Characterization of S-AKAP84, a Novel Developmentally Regulated A Kinase Anchor Protein of Male Germ Cells*

Reigh-Yi Lin‡, Stuart B. Moss§, and Charles S. Rubin‡

From the ‡Department of Molecular Pharmacology, Atran Laboratories, Albert Einstein College of Medicine, Bronx, New York, 10461 and the §Department of Obstetrics and Gynecology, Division of Reproductive Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

In mammalian spermatogenesis, most of the type IIα isoform of cAMP-dependent protein kinase (PKAIIα) is anchored at the cytoplasmic surface of a specialized array of mitochondria in the flagellar cytoskeleton. This places the catalytic subunits of PKAIIα in proximity with potential target substrates in the cytoskeleton. The mechanism by which PKAIIα is anchored at the outer surface of germ cell mitochondria has not been elucidated. We now report the cloning of a cDNA that encodes a novel, germ cell A kinase anchor protein (AKAP) designated S-AKAP84. S-AKAP84 comprises 593 amino acids and contains a centrally located domain that avidly binds regulatory subunits (RIIα and RIIβ) of PKAIIα and PKAIIβ. The 3.2-kilobase S-AKAP84 mRNA and the cognate S-AKAP84 RII binding protein are expressed principally in the male germ cell lineage. Expression of S-AKAP84 is tightly regulated during development. The protein accumulates as spermatids undergo nuclear condensation and tail elongation. The timing of S-AKAP84 expression is correlated with the de novo accumulation of RIIα and RIIβ subunits and the migration of mitochondria from the cytoplasm (round spermatids) to the cytoskeleton (midpiece in elongating spermatids). Residues 1-30 at the NH2 terminus of S-AKAP84 constitute a putative signal/anchor sequence that may target the protein to the outer mitochondrial membrane. Immunofluorescence analysis demonstrated that S-AKAP84 is co-localized with mitochondria in the flagellum.

Multiple isoforms of cAMP-dependent protein kinase (PKA)1 are expressed during mammalian spermatogenesis (1–3). PKA isoforms are generated by the synthesis of four types of cAMP-binding or regulatory (R) subunits that are encoded by distinct genes (4, 5). R subunits undergo homodimerization and confer unique physical and functional properties on heterotetrameric (R1R2C1C2) PKAs that contain similar or identical catalytic (C) subunits (reviewed in Refs. 4 and 5). Individual PKA isoforms, which are named according to their R subunits, are thought to play specialized physiological roles at certain stages of male germ cell differentiation (1-3). Both the temporal patterns of expression and distinct intracellular locations of R subunit isoforms are consistent with this idea. RIIα and its cognate mRNA are expressed in premeiotic and early postmeiotic germ cells (1, 2). Moreover, the RIIα polypeptide persists in elongating spermatids and mature sperm. In contrast, RIIβ mRNA and protein are initially expressed at a relatively late postmeiotic stage. As spermatocytes proceed through nuclear condensation and elongation the level of RIIβ polypeptide declines, and this isoform is not detected in mature sperm (1, 2). RIIα expression is initiated in parallel with RIIβ accumulation. However, the level of RIIα polypeptide becomes maximal in late spermiogenesis, and the protein persists in mature, epididymal sperm (1–3, 6).

Activation of PKA in mature spermatids elicits the initiation and maintenance of flagellar movement (7, 8). This well established physiological response to cAMP is correlated with the cyclic nucleotide-stimulated sliding of a cytoskeletal structure (the fibrous sheath) over an internal microtubular structure called the axoneme (9). In mature spermatids PKAIα (RIIαC1αC2) is evident in the cytoplasm and plasma membrane (3). In contrast, a high proportion of RIIα and (PKAIIα) is sequestered in the cytoskeleton. Thus, the C subunit of PKAIIα may catalyze selectively the phosphorylation of proximal regulatory and/or structural proteins in the cytoskeleton. These phosphoproteins may, in turn, mediate the dynamic reorganization of the flagellar cytoskeleton that is essential for motility and fertilization.

Type IIα and type IIβ PKAs are often attached to the cytoskeleton or other organelles via the binding of RIIα or RIIβ with A kinase anchor proteins (AKAPs) (reviewed in Refs. 10 and 11). AKAP5, as typified by neuronal AKAPs 75, 79, and 150 (12–14), are proteins that possess both a high affinity binding site for RIIβ/RIIα and an independent domain that targets and links RII-AKAP complexes to specific intracellular sites (15). This arrangement can place PKAIα and/or PKAIIβ in proximity with organelle-bound substrates, thereby creating a target site for cAMP action (10–14). RIIα (PKAIIα) accumulates at two sites in the sperm flagellum (16). The bulk of the RIIα polypeptide is attached to the outer membrane of specialized "germ cell" mitochondria that form a helical array around the outer dense fibers and axoneme in the 'midpiece' of the flagellum. A lesser fraction of RIIα is associated with the fibrous sheath in a distal portion (the 'principal piece') of the flagellum.

