromatography on a Ni²⁺-chelate resin (Fig. 5A).

Antisera against the partial S-AKAP84 protein were generated in rabbits. The resulting antibodies produced robust signals with purified antigen and S-AKAP84 protein which is expressed predominantly in condensing spermatids (Fig. 5B). The IgGs also recognized a 121-kDa protein of low abundance in condensing spermatids (Fig. 5B, lane 4). Recombinant and endogenous proteins visualized with the antibodies bound ³²P-labeled RIIβ in standard overlay assays (4, 5) (data not shown).

Western blot analyses performed in the presence of excess recombinant antigen yielded no signals, thereby verifying the specificity of the antibodies. Scanning densitometry of chemiluminescence-derived signals obtained from the Western blots indicates that the ratio of S-AKAP84:AKAP121 proteins in developing germ cells is ~20, despite the presence of similar amounts of the cognate mRNAs (Fig. 4A). This suggests that either AKAP121 is highly labile in spermatids or the efficiency of anchor protein translation may be greatly increased by nucleotide sequences within the alternative exon that is retained in S-AKAP84 mRNA. The formal possibility that AKAP121 is...
and the signals were recorded on x-ray film. Nonspecific blots were developed by an enhanced chemiluminescence procedure as described under “Experimental Procedures.” Samples (25 µg) of total soluble E. coli proteins (lane 2) and proteins in the column flow-through (lane 1) were size-fractionated in a 0.1% SDS-10% polyacrylamide gel. Aliquots (10 µl) of fractions containing purified recombinant protein (apparent Mr = 40,000) eluted with 1 M imidazole were assayed in lanes 4–6. An aliquot (10 µl) of proteins eluted with buffer containing 20 mM imidazole was applied to lane 3. The gel was stained with Coomassie Blue. A, a Western immunoblot was prepared as described under “Experimental Procedures.” Lanes 1 and 6 received 25 ng of purified, recombinant S-AKAP84 fusion protein. Lanes 2–4 contained 30 µg of total protein isolated from purified populations of pachytene, round and condensing spermatids, respectively. Lane 5 is a duplicate of lane 4. The filter was probed with antiserum (1:1500 dilution) directed against epitopes shared by S-AKAP84 and AKAP121, as indicated under “Experimental Procedures.” Excess purified antigen (2 µg) was present when lanes 5 and 6 were probed with antibodies. The immunoblots were developed by an enhanced chemiluminescence procedure and the signals were recorded on x-ray film.

Fig. 5. Preparation and characterization of antibodies directed against murine S-AKAP84 and AKAP121. A, a His-tagged, partial AKAP84/121 protein (amino acid residues 205–451, Fig. 1A) was synthesized in E. coli BL21 and purified and Ni2+ chelate chromatography as described under “Experimental Procedures.” Samples (25 µg) of total soluble E. coli proteins (lane 2) and proteins in the column flow-through (lane 1) were size-fractionated in a 0.1% SDS-10% polyacrylamide gel. Aliquots (10 µl) of fractions containing purified recombinant protein (apparent Mr = 40,000) eluted with 1 M imidazole were assayed in lanes 4–6. An aliquot (10 µl) of proteins eluted with buffer containing 20 mM imidazole was applied to lane 3. The gel was stained with Coomassie Blue. B, a Western immunoblot was prepared as described under “Experimental Procedures.” Lanes 1 and 6 received 25 ng of purified, recombinant S-AKAP84 fusion protein. Lanes 2–4 contained 30 µg of total protein isolated from purified populations of pachytene, round and condensing spermatids, respectively. Lane 5 is a duplicate of lane 4. The filter was probed with antiserum (1:1500 dilution) directed against epitopes shared by S-AKAP84 and AKAP121, as indicated under “Experimental Procedures.” Excess purified antigen (2 µg) was present when lanes 5 and 6 were probed with antibodies. The immunoblots were developed by an enhanced chemiluminescence procedure and the signals were recorded on x-ray film.

converted to S-AKAP84 by post-translational, proteolytic processing in spermatids has not been excluded. Application of the 5'-rapid amplification of cDNA ends procedure (Fig. 1B and text above) revealed that all S-AKAP84 gene transcripts in germ cells are initiated at a single site. This observation and the identity of nucleotides 1–1773 in S-AKAP84 and AKAP121 cDNAs indicate that a single promoter activates transcription of the S-AKAP84 gene in spermatids. Therefore, selective accumulation of S-AKAP84 protein in spermatids is due (in part) to suppression of the splicing of the alternative exon bound by nucleotides 1774 and 1880 (Fig. 1A). Either diminished levels of general splicing factors or the presence of spermatid-specific splicing suppressors could account for this. S-AKAP84 was not detected on immunoblots of proteins from mouse1 and human12 (34) somatic cells and tissues. Instead, AKAP121 (AKAP149 in man) was evident in intestine, skeletal muscle, and several other tissues. Enhancement of differential splicing may be essential for directing the accumulation of AKAP121 in somatic cells.

