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Regulation, Localization, and Anchoring of Protein Kinase A Subunits during Mouse

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The molecular basis of mammalian sperm capacitation, defined as those biochemical and functional changes that render the sperm competent to fertilize the egg, is poorly understood. This extratesticular maturational process is accompanied by the activation of a unique signal transduction pathway involving the cAMP-dependent up-regulation of protein tyrosine phosphorylation presumably through the activation of protein kinase A (PK-A). We demonstrate in this report that capacitation of cauda epididymal mouse sperm *in vitro* was accompanied by a time-dependent increase in PK-A activity. This increase in PK-A activity did not occur in a medium that does not support capacitation. While PK-A catalytic and RI/RII regulatory subunits, as well as PK-A enzyme activity, were found in both the Triton X-100-soluble and -insoluble fractions of the sperm, the increase in PK-A activity accompanying capacitation was associated with enzyme activity found in the soluble fraction. Moreover, the regulatory and catalytic subunits of PK-A were observed by indirect immunofluorescence to be present throughout the head, midpiece, and principal piece of the sperm. Thus, PK-A appears to be functional in multiple compartments of this highly differentiated cell. A fraction of the Triton X-100-insoluble PK-A is presumably tethered by AKAP82, the major protein of the fibrous sheath of the sperm flagellum which anchors and compartmentalizes PK-A to the cytoskeleton via the RII subunit of PK-A. Using various recombinant truncated AKAP82 constructs in a gel overlay assay, the RII subunit-binding domain of this protein was mapped to a 57-amino-acid residue region at its N-terminus. Computer analysis revealed a 14-amino-acid region that resembled the RII-binding domains of other *A Kinase Anchor Proteins*. A synthetic peptide corresponding to this domain inhibited AKAP82–RII binding in a gel overlay assay, providing further support that AKAP82 is an anchoring protein for the subcellular localization of PK-A in the mouse sperm fibrous sheath. This work, along with previous findings that cAMP is a key intermediary second messenger in regulating protein tyrosine phosphorylation and capacitation, further supports the importance of PK-A in these processes and necessitates a further understanding of the contribution of both the soluble and insoluble forms of PK-A, as well as AKAP82, to sperm function. © 1997 Academic Press

Key Words: **mouse; sperm; capacitation; protein kinase A; AKAP82.**

INTRODUCTION

Following ejaculation, mammalian sperm are not immediately able to fertilize eggs. Rather, sperm require a finite period of time in the female reproductive tract to become fertilization-competent. This time-dependent acquisition of fertilization competence was defined as ''capacitation'' more than 40 years ago (Chang, 1951; Austin, 1952). Sperm capacitation has been shown to be correlated with a number of parameters including changes in motility, metabolism, and intracellular ion concentrations (Yanagimachi, 1994).

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However, the molecular changes that are responsible for the capacitation process are poorly understood. Capacitation can be mimicked *in vitro* in a defined medium with an absolute requirement for $NaHCO_3$ and $CaCl_2$ and a source of protein (e.g., serum albumin). Recently, it has been demonstrated that changes in protein tyrosine phosphorylation are tightly correlated with capacitation (Visconti *et al.*, 1995a).

Cyclic AMP appears to be an important regulator of sperm capacitation and protein tyrosine phosphorylation. In the absence of $NaHCO_3$, $CaCl_2$, or BSA, both events can be achieved by adding cAMP agonists (Visconti *et al.*, 1995b). Moreover, human sperm and protein tyrosine phosphorylation are regulated by cAMP via an intermediary phosphorylation of a protein(s) on serine/threonine residues (Leclerc *et al.*, 1996). The intracellular concentration of cAMP increases in a number of species during this maturational process (White and Aitken, 1989; Parrish *et al.*, 1994). However, decreases in cAMP have also been reported (Rogers and Garcia, 1979; Stein and Fraser, 1984). Inhibitors of cyclic nucleotide phosphodiesterase activity or cAMP analogues accelerate mouse sperm capacitation (Fraser, 1979, 1981), and the presence of these compounds in the media appears to be essential for capacitation of nonhuman primate sperm (Boatman and Bavister, 1984). In addition, these reagents induce protein tyrosine phosphorylation in bovine sperm of an identical set of proteins as that seen when bovine sperm are capacitated with heparin *in vitro* (Galantino-Homer *et al.*, 1997).

The only known downstream target for cAMP found thus far in sperm is protein kinase A (PK-A). This enzyme is composed of two regulatory (R) subunits that bind cAMP and two catalytic (C) subunits (e.g., R_2C_2) which can form either a type I or type II holoenzyme, depending on the particular subclass of R subunit (RI and RII) present. When the intracellular concentration of cAMP increases, cAMP binds to each R subunit causing the dissociation of the inactive holoenzyme into an $(R{-}cAMP)_2$ complex and active catalytic monomers. These latter subunits can then phosphorylate substrate proteins, altering their functional properties. Individual PK-A isoforms may play specialized physiological roles during spermatogenesis. This is suggested by the observations that the temporal patterns of expression of the individual R subunits within a subclass differ during germ cell development (Lonnerberg *et al.*, 1992; Landmark *et al.*, 1993). For example, while RIIβ mRNA and protein are expressed during postmeiotic stages of spermatogenesis, the protein is not detected in mature sperm. In contrast, RIIα remains in epididymal sperm (Pariset and Weinman, 1994; Johnson *et al.*, 1997).

Most PK-A activity is found in the soluble fraction of somatic cells where, after activation, the C subunit can rapidly diffuse to its site of action. However, enzyme diffusion may not be the most efficient and effective means for the targeting of intracellular signals via kinases. Recently, it has been demonstrated that sequestration of PK-A isoforms to the cytoskeleton or organelles via anchoring proteins can place the kinases in close proximity to their substrates, thus facilitating the propagation of second-messenger signals by activating specific pools of protein kinases. One family of proteins, the *A-K*inase *A*nchor *P*roteins (AKAPs), has been demonstrated to tether PK-A via the RII subunit at specific intraneuronal sites (Coghlan *et al.*, 1993; Rubin, 1994). These multifunctional AKAPs contain a high-affinity binding site for the RII subunit of PK-A, as well as a targeting domain(s) that mediates their attachment to the cytoskeleton and/or intracellular organelles.