Recently, Carrera et al. (17) cloned and sequenced cDNAs encoding a major fibrous sheath protein p82. p82 binds RIIα and contains two short segments that share sequence similarity with the targeting domains of neuronal AKAP75 (15). It is likely that p82 mediates the binding of PKAIα to the fibrous sheath.

Major questions regarding the targeting of signals carried by cAMP in germ cells remain unanswered. How is the bulk of...
PKAIIα anchored on the outside surface of mitochondria? Does an AKAP mediate this localization? If so, is a previously characterized or a novel AKAP polypeptide used? Is the anchor protein developmentally regulated and targeted principally to mitochondria?

We now describe the cloning and characterization of cDNAs that encode a unique germ cell anchor protein designated spermatid AKAP84 (S-AKAP84). S-AKAP84 contains an RII binding site and a putative mitochondrial targeting sequence. Moreover, S-AKAP84 accumulates as spermatids undergo nuclear condensation and flagellar elongation. The timing of expression of S-AKAP84 appears to be synchronized with the accumulation of RII subunits and the migration of mitochondria from the cytoplasm (round spermatids) to the cytoskeleton (midpiece in elongating spermatids).

Immunofluorescence analysis documented directly that the RII binding protein is colocalized with the helical array of mitochondria in the midpiece of the flagellum.

**EXPERIMENTAL PROCEDURES**

**Screening of cDNA Libraries—**A human testis cDNA library in the expression vector agt11 (Clontech, Palo Alto, CA) was screened by the procedure described by Carrera et al. (12, 13). This method detects the 32P-labeled RII-binding proteins in phage plaques by their ability to bind 32P-labeled RIIβ. Two recombinant phage clones (λT1 and λT2) that contained overlapping cDNA sequences were purified from a population of 5 × 106 plaques. A 5 fragment of the larger (18-kilobase pair) cDNA (λT1) was employed as a template to synthesize random-primed, 32P-labeled cDNA for further screening (see "Results"). Eight independent cDNA clones (λT3–T10) were obtained from a human testis 5‘ stretch ( oligo(dT)- and random-primed) cDNA library in bacteriophage agt10 (Clontech) using procedures and conditions described in a previous publication (18).

**DNA Sequence Analysis—**cDNAs isolated from phage clones λT1–T10 were sequenced using plasmid pGEMTZ (Promega) to generate the plasmid series pG11–pGT10. DNA inserts were sequenced by the dideoxynucleotide chain termination procedure of Sanger et al. (19) as described previously (18, 20). Sequencing reagents were obtained from U.S. Biochemical Corp.

**Computer Analysis—**Analyses of sequence data, sequence comparisons, and data base searches were performed using PCGENE-IntelliGenetics software (IntelliGenetics, Mountainview, CA) and the BLAST program (21) provided by the NCBI server at the National Library of Medicine/National Institutes of Health.

**Expression and Purification of a Partial S-AKAP84 Polypeptide—**A DNA insert that encodes amino acids 193-357 in S-AKAP84 was amplified with primers designed from cDNA sequences (18). The 5‘ primer (5’-CTGACATATGGGGGAGAGGGGAAGAGAGA-3’) introduced an ATG codon in the context of an NdeI restriction site. This sequence preceded 21 nucleotides that encode the first 7 residues at the NH2 terminus of the truncated protein. Each 3‘ primer (5’-ATCGGGATCCTCCACCTGT-3’) contained a 3‘ terminal BamHI site and created a stop codon after the desired COOH-terminal residue. The remaining 21 nucleotides in the 3‘ primer complement the last 7 codons at the COOH terminus of the truncated protein. Mutant-nucleotided-directed truncated cDNAs were cloned into pET14b, and truncated proteins were expressed in E. coli BL21 (DE3) as described by Li and Rubin (22). Two recombinant clones (DE3) were selected for their ability to bind 32P-labeled RIIβ with high affinity. Positive recombinant phage clones (λT1 and λT2) were purified by selecting for the size-fractionated polypeptides and molecular weight standards to an immobilon P (polyvinylidene difluoride) membrane (Millipore Corp.), as described by Bregman et al. (12).

**Assay for RIIβ Binding Activity—**Western blots were probed with 0.3 ng 32P-labeled RIIβ (2 × 105 cpm 32P radioactivity/ml), and RIIβ binding proteins were visualized by autoradiography as described in previous papers (12, 13, 15). Results were quantified by scanning and integrating with a PhosphorImager (Molecular Dynamics) coupled to a computer running ImageQuant software (Molecular Dynamics).