Strategy and a System for Analyzing the Expression, Intracellular Targeting, and Function of S-AKAP84—Human HEK293 cells express both RIα (the predominant isoform) and RIβ subunits (12). Nearly all (~95%) of the RI subunits are incorporated into PKAII holoenzymes that are dispersed in the cytoplasm. The cells also contain a low level of AKAP79, which anchors ~5% of total PKAII (12, 28). When an AKAP75 transgene is introduced and the level of anchor protein is raised by

more than an order of magnitude, most of the RI subunits (and PKAIIα and β) are tethered to AKAP75 and immobilized in the cortical actin cytoskeleton (9, 12). Thus, abilities of candidate AKAPs to bind and translocate RI subunits to target organelles can be directly assessed in the context of a well characterized, intact cell system. Neither the cytosol nor total particulate fraction of homogenates of HEK293 cells contain proteins that bind antibodies directed against mouse S-AKAP84 and AKAP121 (Fig. 6A, lanes 3 and 4). Similar results were obtained with anti-human S-AKAP84 IgGs. A cDNA fragment encompassing nucleotides 53–1890 in Fig. 1A was inserted into the expression vector pCEP4 (see “Experimental Procedures”). Anti-S-AKAP84 IgGs bound an 84-kDa polypeptide produced by HEK293 cells that were transiently transfected with recombinant vector (Fig. 6A, lanes 1 and 2). HEK293 cells were also transfected with a recombinant pCEP4 vector that contains a transgene encoding the 857-residue mouse AKAP121 protein (alternative exon deleted). A 121-kDa polypeptide that binds antibodies directed against epitopes shared by S-AKAP84 and AKAP121 was evident in the particulate fraction of the transfected cells (Fig. 6B). The sizes of anchor proteins synthesized in transfected cells match the sizes of the major (84 kDa) and minor (121 kDa) RI-binding proteins observed in germ cells in vivo (Fig. 5B). The S-AKAP84 and AKAP121 proteins bound 32P-labeled RIβ and RIα subunits in overlay assays (data not shown).

Since HEK293 cells synthesize and assemble mitochondria in the absence of S-AKAP84 and AKAP121 (Fig. 6A, lanes 3 and 4), these organelles constitute “simple” target sites that lack endogenous anchor proteins. This allows the determination of the properties of transfected AKAPs in the absence of epitope tags or other modifications. Since S-AKAP84 (547 amino acids) is the predominant RI anchoring protein in spermatids, subsequent studies focused on the properties of this protein in situ.
Targeting and Tethering RII Subunits to Mitochondria

S-AKAP84 Is Targeted to the Outer Membrane of Mitochondria—Recombinant pCEP4 plasmid that contains cDNA encoding S-AKAP84 was introduced into HEK293 cells and stable transfectants were selected by their resistance to 0.2 mg/ml hygromycin. Cells named H-547.1 were used for studies presented below. H-547.1 cells accumulate S-AKAP84 and a small amount of 48-kDa polypeptide, which presumably corresponds to a proteolytic fragment of the anchor protein (Fig. 7A, lane 1). Cells were gently disrupted and highly-purified mitochondria were prepared by differential and Percoll density gradient centrifugation (29). More than 90% of S-AKAP84 co-purifies with mitochondria (Fig. 7A, lane 2). In contrast, a different distribution of RII was evident in cells expressing S-AKAP84. Approximately 15% of total RII was found in the mitochondrial membrane; the RII-binding site remains in the cytosolic fraction (Fig. 7A, lane 3). These results were confirmed by 32P-RII overlay binding assays (data not shown).