Sperm motility is also dependent on the cAMP-dependent and PK-A-mediated protein phosphorylation of flagellar proteins (Tash, 1989; Lindemann and Kanous, 1989; San Augustin and Witman, 1994). Although there is extensive evidence supporting the notion that phosphorylation of specific proteins on serine and threonine residues (presumably through PK-A) plays a role in motility, protein tyrosine phosphorylation may be involved in this process in lower species and this occurs in a cAMP-dependent fashion (Hayashi *et al.*, l987). Sliding of a flagellar accessory structure, the fibrous sheath (FS), in a demembranated mouse sperm model system is also cAMP-dependent (Si and Okuno, 1993), suggesting that the FS may be involved in the regulation of sperm motility. While the precise function of the FS is under investigation, we have recently shown that the major FS protein is a novel AKAP (Carrera *et al.*, 1994). This protein, AKAP82, is present only in the principal piece of the tail where the FS surrounds both the axoneme and outer dense fibers and is likely to be involved in the cAMP regulation of sperm motility by tethering PK-A to the FS (Carrera *et al.*, 1994; MacLeod *et al.*, 1994). This hypothesis is further supported by the recent observation that stearylated Ht31, a peptide derived from a thyroid tissue AKAP, interferes with RII binding of different AKAPs and inhibits bovine sperm motility (Vijayaraghavan *et al.*, 1997). However, the Ht31 sequence is not present in AKAP82 and the domains in AKAP82 that mediate RII binding have not been defined.

Since capacitation and changes in motility are correlated with one another and both appear to rely on a PK-A regulatory system, our laboratories are collaborating to determine how PK-As and AKAPs are involved in regulating mammalian sperm capacitation and motility. We are particularly interested in how cAMP and PK-A regulate these events given the highly differentiated nature of the mature spermatozoon. In this report we demonstrate that capacitation of caudal epididymal mouse sperm *in vitro* was accompanied by a time-dependent increase in PK-A activity. The C, RI, and RII subunits of PK-A, as well as PK-A activity, were found in both the Triton X-100-soluble and -insoluble fractions of sperm, suggesting that some PK-A was anchored to the cytoskeleton. However, the increase in PK-A activity that accompanied capacitation was found only in the Triton X-100-soluble portion of the cell. The localization of PK-A to soluble and insoluble fractions of the sperm indicates that PK-A is compartmentalized and implies that insoluble PK-A may be tethered to the cytoskeleton with an AKAP such as AKAP82. To examine this issue further, we mapped

the RII binding site to a 14-amino-acid residue region at the N-terminus of AKAP82. The results from these experiments provide a mechanism for tethering RII to the insoluble FS via AKAP82.

MATERIALS AND METHODS

Preparation of Sperm

Uncapacitated cauda epididymal mouse sperm were collected either in an ice-cold buffer containing 20 mM Tris–HCl, pH 7.5, 130 mM NaCl, 2 mM EGTA (TN/EGTA) (Kalab *et al.,* 1994) or in a modified Kreb's–Ringer bicarbonate medium (HMB–Hepes buffered) (Lee and Storey, 1986). HMB medium, which supports sperm capacitation, was first prepared in the absence of Ca^{2+}, BSA, and pyruvate (Visconti *et al.,* 1996). HM medium, which does not support capacitation, was prepared by replacing the 10 mM $NaHCO_3$ with 10 mM NaCl, but maintaining the pH at 7.3. Culture media were prepared using culture water from ICN Biomedicals (No. 16-960-54; Costa Mesa, CA).

Preparation of Sperm Fractions

Sperm (4×10^7 cells) were homogenized using 10 strokes with a Teflon dounce homogenizer in TE buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA) and centrifuged at 1000*g* for 10 min. The pellet was saved and the supernatant was centrifuged at 10,000*g*. Again the resultant pellet was saved and the supernatant then was centrifuged at 100,000*g* for 1 h. The pellet and supernatant from this final centrifugation were also saved.

Triton X-100-soluble and -insoluble proteins were prepared by recovering sperm in TN/EGTA buffer (25 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, pH 7.4) by centrifugation at 600*g*, followed by resuspension in the same buffer plus 1% (v/v) Triton X-100. The suspensions were then incubated on ice for 30 min. The pellet and supernatant fractions were collected after centrifugation and resuspended in SDS sample buffer. The proteins were analyzed by immunoblotting with the different PK-A subunit antibodies and/or the fractions were assayed for PK-A activity as described below.

Assay of Protein Kinase A Activity

PK-A activity was measured using Kemptide (Sigma Chemical Co., St. Louis, MO) as a specific substrate. Sperm were adjusted to a final concentration of 10^7 cells/ml and incubated for various periods of time in either HMB medium or HM medium. At the appropriate time points, 10 μl of the sperm suspension was added to 10 μl of 2× assay cocktail so that the final concentration of the assay components was 100 μM Kemptide, [γ-^{32}P]ATP (3000 Ci/mmol) (3×10^6 cpm/assay), 100 μM ATP, 1% (v/v) Triton X-100, 1 mg/ml BSA, 10 mM $MgCl_2$, 40 mM β-glycerophosphate, 5 mM *p*-nitrophenyl phosphate, 10 mM Tris–HCl, pH 7.4, 10 μM aprotinin, and 10 μM leupeptin. This assay cocktail was also supplemented (final concentrations) with (i) H_2O, (ii) 100 μM isobutylmethylxanthine (IBMX) plus 1 mM dibutyryl-cAMP, or (iii) 10 μM H-89. The samples then were incubated for 15 min at 37°C; the assay was linear at this time at a variety of sperm protein concentrations. The reactions were stopped by adding 20 μl 20% TCA, cooled on ice for 20 min, and centrifuged at room temperature for 3 min at 10,000*g*. Thirty microliters of the resultant supernatant was then spotted onto phosphocellulose papers (2 × 2 cm) (Whatman P81). The phosphocellulose papers were washed 5 × 5 min in 5 mM phosphoric acid with agitation, dried, placed in vials with 2.5 ml of scintillation fluid (ICN; EcoLite), and subjected to liquid scintillation counting. When the PK-A activity was measured in the Triton X-100-soluble or -insoluble sperm fractions, the same method was used except that 10 μl of the different fractions was assayed. All of the experiments were repeated at least three times and were performed in triplicate; the means ± SE are shown from a representative experiment.