Western Immunoblot Assays—Western blots of proteins from fractionated germ cell tests were blocked, incubated with antisem (1:1000 relative to serum), and washed as described previously (15, 26). S-AKAP84 and partial S-AKAP84 polypeptides were visualized by indirect chemiluminescence using an ECL kit from Amersham Corp. as previously reported (26, 27). Relative amounts of antigen were determined by scanning densitometry (Molecular Dynamics) of chemiluminescence signals on x-ray film. Standard curves were prepared with 0.5–100 ng of purified S-AKAP84. Amounts of S-AKAP84 protein in experimental samples were obtained from the linear portion of the standard curve.

Northern Gel Analysis—Total RNA was isolated from purified germ cells and mouse testis as described by Carrera et al. (17). RNAs were fractionated by electrophoresis in a denaturing agarose gel, transferred to nitrocellulose, and probed with the 32P-labeled cDNA. Hybridization, washing, and autoradiography were performed as described previously (28).

**Immunofluorescence Microscopy—**A mixed germ cell population was prepared by mincing testis with a razor blade and then gently pipetting to release cells from the seminiferous tubules. Germ cells were fixed with high affinity. Two positively stained populations of each cell type were obtained by scanning densitometry (Molecular Dynamics) of chemiluminescence signals on x-ray film. Standard curves were prepared with 0.5–100 ng of purified S-AKAP84. Amounts of S-AKAP84 protein in experimental samples were obtained from the linear portion of the standard curve.

Northern Gel Analysis—Total RNA was isolated from purified germ cells and mouse testis as described by Carrera et al. (17). RNAs were fractionated by electrophoresis in a denaturing agarose gel, transferred to nitrocellulose, and probed with the 32P-labeled cDNA. Hybridization, washing, and autoradiography were performed as described previously (28).

**Immunofluorescence Microscopy—**A mixed germ cell population was prepared by mincing testis with a razor blade and then gently pipetting to release cells from the seminiferous tubules. Germ cells were fixed and permeabilized as described by Carrera et al. (17). The specimens were incubated overnight with affinity-purified anti-S-AKAP84 IgGs (1:1000 relative to serum), and washed as described previously (15, 26). S-AKAP84 and partial S-AKAP84 polypeptides were visualized by indirect chemiluminescence using an ECL kit from Amersham Corp. as previously reported (26, 27). Relative amounts of antigen were determined by scanning densitometry (Molecular Dynamics) of chemiluminescence signals on x-ray film. Standard curves were prepared with 0.5–100 ng of purified S-AKAP84. Amounts of S-AKAP84 protein in experimental samples were obtained from the linear portion of the standard curve.

**RESULTS**

Cloning and Sequence Analysis of cDNAs Encoding a Novel AKAP—A human testis cDNA library in bacteriophage agt11 was screened for the expression of β-galactosidase fusion proteins that bind 32P-labeled RIIβ with high affinity. Two positive recombinant phage clones (λT1 and λT2) were purified to ho-
mogeneity. The cDNA inserts from λT1 and λT2 were subcloned into the EcoRI site of pGEM7Z and sequenced. The larger λT1 cDNA insert included the DNA sequence from λT2 and began with an open reading frame for 401 amino acids. The coding region was followed by a translation termination signal and 5'-untranslated sequence. These observations and the absence of a consensus initiator ATG indicated that the cDNAs were incomplete and lacked upstream coding and 5'-untranslated sequences.

Subsequently, a 499-base pair Apal fragment, corresponding to nucleotides 123–621 at the 5' end of the λT1 cDNA, was used to generate 32P-labeled cDNA for further screening. A human testis cDNA library enriched in 5'-sequences yielded eight cDNA clones that were analyzed by sequencing. The cDNA designated pGT7 contained novel 5'-coding and 5'-untranslated sequences linked to the complete downstream coding and untranslated sequences determined for λT1 cDNA. The remaining phage clones contained overlapping partial cDNA sequences that were identical to portions of the cDNAs in pGT7 and λT1. The net result from multiple rounds of analysis is that each segment of the testis AKAP cDNA was sequenced from both strands in at least three independent cDNA clones. The data are presented in Fig. 1A.