Thus, S-AKAP84 is targeted to the external surface of the outer mitochondrial membrane (as predicted by the current model) and mediates its tethering to the outer mitochondrial membrane. The quality of the mitochondria was monitored by assaying the activity and accessibility of cytochrome c oxidase, a component of the inner membrane of the organelle (30). Nearly all of the cytochrome oxidase activity was recovered in purified mitochondria (Fig. 8A). In the absence of detergent, cytochrome oxidase was unaffected by incubation of mitochondria with trypsin. However, cytochrome oxidase was inactivated by the simultaneous addition of Triton X-100 and trypsin (Fig. 8B). Thus, the integrity of the purified mitochondria was preserved, thereby permitting an assessment of the orientation of the RII-binding site of S-AKAP84. If the N terminus of the anchor protein is the sole portion of AKAP inserted into the outer mitochondrial membrane (as predicted by the current model) then epitopes included between residues 205 and 451 should be cleaved by external protease. S-AKAP84 immunoreactivity was visualized by Western immunoblot analysis as described under “Experimental Procedures.” In A, lane 1 received a sample of total particulate proteins from H-547.1 cells; lanes 2–5 contained proteins derived from the mitochondrial membrane, nuclear, and cytosolic fractions, respectively, of H-547.1 cells. Lanes 6 and 7 received samples of total particulate and purified mitochondrial proteins, respectively, isolated from control HEK293 cells. Parallel lanes that contained cytosolic, membrane, and nuclear proteins from control cells yielded no signals with anti-S-AKAP84 serum (data not shown). Lanes 8 and 9 contained proteins derived from mitochondria that were incubated with external trypsin (trypsin:mitochondrial protein, 1:50 (w/w)) in the absence and presence of 0.2% Triton X-100, respectively. In B, lanes 1–4 received proteins from the cytosolic (c), nuclear pellet (n), mitochondria-depleted membranes (m), and purified mitochondria (mt) fractions of control cells; lanes 5–8 contained proteins obtained from subcellular fractions of H-547.1 cells. Only the relevant portions of the immunoblots are shown. No other signals were observed.

FIG. 7. Distribution of S-AKAP84 and endogenous RII subunits in HEK293 and H-547.1 cells. Cells were disrupted and fractionated into cytosol, low speed pellet (nuclei), mitochondria, and a mixture of organelles depleted of mitochondria (“membranes”) as indicated under “Experimental Procedures.” Samples of proteins (30 µg) from each fraction were assayed for S-AKAP84 (panel A) and RII subunit (panel B) content by Western immunoblot analysis as described under “Experimental Procedures.” In A, lane 1 received a sample of total particulate proteins from H-547.1 cells; lanes 2–5 contained proteins derived from the mitochondrial membrane, nuclear, and cytosolic fractions, respectively, of H-547.1 cells. Lanes 6 and 7 received samples of total particulate and purified mitochondrial proteins, respectively, isolated from control HEK293 cells. Parallel lanes that contained cytosolic, membrane, and nuclear proteins from control cells yielded no signals with anti-S-AKAP84 serum (data not shown). Lanes 8 and 9 contained proteins derived from mitochondria that were incubated with external trypsin (trypsin:mitochondrial protein, 1:50 (w/w)) in the absence and presence of 0.2% Triton X-100, respectively. In B, lanes 1–4 received proteins from the cytosolic (c), nuclear pellet (n), mitochondria-depleted membranes (m), and purified mitochondria (mt) fractions of control cells; lanes 5–8 contained proteins obtained from subcellular fractions of H-547.1 cells. Only the relevant portions of the immunoblots are shown. No other signals were observed.

FIG. 8. Cytochrome c oxidase co-purifies with mitochondria from H-547.1 cells and is unaffected by external protease. A, cytochrome c oxidase activity was measured in subcellular fractions derived from H-547.1 cells as indicated under “Experimental Procedures.” Data are presented as the percentage of cytochrome oxidase associated with the indicated subcellular fractions. Recovery of cytochrome oxidase activity from cell homogenates was ~85% Similar results were obtained from non-transfected cells (data not shown). B, cytochrome oxidase activity in purified H-547.1 mitochondria was measured after incubation with trypsin (1:50 (w/w), trypsin:mitochondrial protein) for 30 min at 30 °C in the presence or absence of 0.2% Triton X-100. A 10-fold molar excess of Pefabloc (Boehringer Mannheim) was added after incubation with trypsin to inactivate the protease. The experiments in A and B were performed three times and very similar results were obtained in each repetition. Typical data are shown.

S-AKAP84 Is Incorporated into Mitochondria in Intact Cells—Confocal fluorescence microscopy was used to directly visualize the intracellular distribution of S-AKAP84 in H-547.1 cells.
cells. Cells were treated with a cell-permeant tetramethylrhodamine dye (Mitotracker Red) that is selectively sequestered in mitochondria (see “Experimental Procedures”). After incubation with 0.2 μM Mitotracker Red for 30 min, mitochondria of H-547.1 cells were clearly evident (Fig. 9B). The same pattern of fluorescence was seen in control HEK293 cells (not shown). Cells containing dye-labeled mitochondria were also probed by sequential incubations with rabbit anti-S-AKAP84 IgGs and fluorescein-tagged goat antibodies directed against rabbit IgGs.