Immunoblot Analysis of Sperm Proteins

Monoclonal antibodies against the regulatory (RI; Cat. No. P19920) and catalytic (C; Cat. No. P28320) subunits of PK-A were obtained from Transduction Laboratories (Lexington, KY). A polyclonal antiserum against the regulatory (RII; Cat. No. 06-411) subunit of PK-A was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Sperm proteins were separated under reducing conditions by SDS–PAGE on 10% polyacrylamide gels (Laemmli, 1970) and electrophoretically transferred to Immobilon-P (Millipore) membranes (Towbin *et al.,* l979). Following blocking in PBS containing 10% (v/v) fish gelatin (Sigma) and 0.1% (v/v) Tween 20, the blots were incubated for 1 h with the various antibodies [anti-RI (1:1000), anti-RII (1:10,000), anti-C (1:1000)] in PBS containing 0.1% (v/v) Tween 20 and 3% (w/v) BSA. The blots were washed 3 × 10 min with PBS containing 0.1% (v/v) Tween 20, incubated for 30 min with the appropriate secondary antibody conjugated to HRP (1:5000; Jackson Immunoresearch Laboratories, West Grove, PA) in PBS containing 0.1% Tween 20 and 3% (w/v) BSA, washed in PBS, developed using an ECL kit (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions, and then exposed to Reflection (New England Nuclear, Boston, MA) film.

Indirect Immunofluorescence of Sperm

Uncapacitated sperm were fixed in suspension with a solution of 3% (w/v) paraformaldehyde–0.05% (v/v) glutaraldehyde in PBS for 1 h, washed in PBS, and then permeabilized with 0.1% (v/v) Triton X-100 in PBS at 37°C for 10 min. The sperm were washed in PBS and incubated overnight with anti-C (1:50) or anti-RI (1:50) antiserum. A monoclonal antibody against hexokinase (antibody 21A; 1:50) (Visconti *et al.,* 1996) was used as a control. After washing the sperm with PBS, they were incubated with anti-mouse FITC-conjugated secondary antibody (1:50) (Pierce, Rockford, IL) and then attached to poly-lysine-coated microscope slides. Fluorescence was viewed with a Zeiss Photomicroscope III equipped with epifluorescence and photographs were taken with Kodak T-Max P3200 film.

Generation of AKAP Fusion Proteins

Oligonucleotide primers containing *Bam*HI restriction sites on the 5′-ends were used to amplify various regions of the pro-AKAP82 cDNA by polymerase chain reaction (PCR). The PCR products were cut with *Bam*HI, ligated into an expression vector, pGEX-2T (Pharmacia Biotech, Inc., Piscataway, NJ), and then transformed. DNA was isolated from colonies and the presence of inserts was verified by *Bam*HI digestion. To ensure that the inserts were in-frame with the glutathione *S*-transferase, samples with appropriately sized inserts were sequenced using an Applied Biosystems Model 373A

automated sequencer (Foster City, CA) using the DyeDeoxy Terminator cycle sequencing kit. Fusion protein synthesis was induced by IPTG treatment and then the recombinant proteins were isolated using glutathione–Sepharose beads according to the manufacturer's instructions. To confirm that the fusion proteins were of the predicted sizes, the proteins were characterized by SDS–PAGE on a 10% polyacrylamide gel.

RII Overlay Assay

The regulatory subunit (RII) of the cAMP-dependent protein kinase from bovine heart (Promega Corp., Madison, WI) was labeled by phosphorylation with [γ-^{32}P]ATP in the presence of the catalytic subunit and cAMP for 30 min at 4°C (Rubin *et al.*, 1979; Carrera *et al.*, 1994). Recombinant fusion proteins (1 μg/lane) and electrophoretically purified AKAP82 were separated by SDS–PAGE on a 10% polyacrylamide gel, blotted onto nitrocellulose membranes, and incubated with 10 mM potassium phosphate buffer, pH 7.4, 0.15 M NaCl, 0.02% (w/v) NaN_3, 5% (w/v) non-fat dry milk, and 0.1% (w/v) BSA (BLOTTO/BSA) overnight at 4°C (Leiser *et al.*, l986). Labeled RII [10 units (~200 ng); 1.3×10^6 cpm] was added and incubated for 4 h at room temperature. Each RII overlay blot was washed 3×10 min with BLOTTO/BSA without the labeled subunit and then 3×5 min with PBS. The membranes were dried and exposed to Reflection film.

A peptide (NH_2-FYVNRLSSLVIQMA-COOH) corresponding to the putative RII-binding domain of AKAP82 was synthesized (Quality Control Biochemicals, Inc., Hopkinton, MA). In addition, a scrambled version of the peptide (NH_2-NLSMYVAVRISLQF-COOH) was made in which the hydrophilic and hydrophobic domains were randomized. Each peptide was dissolved to a final stock concentration of 1 mg/ml in 10% (v/v) DMSO. Electrophoretically purified AKAP82 was run on a 10% SDS polyacrylamide gel in a single lane that spanned the width of the gel, blotted to nitrocellulose membranes, and incubated in BLOTTO/BSA overnight. To determine the specificity of RII binding, the RII subunit (44 pM) was radiolabeled as described above and, prior to the overlay assay, preincubated with either the RII-binding peptide (426 μM) or the scrambled peptide (426 μM). After 1 h at room temperature, the samples were diluted to 100 μl with BLOTTO/BSA. The RII subunit alone, the RII subunit peptide, and the RII subunit-scrambled peptide were incubated in individual lanes of a microblotter containing the nitrocellulose with AKAP82 for 3 h at room temperature. Each blot was washed and developed as described above.