An open reading frame of 1779 base pairs begins at an initiator ATG (nucleotides 63–65) that lies within a consensus translation start site (29). Although the exact size of the 5'-untranslated region is unknown, the occurrence of an in-frame translation stop codon at nucleotides 42–44 precludes the possibility of utilizing a distal, upstream initiator ATG. The AKAP open reading frame terminates with a stop codon at nucleotides 1842–1844 (Fig. 1A). The sequence of >500 base pairs of 3'-untranslated sequence was determined, but neither a poly(A) tail nor a polyadenylation signal was evident. Since a 3.2-kb mRNA hybridized with the newly cloned cDNA (see Fig. 4), it is possible that the total length of the 3'-untranslated region exceeds 1.2 kb. However, the length and complete sequence of the 5'-untranslated region also remain to be determined.

Nomenclature and Structural Features of the S-AKAP84 Polypeptide—The protein encoded by the cDNA sequence shown in Fig. 1A is named spermatid-A kinase anchor protein 84 or S-AKAP84 because it (a) binds RIβ and RIα, (b) exhibits an apparent Mr of 84,000 upon electrophoresis in an SDS-polyacylamide gel, and (c) is expressed in male germ cells. Experiments documenting these properties are presented below.

The open reading frame in Fig. 1A reveals that S-AKAP84 comprises 593 amino acids and has a calculated Mr of 62,912. The amino acid composition of S-AKAP84 is atypical; the protein is enriched in Glu, Pro, and Ser, which account for 31% of the residues in the predicted sequence. The abundance of Glu residues is reflected in the predicted isoelectric point (pI = 4.55) and net charge (−36) of the polypeptide at pH 7.4.

The sequence of S-AKAP84 is not homologous with protein sequences compiled in the SWISS-PROT, GenBank™, and PIR data bases. However, the novel protein includes two short motifs that may correspond to functional domains. Four tandem repeats of the heptapeptide consensus sequence DRN-EEGL are evident between amino acid residues 310 and 338 (Fig. 1A). The beginning and end of the reiterated region are closely bordered by the tripeptide NEE (residues 306–308 and 341–343), which corresponds to the central core of the consensus heptapeptide. The occurrence of a Leu residue at the seventh position in each repeated unit raises the possibility that this segment of S-AKAP84 constitutes a coiled-coil or leucine zipper region (30). Leucine zipper regions often mediate the homo- and/or hetero-oligomerization of protein subunits. An
important caveat is that the heptapeptide repeats of S-AKAP84 lack an internal hydrophobic residue that is conserved in prototypical leucine zipper domains (30, 31).

A lengthy hydrophobic region (amino acid residues 8–26, Fig. 1A) is evident near the NH$_2$ terminus of S-AKAP84. The hydrophobic domain and contiguous NH$_2$- and COOH-terminal flanking sequences were compared with classical “signal sequences” via computer programs (32, 33). The results suggest that this portion of S-AKAP84 does not constitute a classical signal sequence capable of targeting the protein to a secretory pathway or the plasma membrane. Instead, aspects of the organization and properties of the NH$_2$ terminus of S-AKAP84 match more closely with features described for the NH$_2$-terminal segment of NADH-cytochrome $b_6$ reductase (34, 35). The key shared properties in S-AKAP84 are as follows: (a) the first 7 residues include basic and hydroxylated amino acids (Arg$^b$, Ser$^b$); (b) a long, extremely hydrophobic segment (19 residues) provides a contiguous stop-transfer/hydrophobic anchor domain; (c) positively charged downstream residues (Arg$^b$, Lys$^b$) are more evident in Fig. 7, lane 5 (see below), where twice as much protein was analyzed and the time of exposure to x-ray film was increased by a factor of 2.5.

Preparation and Specificity of Anti-S-AKAP84 IgGs—Western blots were prepared as described under “Experimental Procedures.” A, lanes 1 and 2 were loaded with 30 µg of particulate P21 fusion protein; B, lanes 1 and 2 received 30 µg of particulate proteins from mouse testis. The membranes were probed with affinity-purified anti-S-AKAP84 IgGs (1:1000) as described under “Experimental Procedures.” Purified P21 (1 µg/ml) was included in the buffer when membranes containing the proteins fractionated in lane 2, (A) and lane 2 (B) were incubated with affinity-purified IgGs. The immunoblots were developed by the enhanced chemiluminescence procedure, and the signals were recorded on x-ray film. The weakly immunoreactive proteins (120 kDa and <84 kDa in testis (B, lane 1) are more evident in Fig. 7B, lane 5 (see below), where twice as much protein was analyzed and the time of exposure to x-ray film was increased by a factor of 2.5.

An RII Binding Site Lies between Residues 331 and 389 in the S-AKAP84 Polypeptide—A combination of deletion mutagenesis and expression of partial S-AKAP84 polypeptides in E. coli was used to map the RII binding region. The ability of wild type and truncated S-AKAP-84 proteins to bind $^{32}$P-labeled RII$\beta$ was monitored by an “overlay binding assay” (13, 15, 22). (S-AKAP84 also binds RII$\alpha$ avidly, but studies described in this paper were performed with RII$\beta$. RI subunits are not bound by S-AKAP84). Amounts of full-length and partial AKAP polypeptides synthesized were independently assessed by Western immunoblot analysis.