FIG. 9. S-AKAP84 is incorporated into mitochondria in intact H-547.1 cells. H-547.1 cells were grown on coverslips and incubated with the rosamine dye Mitotracker Red (0.2 μM) for 30 min prior to fixation, permeabilization, and blocking with 3% albumin (see “Experimental Procedures” and Ref. 9). Cells were then incubated sequentially with antibodies directed against S-AKAP84 and then fluorescein isothiocyanate-conjugated goat IgGs directed against rabbit immunoglobulins. Fluorescence signals corresponding to antigen-antibody complexes (green, panel A) or mitochondrial-inserted Mitotracker Red (red, panel B) were collected with a Bio-Rad MRC 600 laser scanning confocal microscope (see “Experimental Procedures”). Panel C simultaneously displays the positions of S-AKAP84 and mitochondria thereby yielding a yellow color when the fluorescence signals overlap.

FIG. 10. Residues 1–30 are essential for immobilizing S-AKAP84 at the outer mitochondrial membrane. HEK293 cells were transiently transfected with a transgene encoding amino acids 31–547 of S-AKAP84 as described under “Experimental Procedures.” The distribution of the N-terminally truncated anchor protein among the cytosolic (c), nuclear (p), membrane (m), and mitochondrial (mt) fractions of the cells was visualized by Western immunoblot analysis as described in the legend for Fig. 7 and under “Experimental Procedures.”

| Table I | S-AKAP84 deletion mutants |
|---------|--------------------------|
| Designation* | Segment of S-AKAP84 expressed (residues) | RII binding activity |
| ΔN252-ΔC85 | 253–452 | + |
| ΔN236-ΔC85 | 287–452 | + |
| ΔN298-ΔC85 | 299–452 | + |
| ΔC85 306–452 | 306–452 | + |
| ΔN316-ΔC85 | 317–452 | - |
| ΔN335-ΔC85 | 336–452 | - |
| ΔN181-ΔC227 | 182–319 | - |
| ΔN30 | 31–547 | + |

* Each truncated mutant S-AKAP84 lacks the indicated number of N (ΔN) and/or C (ΔC) terminal amino acids.
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Fig. 11. RIIβ binding activity of truncated S-AKAP84 polypeptides. Deletion mutants of S-AKAP84 were expressed in E. coli BL21 as described under “Experimental Procedures.” Samples (0.5 μg of total protein; 20–50 ng of recombinant protein) of the soluble fraction of bacterial lysates were subjected to electrophoresis in a 0.1% SDS-12% protein; 20–50 ng of recombinant protein) of the soluble fraction of received extracts from E. coli indicated. Only the relevant regions of the blots are shown.

partial S-AKAP84 proteins that were expressed in E. coli is provided in Table I. All fusion proteins that contain residues 306–325 avidly bound 32P-labeled RII in overlay assays (Fig. 11). Fusion proteins lacking portions of this domain were unable to form complexes with RII subunits. Therefore, this 20–residue segment of the germ cell anchor protein governs the tethering of RII (PKAII) isoforms.

Conclusions—S-AKAP84, a prototype for the general class of organellae-associate anchor proteins, was targeted to mitochondria and excluded from other cell compartments. Thus, S-AKAP84 contains sufficient structural information to direct self-association with a target organelle. This excludes the possibility that sperm-specific co-factors (e.g. chaperones, anchor protein subunits etc.) are required to mediate organelle insertion. Previously (10), we observed that the positions of hydrophobic, hydroxylated, and charged amino acids in sequences of the N-terminal region of S-AKAP84 (residues 1–30) and an established N-terminal signal/anchor domain that inserts into the outer mitochondrial membrane in yeast are highly homologous (see Refs. 38 and 39 for details). Together, conservation of N-terminal sequence, exclusive association of wild type S-AKAP84 with mitochondria, and mislocalization of the N-terminally-truncated anchor protein indicate that targeting of RII subunits to the outer mitochondrial membrane is governed principally by a segment of S-AKAP84 composed of amino acid residues 1–30. The destruction of S-AKAP84 epitopes and RII binding activity by external proteases demonstrates that the RII-tethering site is located in the cytoplasm adjacent to the mitochondrial surface. The demonstration that expression of S-AKAP84 elicits association of endogenous RII subunits with mitochondria in H547.1 cells (Fig. 7) provides compelling documentation for both the proper orientation and physiological function of the tethering domain in situ. Since S-AKAP84 expression causes a redistribution of ~15% of total RII subunits to mitochondria in H547.1 cells, it is evident that the tethering domain of the anchor protein has a high-affinity for RII subunits in intact cells.

The current idea that various non-neuronal AKAPs concentrate and immobilize RII isoforms at the surface of organelles bounded by lipid bilayers (Golgi membranes, endosomes, peroxisomes etc.) was derived from indirect, correlative evidence (1–3). To identify organellar AKAP-PIKAII complexes that mediate critical physiological functions, it is essential to demonstrate that a candidate AKAP efficiently tethers and differentially targets RII subunits to a specific organelle within intact cells. Overall, properties documented for S-AKAP84 in intact cells strongly suggest that this protein could govern the localization and influence the functions of PKAII isoforms in vivo.

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