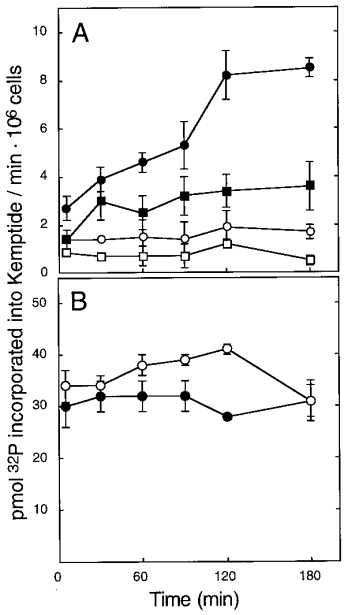

FIG. 1. Changes in protein kinase A activity during mouse sperm capacitation. (A) Sperm were incubated in HMB (●), HMB + H-89 (○), HM (■), or HM + H89 (□) for various time periods and then assayed for PK-A activity in the presence of Kemptide as described under Materials and Methods. Under conditions conducive to capacitation (incubation in the presence of HMB), sperm displayed a time-dependent increase in PK-A activity. This increase in activity was inhibited by H-89, a specific inhibitor of PK-A. In contrast, incubation of sperm in the absence of HCO_3^- (HM medium), which does not support capacitation, did not result in a time-dependent increase in PK-A activity. (B) Sperm incubated in HMB + dbcAMP and IBMX (●) or HM + dbcAMP and IBMX (○) for various time periods and then assayed for PK-A activity. The total PK-A activity did not differ between capacitated and noncapacitated sperm, demonstrating that equal amounts of sperm protein were assayed in A. Shown are the means ± SE of a representative experiment which was performed in triplicate.

RESULTS

Measurement of Sperm Protein Kinase A Activity under Conditions Conducive to Sperm Capacitation

Previously, we demonstrated that both capacitation and the capacitation-related changes in protein tyrosine phosphorylation levels of sperm proteins are regulated by a cAMP pathway (Visconti *et al.*, 1995a,b). Based on these results, an increase in cAMP levels and PK-A activity would be predicted to occur during capacitation. To monitor changes in PK-A activity during capacitation, sperm were incubated in media that support (HMB) or do not support (HM) capacitation (HM is identical to HMB except that it is devoid of $NaHCO_3$). At different time points, sperm extracts were prepared and measured for PK-A activity using the PK-A specific substrate Kemptide. PK-A activities were measured in either the absence or presence of the PK-A inhibitor H-89 (Fig. 1A) and in the presence of its agonists, dbcAMP and IBMX (Fig. 1B), to measure baseline and maximal enzyme activity, respectively.

When epididymal sperm were incubated for various periods of time under conditions conducive to capacitation, PK-A activity increased in a time-dependent manner (Fig. 1A). PK-A activity reached a maximum level after 2 h. Mouse sperm capacitation is complete at 2 h under these incubation conditions (Ward and Storey, 1984; Visconti *et al.*, l995a) and protein tyrosine phosphorylation is maximal during this same time frame (Visconti *et al.*, 1995a). Enzyme activity did not change from that level over the next hour. In contrast, when sperm were incubated in HM medium,

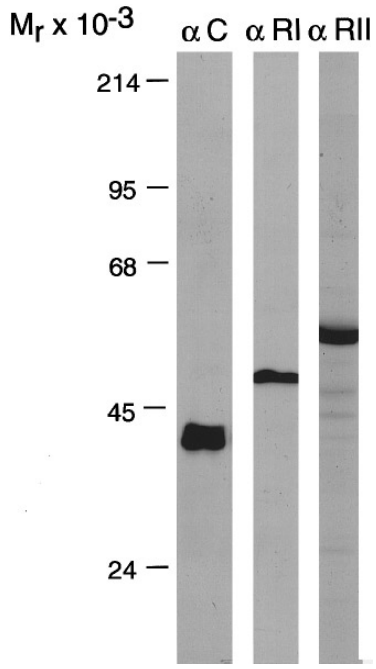

FIG. 2. The presence of protein kinase A subunits in mouse sperm. Proteins from cauda epididymal sperm were extracted and separated by SDS–PAGE, transferred to Immobilon-P membranes, and probed with antisera against the catalytic subunit (αC), the RI subunit (αRI), or the RII subunit (αRII) of PK-A. Each antiserum recognized a single polypeptide of the predicted molecular weight. Numbers to the left of the blots represent the positions of the molecular weight standards ($\times 10^{-3}$).

they did not show a time-dependent increase in PK-A activity. Rather, activity remained relatively constant at the various time points that were assayed (Fig. 1A). PK-A activities in both capacitated and noncapacitated sperm were inhibited by H-89, a specific inhibitor of PK-A (Fig. 1A). To eliminate the possibility that the differences in PK-A activity between the noncapacitated and capacitated sperm were due to different amounts of enzyme activity present in the *in vitro* PK-A assays (i.e., different amounts of sperm assayed), samples from both populations were measured in the presence of the PK-A agonists dbcAMP and IBMX; these assay conditions would monitor total PK-A activity in the sperm extracts. The amount of total PK-A activity in noncapacitated and capacitated sperm was the same and did not change over time (Fig. 1B; note differences in the ordinates of Figs. 1A and 1B).

Protein Kinase A Distribution in the Triton X-100-Soluble and -Insoluble Fractions of Sperm

The identity and distribution of the PK-A subunits present in mouse sperm were evaluated next. The catalytic subunit (C) and both subclasses of the regulatory subunits, RI and RII, of PK-A were detected in epididymal sperm using specific antibodies (Fig. 2). Each antibody recognized a single protein at the predicted molecular weight. Previously, we demonstrated that AKAP82, the major FS protein of the mouse sperm flagellum, binds the RII regulatory subunit of PK-A in a ligand blotting assay (Carrera *et al.*, 1994). To examine the potential association of PK-A with AKAP82 or with other structures in sperm in greater detail, the localization of the different PK-A subunits after sperm fractionation was investigated. Cauda epididymal mouse sperm were homogenized and four fractions were prepared: (1) a low-speed pellet fraction (1000*g*) containing organelles such as nuclei, mitochondria, and accessory structures of the flagellum including the FS (P1); (2) a second low-speed pellet (10,000*g*) to pellet the remaining organelles further (P10); (3) a high-speed pellet fraction (100,000*g*) containing primarily membranes (P100); and, (4) a high-speed supernatant fraction (100,000*g*) containing soluble proteins (S100). When proteins from these different fractions were probed with antibodies against the different PK-A subunits, the immunoblots revealed that each PK-A subunit was present in both the S100 and P1 fractions (Fig. 3A), indicating that PK-A was present in both the soluble and particulate fractions of sperm (see also Johnson *et al.*, 1997).