Partial S-AKAP84 proteins were generated by NH$_2$- and/or COOH-terminal truncation as indicated in Table I. Signals indicating expression levels and RII$\beta$ binding activities for a representative subset of mutant proteins are shown in Fig. 3. Deletions of ~200 amino acids from the COOH and NH$_2$ termini of S-AKAP84 did not markedly alter specific RII$\beta$ binding activity (i.e. binding of $^{32}$P-labeled ligand/mol of expressed protein) (Fig. 3A, lanes 1–7). However, when residues 358–389 were eliminated, binding activity was extinguished (Fig. 3A, lane 8). The absence of RII$\beta$ binding activity in a partial S-AKAP84 protein that contains residues 358–593 (Fig. 3A, lane 9) suggests that Glu$^{358}$ and adjacent NH$_2$-terminal residues (Fig. 1A) constitute a portion of the RII$\beta$ tethering domain. Inspection of residues 355–376 in the S-AKAP84 sequence revealed that the positions of branched aliphatic side chains (VIX$_3$VX$_3$VX$_3$VX$_3$VX$_2$V) can be aligned in register with bulky aliphatic residues that mediate the high affinity binding of RII$\beta$ by neuronal AKAP75 (LLX$_6$LVX$_3$VX$_3$VX$_3$LV) (15). The RII$\beta$ tethering site in S-AKAP75 comprises amino acids 392–413 and functions as an independent domain (15). Only one potentially significant difference is noted in the comparison. The occurrence of Ala$^{372}$ in S-AKAP75 conserves the hydrophobic character and $\alpha$-helix-forming propensity of Ile$^{389}$ in AKAP 75, but the methyl side chain contributes a smaller apolar surface. Substitution of branched chain amino acids with Ala diminishes the avidity of AKAP75 for RII$\beta$. However, the presence of neighboring Val$^{371}$ in S-AKAP84 may compensate for this

| Designation* | Segment of S-AKAP84 expressed (residues) |
|--------------|-----------------------------------------|
| $\Delta$0 (wild type) | 1–593 |
| $\Delta$N34 | 35–593 |
| $\Delta$N115 | 116–593 |
| $\Delta$N190 | 191–593 |
| $\Delta$N190–$\Delta$C97 | 191–496 |
| $\Delta$N190–$\Delta$C124 | 191–469 |
| $\Delta$N190–$\Delta$C151 | 191–443 |
| $\Delta$N190–$\Delta$C177 | 191–416 |
| $\Delta$N190–$\Delta$C204 | 191–389 |
| $\Delta$N190–$\Delta$C235 | 191–358 |
| $\Delta$N358 | 359–593 |
| $\Delta$N358–$\Delta$C97 | 359–496 |

*Each truncated mutant S-AKAP84 lacks the indicated number of NH$_2$-($\Delta$N) and/or COOH-($\Delta$C) terminal amino acids.
deficiency.

Based on the preceding considerations a truncated S-AKAP84 protein that corresponds to amino acids 331–443 was expressed in E. coli. Twenty-four residues that precede the putative RII binding site were retained because (a) this region is flanked by a long NH$_2$-terminal segment in wild-type S-AKAP84 and (b) there is no precedent for an RII binding domain that is congruent with the NH$_2$ terminus of an AKAP (10–15). The RII$\beta$ binding activity of this mutant was similar to that observed for S-AKAP84 and partial S-AKAP84 proteins in lanes 1–7 of Fig. 3A. Thus, amino acid residues that govern RII binding activity in S-AKAP84 lie between residues 331 and 389.

Determination of the Size and Relative Abundance of S-AKAP84 mRNA in Various Tissues—A Northern blot containing poly(A$^+$) RNA from several human tissues was probed with $^{32}$P-labeled S-AKAP84 cDNA. A 3.2-kb mRNA from testis hybridized strongly with a 3.2-kb mRNA from mouse germ cells (Figs. 5 and 6). This mRNA encodes a murine RII$\beta$ binding protein that is homologous with human S-AKAP84. Murine S-AKAP84 mRNA was not detected in the population of total testis poly(A$^+$) RNAs during early postnatal and juvenile development (Fig. 5). The transcript is not evident even when postmeiotic round spermatids are present (days 20 and 24; Fig. 5). However, a substantial level of S-AKAP84 mRNA accumulates as the animals gain the capacity to reproduce (Fig. 5, lane A).

The expression pattern is consistent with the appearance of S-AKAP84 mRNA during a late phase of spermatid development. This possibility was addressed by monitoring the expression of S-AKAP84 and its cognate mRNA in purified populations of developing germ cells that correspond to meiotic pachytene spermatocytes, postmeiotic round spermatids, condensing spermatids (which undergo nuclear condensation and assume the elongated morphology of mature sperm), and epididymal sperm. S-AKAP84 mRNA was not detected in either pachytene spermatocytes or round spermatids (Fig. 6, lanes 2 and 3). In contrast, the 3.2-kb S-AKAP84 transcript is clearly evident in an enriched population of condensing/elongating spermatids (Fig. 6, lane 4). S-AKAP84 mRNA is not retained in mature sperm. The expression pattern of an 84-kDa RII$\beta$ binding protein parallels the developmentally controlled accumulation and disappearance of S-AKAP84 mRNA (Fig. 7A). A can-

3 Q. Chen, R. Y. Lin, and C. S. Rubin, unpublished observations.
The measurements. It is also possible that the 84-kDa protein and RII binding activity (Fig. 7A) are under denaturing conditions and transferred to nitrocellulose as described under “Experimental Procedures.” The Northern blot was probed and calibrated as described in “Experimental Procedures” and Fig. 4. The size of the S-AKAP84 transcript is indicated. An autoradiogram is shown.

Fig. 6. Expression of S-AKAP84 mRNA in fractionated germ cells. Total RNA (20 μg/lane) from spleen (a negative control, lane 1), pachytene spermatocytes (lane 2), round spermatids (lane 3), and condensing spermatids (lane 4) were subjected to Northern gel analysis as described in the legend to Fig. 5 and under “Experimental Procedures.” An autoradiogram is presented, and the size of S-AKAP84 mRNA is indicated.

The apparent Mr of the murine RIIβ binding protein is estimated to be 84,000 when it is compared with protein standards. Proteins of similar size are observed when extracts of rat, human, and bovine testis are subjected to RIIβ binding and immunoblot analyses. Other tissues do not contain the 84-kDa protein. The discrepancy between the calculated Mr (63,000) and the apparent Mr is probably due to the highly acidic and Pro-rich nature of the protein (Fig. 1A). Proteins with these properties often bind SDS poorly and assume atypical conformations under denaturing conditions. Other AKAPs exhibit similar properties and have aberrantly large apparent Mr values (10, 26).

A very low level of a 120-kDa protein is also evident in testis and fractionated germ cells (Fig. 7B, lanes 2, 3, and 5). This protein (917 residues) is encoded by a 3.1-kb, alternatively spliced S-AKAP84 mRNA. Structure/function analysis of the 120-kDa protein is in progress.

S-AKAP84 Is Targeted to Mitochondria in the Midpiece of Condensing Spermatids—The intracellular location of S-AKAP84 was established by immunofluorescence microscopy. When a mixed population of developing germ cells was probed with antibodies directed against S-AKAP84 only condensing spermatids (Fig. 8) and their developmental by-product, anucleate residual bodies (not shown), emitted intense fluorescence signals. The fluorescence signal from condensing spermatids was precisely congruent with the previously described (30) col of ∼70–80 mitochondria that form a helical array in the midpiece (Fig. 8). Staining was excluded from the head, the tail, and the remainder of the flagellum including the axoneme, the fibrous sheath, the outer dense fibers, and the plasma membrane. Thus, S-AKAP84 accumulates de novo in a late stage of spermatogenesis. In addition, this anchor protein is targeted selectively to the major RII binding site in murine spermatozoa, the mitochondrial sheath of the midpiece region.

DISCUSSION

A cDNA encoding a novel, spermatid AKAP (S-AKAP84) has been cloned and characterized. Previous investigations documented that PKAIIα is associated with two distinct components of the spermatozoan cytoskeleton: the fibrous sheath in the principal piece and the outer membranes of an array of mitochondria located in the midpiece (16). Most of the RIIα subunits are sequestered by the mitochondria. Recently, Carrera et al. (17) demonstrated that an abundant cytoskeletal protein, p82, mediates the anchoring of RII (PKAII) in the fibrous sheath. Experiments described herein strongly implicate S-AKAP84 in the delivery (targeting) of RII (PKAII) to the...
midpiece. The NH₂-terminus of S-AKAP84 has several structural features that parallel the properties of signal/anchor domains (see "Results"). Such domains target NADH-cytochrome b₅ reductase and several other proteins to the outer mitochondrial membrane (35). The properties of the NH₂-terminal segment of S-AKAP84 (residues 1–30) are consistent with the location of the protein in situ. Immunofluorescence microscopy demonstrated that S-AKAP84 is targeted selectively to a large, helical structure in the midpiece of spermatids undergoing nuclear condensation and tail elongation (Fig. 8). The helical superstructure corresponds to a precisely arranged assembly of 70–80 mitochondria, otherwise known as the mitochondrial sheath (36). Thus, S-AKAP84 accumulates in a flagellar compartment that also contains a high proportion of the anchored RIIα (PKIα) in mature spermatids. The highly specialized germ cell mitochondria are components of the midpiece cytoskeleton. In late postmeiotic stages of spermatogenesis, a portion of the outer membrane of each mitochondrion closely apposes the outer dense fibers (another cytoskeletal structure), whereas the remaining mitochondrial surface directly contacts the cytoplasm.