The nature of the association of the PK-A subunits in the P1 fractions was further delineated by extracting this fraction under different conditions and analyzing the fractions by immunoblotting with the various PK-A subunit antibodies. Extraction with reagents that solubilize peripheral (NaCl and Na_2CO_3, pH 11) and integral (Triton X-100) membrane proteins failed to completely solubilize the C, RI, and RII subunits (Fig. 3B); in fact, a significant amount of these subunits remained in the pellet fraction after these extractions, consistent with the sequestration of PK-A to the cytoskeleton (e.g., to the FS via AKAP82). The release of a fraction of these PK-A subunits following treatment with Na_2CO_3, pH 11, suggested that a portion of the subunits associated with the particulate fraction appeared to be through ionic interactions (Fig. 3B). Although it has been thought that RI is a soluble protein, its presence in the Triton-insoluble fraction also suggested that it is associated with the cytoskeleton; however, the nature of this association is not known.

Capacitation-Dependent Increases in Protein Kinase A Activity Associated with the Triton X-100-Soluble Sperm Fraction

As shown in Fig. 1, PK-A activity increased during sperm capacitation. In addition, PK-A was found in both the soluble (i.e., freely soluble and Triton X-100-soluble) and cytoskeleton-associated fractions of the sperm (Fig. 3). To examine whether the capacitation-dependent increase in PK-A enzyme activity was Triton X-100-soluble or cytoskeleton-associated (or both), cauda epididymal sperm were incubated in either HMB medium or HM medium for 90 min. After the incubation, the sperm were extracted with Triton X-100, and the pellet and supernatant fractions were analyzed for PK-A activity. The Triton-soluble fraction

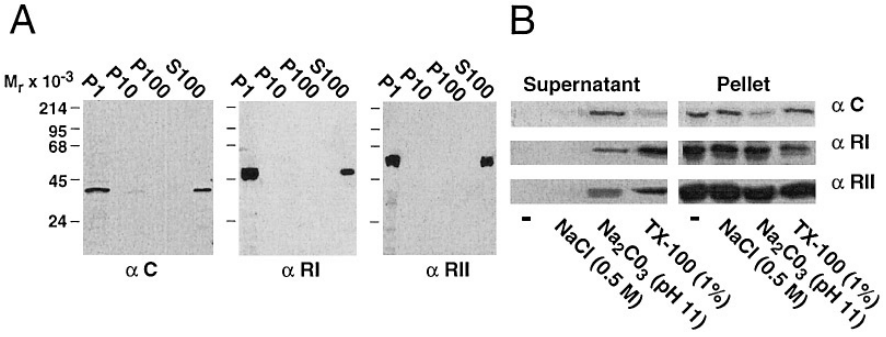

FIG. 3. The presence of protein kinase A subunits in both the soluble and cytoskeletal fractions of sperm. (A) Sperm were homogenized and four fractions prepared: a low-speed pellet fraction (1000*g*) (P1), a second low-speed pellet (10,000*g*) (P10), a high-speed pellet fraction (100,000*g*) (P100), and a high-speed supernatant fraction (100,000*g*) (S100). When proteins from these fractions were separated and probed with the antisera to the catalytic subunit (αC), the RI subunit (αRI), and the RII subunit (αRII), each PK-A subunit was present in the fraction containing organelles such as the accessory structures of the flagellum (P1) and in the soluble fraction (S100). Numbers to the left of the blots represent the positions of the molecular weight standards ($\times 10^{-3}$). (B) The P1 fraction either was not treated (−) or treated with 0.5 M NaCl (a treatment that dissociates peripheral membrane proteins); 100 mM Na_2CO_3, pH 11 (a treatment that dissociates peripheral membrane proteins); or 1% Triton X-100 (a treatment that solubilizes integral membrane proteins). After separating the supernatant from the pellet, proteins were analyzed by immunoblotting with the various antisera against the PK-A subunits. A significant fraction of each of the three subunits remained in the pellet, indicating that they were associated with the sperm cytoskeleton.

from capacitated sperm contained a significantly greater amount of PK-A activity when compared to noncapacitated sperm (Fig. 4A). In contrast, no differences in activity were apparent when the Triton-insoluble fractions were compared between capacitated and noncapacitated sperm. These results indicated that the majority of the increase in PK-A activity observed during capacitation was associated with a freely soluble and/or Triton X-100-soluble fraction of the sperm but not with the cytoskeleton (Triton X-100-insoluble). As expected, the total amount of PK-A activity in the supernatant and pellet fractions measured in the presence of dbcAMP and IBMX did not change with capacitation (Fig. 4B); these experiments also confirmed that the amounts of sperm (and enzymatic activity in the sperm extracts) measured from the noncapacitated and capacitated sperm populations in Fig. 4A were similar.

While these results indicated that the increase in PK-A activity during capacitation was due to a soluble PK-A activity, it was possible that cytoskeleton-associated PK-A also was activated and would result in the release of the free catalytic subunit from the cytoskeleton-associated regulatory subunit. If this were the case, the total PK-A activity (measured in the presence of dbcAMP and IBMX) should increase in the Triton-soluble fraction and decrease in the Triton-insoluble fraction after capacitation since the catalytic subunit would become soluble after activation. Although this was not apparent (Fig. 4B), it remained to be shown that the catalytic subunit (associated with the insoluble PK-A) could become soluble in the presence of dbcAMP and IBMX. To test this possibility, sperm were extracted with Triton X-100 in the presence of dbcAMP and IBMX. Under these conditions, the PK-A activity was maximal and was found only in the supernatant fraction (Fig. 4C). Furthermore, subsequent addition of dbcAMP and IBMX to the enzyme assay under the extraction conditions outlined in the legend to Fig. 4C did not change the PK-A activity measured (Fig. 4D), indicating that the C subunit that was associated with the cytoskeleton could become soluble in the presence of cAMP agonists. Taken together, these results demonstrated that the increase in PK-A activity following capacitation was due to the activation of the Triton X-100-soluble PK-A.