The putative signal/anchor domain of S-AKAP84 is predicted to insert into the outer membrane bilayer post-translationally (35). Thus, all other functional domains in organelle-sequenced S-AKAP84 molecules would be concentrated in a thin layer of cytoplasm adjacent to the mitochondrial surface. Therefore, the RII binding region located between residues 331 and 389 in S-AKAP84 would be accessible to mediate the tethering of cytoplasmic PKAII isoforms. PKAII captured by this mechanism could control or modulate flagellar activities by phosphorylating co-localized target substrates in the cytoskeleton, thereby altering their functional and/or structural properties.

The sequence of S-AKAP84 diverges markedly from the primary structures of all other AKAPs, with the exception of one short but potentially important domain. A 22-residue segment of the RII binding region of S-AKAP84 (amino acids 355–376) contains a group of hydrophobic, branched chain amino acids that align precisely with Ile, Leu, or Val residues in the 22-amino acid sequence that constitutes the RIIβ binding domain of the neuronal AKAP, AKAPE75 (15). Moreover, truncated S-AKAP84 polypeptides that lack residues 355–358 or 359–376 have no RII binding activity (Fig. 3, and "Results"). Since (a) the long, aliphatic side chains in the tethering domain of AKAP75 are essential for high affinity RII binding activity and (b) similar patterns of hydrophobic, aliphatic side chains are evident in non-neuronal AKAPs (10, 11), it appears that the sequence encompassed by residues 355–376 in S-AKAP84 may constitute a tethering site. The inclusion of a 20–22-residue module that conserves the spacing among long aliphatic side chains (and presumably a precise orientation of hydrophobic surfaces in a higher order structure) in otherwise divergent proteins, provides a mechanism for targeting PKAII isoforms to a diverse array of intracellular target sites (see Refs. 10 and 11).

Overall, the properties of S-AKAP84 fulfill criteria that are used in establishing the physiological significance of an anchor protein. The polypeptide contains structural features that appear to account for both the sequestration of RII and the targeting/tethering of AKAP-RII (or PKAII) complexes at a previously established target site for cAMP action. Further experiments, involving site-directed mutagenesis and transfection/expression analysis, will be required to (a) verify the proposed roles of the NH₂-terminal targeting and centrally located tethering domains of S-AKAP84 and (b) elucidate the exact roles of individual amino acids in each functional domain.

The pattern of the developmental regulation of S-AKAP84 expression is quite atypical and provides additional, albeit indirect, cues regarding the function of the anchor protein. The RII binding protein and its cognate mRNA are apparently expressed de novo during late spermiogenesis (Figs. 5–7). Since the transcription of most germ cell genes is silenced at or before this stage of development (37), the data suggest that S-AKAP84 expresses a "late" developing, cAMP-mediated signaling pathway that emerges with temporal precision. Furthermore, S-AKAP84 accumulation occurs as the RIIα and RIIβ polypeptides reach their maximal levels (1, 2) and in parallel with the remodeling and reorganization of spermatid mitochondria (36). In the latter instance, classicaly shaped mitochondria are dispersed in the cytoplasm of round spermatids; as the cells undergo nuclear condensation and tail elongation the mitochondria migrate to the outer dense fibers of the midpiece cytoskeleton and assume a crescent shape (36). These mitochondria align end-to-end to form a helical array. It is thought that preexisting mitochondria are modified by the insertion of novel proteins during this period of transformation. Our results indicate that S-AKAP84 and possibly S-AKAP84 complexed with PKAII are incorporated into the outer mitochondrial membrane during this major developmental transition. These observations raise the possibility that S-AKAP84-
PKAII complexes play a crucial role in initiating or modulating this key step in sperm development. Ultimately, it will be necessary to disrupt the S-AKAP84 gene or otherwise compromise S-AKAP84 expression to establish the exact physiological role of the anchor protein.