The prediction from the results of the experiments in Fig. 4A was that the catalytic subunit would remain partially Triton-insoluble after capacitation. When the different fractions (Triton-soluble and -insoluble) were analyzed by immunoblotting with specific antibodies against RI, RII, and C subunits, a portion of these subunits remained in the Triton-insoluble fraction (Fig. 4E). Consistent with the enzyme assays presented above (Figs. 4C and 4D), the catalytic subunit was dissociated totally from the Triton-insoluble fraction when the extraction was performed in the presence of dbcAMP and IBMX.

Localization of the RI and C Subunits of Protein Kinase A to the Head and the Flagellum of the Mouse Sperm

While previous work has demonstrated that the RII subunit of PK-A is localized to the mitochondrial and FS of the sperm flagellum (Lieberman *et al.*, l988; Pariset *et al.*, l989; Pariset and Weinman, 1994; Johnson *et al.*, 1997), the locations of the C and RI subunits in sperm are less defined. To localize these subunits in mouse sperm, indirect immunofluorescence was performed using antibodies against the C and RI subunits (both antibodies recognized a single polypeptide on Western immunoblots as shown in Fig. 2). Indirect immunofluorescence indicated that the C subunit was

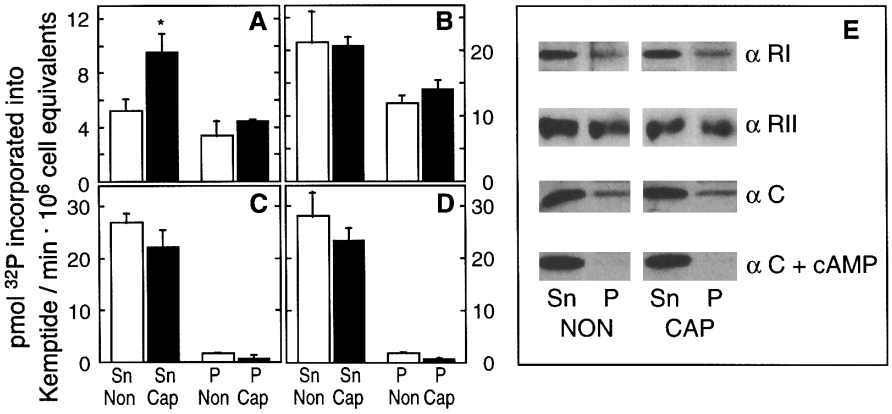

FIG. 4. Changes in protein kinase A activity in the soluble and cytoskeleton fractions of mouse sperm during capacitation. (A) Sperm, after incubation in a medium that either supports capacitation (Cap) or does not support capacitation (Non), were extracted with Triton X-100, and the supernatant (Sn) and pellet (P) fractions assayed for PK-A activity. The Triton X-100-soluble fraction from capacitated sperm contained a greater amount of PK-A activity when compared to noncapacitated sperm, while no differences in activities were observed in the pellets. (B) Sperm were treated as described in A except that PK-A was measured in the presence of dbcAMP and IBMX. The total amount of PK-A activity in the supernatant and pellet measured in the presence of dbcAMP and IBMX did not change with capacitation, suggesting that the cytoskeleton-associated PK-A was not activated and thus did not release free catalytic activity into the supernatant fraction. Moreover, these results also demonstrated that equal amounts of enzyme protein were measured under both capacitation and noncapacitation conditions. (C) Sperm were treated as described in A except that they were extracted with Triton X-100 in the presence of dbcAMP and IBMX prior to measuring their PK-A activities. The total PK-A activity increased in the Triton-soluble fraction and decreased in the Triton-insoluble fraction, indicating that the catalytic subunit was solubilized under these conditions. (D) Sperm were treated as described in C except that PK-A activity also was measured in the presence of dbcAMP and IBMX. (E) Sperm were treated as described in A except that the supernatants and pellets were assayed for the presence of the various PK-A subunits by immunoblotting with antisera against RI (αRI), RII (αRII), and C (αC). Some of all three subunits including C remained associated with the Triton X-100-insoluble fraction. When the extraction was performed in the presence of dbcAMP and IBMX (αC + cAMP), all of the catalytic subunit was dissociated from the Triton-insoluble fraction, demonstrating that catalytic subunit activity associated with the Triton X-100-insoluble fraction could be solubilized.

distributed throughout the entire sperm (Fig. 5B). This localization pattern supports the idea that PK-A is involved in signal transduction events related to both sperm head and tail functions. The RI subunit displayed a similar distribution as that seen with the C subunit (Fig. 5D). A monoclonal antibody (mAb21; gift of Dr. John Wilson, Michigan State University) against rat brain hexokinase, which does not recognize mouse sperm hexokinase on immunoblots (Visconti *et al.*, 1996), was used as a negative control in these experiments and did not display any staining (Fig. 5F). It should also be noted that there was a significant amount of both the C and RI subunits present in the cytosolic droplet of sperm containing these structures, as assessed using immunofluorescence with the specific antibodies (data not shown).

Mapping of the RII-Binding Domain of AKAP82

As shown above, PK-A was present in both the soluble and cytoskeletal fractions of the sperm cell. The association of PK-A with the cytoskeleton presumably occurs via AKAPs. Since AKAP82 (the predominant protein of the FS) and p109 (its phosphorylated precursor; see Johnson *et al.*, 1997) are the only detectable mouse sperm proteins that bind RII *in vitro* (data not shown), and the flagellum-associated PK-A activity most likely plays a key role in motility, PK-A may anchor to these domains. Therefore, experiments were performed to map the RII-binding domain of AKAP82. Several DNA fragments encoding selected regions of both the amino- and carboxyl-terminal regions of pro-AKAP82 were ligated into the pGEX vector and expressed as GST fusion proteins. These proteins were purified and detected by SDS–PAGE (data not shown). None of the fusion proteins containing the carboxyl-terminal fragments of pro-AKAP82 bound ^{32}P-labeled RII as assessed by the direct overlay method (data not shown). However, a fusion protein comprising amino acids 5–306 of pro-AKAP82 (pro-AKAP5-306, Fig. 6) demonstrated RII-binding ability. These data indicated that the RII-binding domain was located in the N-terminal region of the protein.

To define the RII-binding domain further, proAKAP5-306 was systematically truncated, resulting in recombinant proteins containing smaller fragments of pro-AKAP82 (Fig. 6). Elimination of 67 amino acids from the carboxyl-terminal and 113 amino acids from the N-terminal region of pro-AKAP5-306, resulting in pro-AKAP118-239, had no effect on the ability of the fusion protein to bind

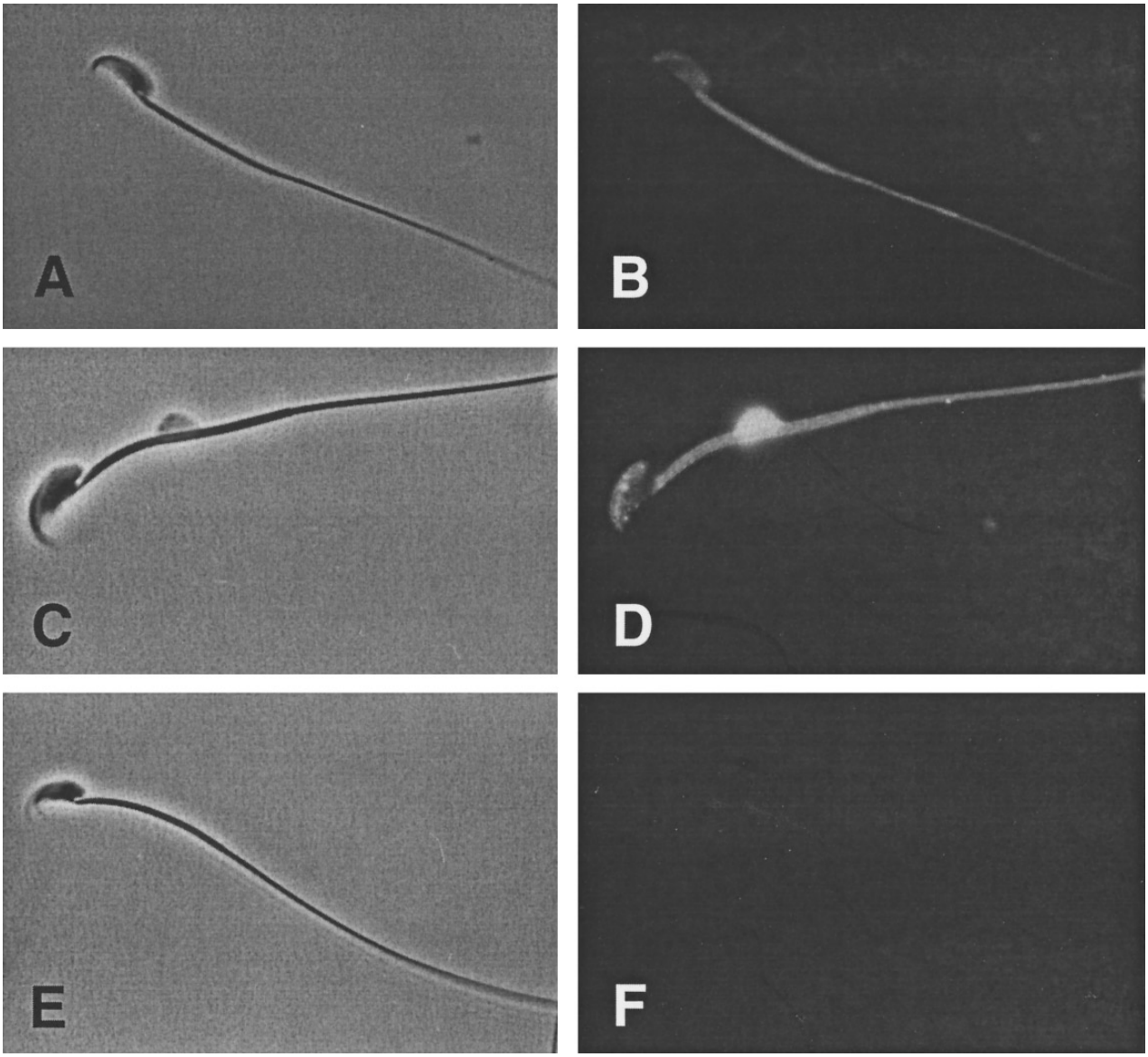

FIG. 5. Localization of the RI regulatory and catalytic subunits of protein kinase A in mouse sperm by indirect immunofluorescence. Uncapacitated cauda epididymal sperm were fixed and processed for indirect immunofluorescence using antibodies to the catalytic subunit (B), RI subunit (D), or rat brain hexokinase type I (F) as described under Materials and Methods. A, C, and E represent the corresponding phase-contrast photomicrographs of B, D, and F, respectively. Note that with both RI and C immunostaining was observed in all regions of the sperm. A negative control (monoclonal antibody to rat brain hexokinase, type I) is shown in F.

RII. However, when an additional 57 amino acids from the carboxyl-terminal end of pro-AKAP118-239 were removed to generate pro-AKAP118-182, RII-binding activity was abolished. RII-binding activity was restored in the presence of pro-AKAP169-239, a recombinant protein containing the 57-amino-acid putative RII-binding domain (Fig. 6). As expected, a fusion protein containing only the pro region of pro-AKAP82 did not bind RII. Thus, the RII-binding domain was localized within a 57-amino-acid segment of the NH_2-terminal region of AKAP82, the mature polypeptide.

Computer-assisted analysis of the 57-amino-acid RII-binding fragment of AKAP82 revealed a 14-amino-acid region that resembled the RII-binding domains of other AKAPs (Fig. 7). This region consisted of several hydrophobic amino acids with long aliphatic side chains (i.e., leucine, isoleucine, and valine) and was predicted by computer modeling to form an amphipathic α-helix (Fig. 8A). On the basis