A puzzling aspect of the results is that S-AKAP84 is not detected in mature mouse sperm. In contrast, the association of RIIα with the mitochondrial sheath has been documented by electron microscopy in mature bovine sperm (16). Several possible explanations may account for this apparent discrepancy. Murine S-AKAP84 may be labile and subject to artifactual degradation during the preparation of murine spermatozoa. Alternatively, S-AKAP84 may target and retention in mature sperm is species-specific and not physiologically relevant. Alternatively, S-AKAP84 may play a central physiological role only in condensing spermatids and its vestigial significance of the low level RII binding activity associated with the 105-kDa protein remain to be investigated. Irrespective of whether S-AKAP84 functions only in condensing spermatids or over a broader span of development, it represents the first example of a developmentally regulated, mitochondrially targeted RII anchor protein.

Acknowledgement—We thank Ann Marie Alba for expert secretarial services.

REFERENCES
1. Lonnerberg, P., Parvinen, M., Jahnson, T., Hansson, V., and Persson, H. (1992) Biol. Reprod. 46, 1057–1068
2. Landmark, B. F., Oyen, O., Skalhegg, B. S., Fauske, B., Jahnson, T., and Hansson, V. (1993) J. Reprod. Fertil. 99, 323–334
3. Horowitz, J. A., Toeg, H., and Orr, G. A. (1984) J. Biol. Chem. 259, 832–838
4. Taylor, S. S., Buechler, J. A., and Yonemoto, Y. (1990) Annu. Rev. Biochem. 59, 971-1005
5. Francis, S. H., and Corbin, J. D. (1994) Annu. Rev. Physiol. 56, 237–272
6. Horowitz, J. A., Wasco, W., Leiser, M., and Orr, G. A. (1988) J. Biol. Chem. 263, 2098–2104
7. Tash, J. S., and Means, A. R. (1982) Biol. Reprod. 26, 745–763
8. Brokaw, C. J. (1987) J. Cell Biol. 105, 175–184
9. Li, Y., and Okuno, M. (1993) Exp. Cell. Res. 208, 170–174
10. Rubin, C. S. (1994) Biochim. Biophys. Acta 1224, 467–479
11. Scott, J. D., and McCartney, S. (1994) Mol. Endocrinol. 8, 5–11
12. Bregman, D. B., Bhattacharyya, N., and Rubin, C. S. (1989) J. Biol. Chem. 264, 4648–4656
13. Bregman, D. B., Hirsch, A. H., and Rubin, C. S. (1991) J. Biol. Chem. 266, 7107–7113
14. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Cone, R. D., and Scott, J. D. (1992) J. Biol. Chem. 267, 16816–16823
15. Glantz, S. B., Li, Y., and Rubin, C. S. (1993) J. Biol. Chem. 268, 12796–12804
16. Lieberman, S. J., Wasco, W., MacLeod, J., Satir, P., and Orr, G. A. (1988) J. Cell Biol. 107, 1809–1816
17. Carrera, A., Gerton, G. L., and Moss, S. B. (1994) Dev. Biol. 165, 272–284
18. Lu, X., Cross, R. E., Bagchi, S., and Rubin, C. S. (1996) J. Biol. Chem. 265, 3293–3303
19. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci., U. S. A. 74, 5463–5467
20. Hu, E., and Rubin, C. S. (1990) J. Biol. Chem. 265, 5072–5080
21. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
22. Li, Y., and Rubin, C. S. (1995) J. Biol. Chem. 270, 1935–1944
23. Land, M., Islas-Trejo, A., Freedman, J. H., and Rubin, C. S. (1994) J. Biol. Chem. 269, 9234–9244
24. Ronnert, L. Y., Belive, A. R., and Favocott, D. W. (1976) Dev. Biol. 40, 119–131
25. Belive, A. R., Milette, C. F., Bhatnager, Y. M., and O'Brien, D. A. (1977) J. Histochem. Cytochem. 25, 480–494
26. Hirsch, A. H., Glantz, S. B., Li, Y., You, Y., and Rubin, C. S. (1993) J. Biol. Chem. 267, 2131–2134
27. Nubukka, C., Li, Y., and Rubin, C. S. (1993) J. Biol. Chem. 268, 7621–7624
28. Sul, H. S., Wise, L. S., Brown, M. L., and Rubin, C. S. (1991) J. Biol. Chem. 259, 1201–1205
29. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
30. Landschultz, W. H., Johnson, P. F., and McKnight, S. L. (1988) Science 240, 1759–1764
31. O'Shea, E., Klimrm, J. D., Kim, P. S., and Auer, T. (1991) Science 254, 539–544
32. von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690
33. Gavel, Y., and von Heijne, G. (1990) Protein Eng. 4, 33–37
34. Biehler, E. M., Muller, S., and Schatz, G. (1993) J. Biol. Chem. 268, 1203–1210