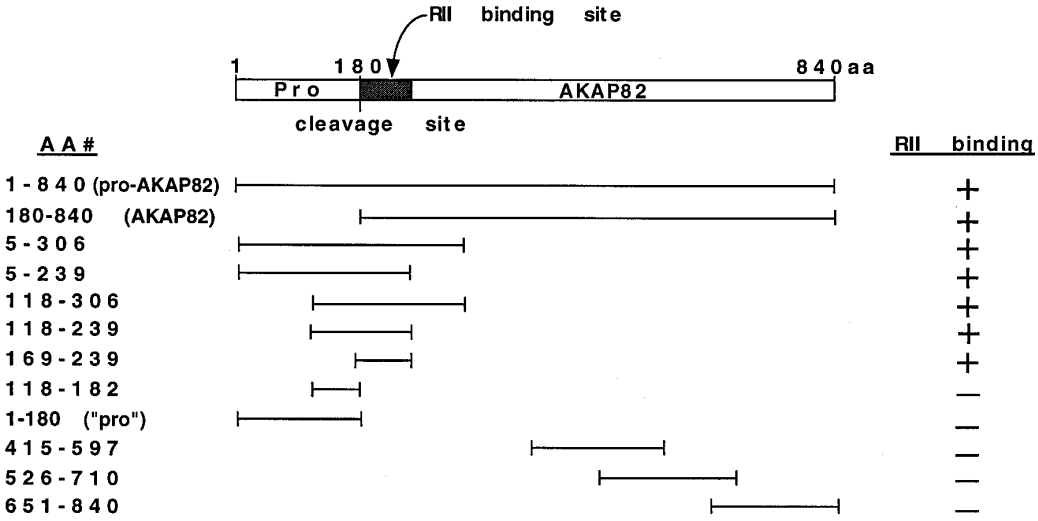

FIG. 6. Schematic diagram indicating the RII-binding domain of pro-AKAP82 following the assessment of the RII-binding activity of different recombinant polypeptides encoding truncated forms of pro-AKAP82. RII-binding activity (positive RII binding indicated by +) was initially detected with pro-AKAP5-306 in a RII overlay assay. With systematic N- and C-terminal truncations of pro-AKAP5-306, the RII-binding domain mapped to 57 amino acids in the N-terminal domain of AKAP82.

of these data, we hypothesized that this region was important for RII binding to AKAP82. To test this hypothesis, a 14-amino-acid peptide encompassing the putative amphipathic α-helix (Fig. 8A) and a scrambled version of the peptide where the hydrophilic and hydrophobic domains were randomized (destroying the amphipathic α-helical structure) (Fig. 8B) were synthesized. In competition experiments where the RII subunit was preincubated with the synthetic peptides, RII binding to AKAP82 was inhibited when compared to the control (Fig. 8C; compare control lane C with peptide lane P). There was no effect on RII binding in the presence of the scrambled peptide (Fig. 8C; compare control lane C with peptide lane P and scrambled peptide lane S). These results support the concept that RII binding by AKAPs is mediated by conserved features of protein secondary structure.

AKAP82	(210-223)	F Y V N R L S S L V I Q M A
AKAP75	(392-405)	L L I E T A S S L V K N A I
AKAP150	(428-441)	L L I E T A S S L V K N A I
S-AKAP84	(305-318)	Q I K Q A A F Q L I S Q V I
Ht31	(493-506)	D L I E E A A S R I V D A V
AKAP100	(397-410)	E I I D M A S T A L K S K S
MAP2	(85-98)	E T A E E V S A R I V Q V V

FIG. 7. Comparison of the RII-binding domains of a variety of AKAPs. A computer analysis of several RII-binding proteins revealed possible structural homologies. Shown is a 14-amino-acid region present in the RII-binding site of several RII-binding proteins. Sequences were compared for AKAP82 (Carrera *et al.*, 1994), AKAP75 (Glantz *et al.*, 1993), AKAP150 (Bregman *et al.*,1989), S-AKAP84 (Chen *et al.*, 1997), Ht31 (Rosenmund *et al.*, 1994), AKAP100 (McCartney *et al.*, 1995; Carr *et al.*, 1991), and MAP2 (Rubino *et al.*, 1989; Obar *et al.*, 1989). Regions of amino acid identity are highlighted.

DISCUSSION

In previous work we demonstrated that capacitation was accompanied by a time-dependent increase in protein tyrosine phosphorylation (Visconti *et al.*, 1995a). We also demonstrated that both capacitation and the capacitation-associated increase in protein tyrosine phosphorylation were downstream of a cAMP pathway (Visconti *et al.*, 1995b). These results were confirmed in spermatozoa from other mammalian species, including human (Leclerc *et al.*, 1996; Carrera *et al.*, 1996) and bovine (Galantino-Homer *et al.*, 1997). On the basis of these observations, it would be predicted that cAMP concentrations should increase during capacitation. Although some authors have observed elevations in cAMP levels when spermatozoa were incubated under conditions that support capacitation (White and Aitken, 1989; Tajima and Okamura, 1990; Parrish *et al.*, 1994), others have reported decreasing levels of cAMP under these conditions (Rogers and Garcia, 1979; Stein and Fraser, 1984). The origins of this discrepancy might be related to the steady-state concentration of cAMP, which is a function of its rate of synthesis by adenylyl cyclase and its rate of degradation by phosphodiesterases; however, small changes in the activity of these enzymes could result in drastic changes of cAMP concentrations. Another problem is that

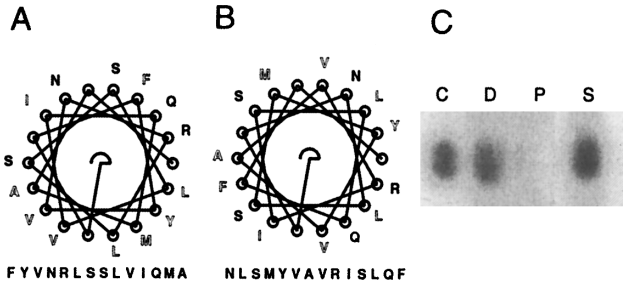

FIG. 8. RII-binding activity of a 14-amino-acid peptide within the predicted RII-binding domain of AKAP82. (A) Pinwheel diagram of the proposed helical structure of the 14-amino-acid AKAP82 putative RII-binding peptide demonstrating its hydrophilic and hydrophobic domains. Hydrophobic residues are indicated by outlined letters. The sequence of this peptide is based on the sequence of AKAP82 shown in Fig. 7. (B) Pinwheel diagram of the scrambled version of this peptide with the hydrophilic and hydrophobic domains disrupted. (C) Electrophoretically purified AKAP82 was subjected to SDS–PAGE and electroblotting. With a microblotter, the blot was incubated with ^{32}P-labeled RII alone (C) or RII preincubated with either the AKAP82 RII-binding peptide (P) or the scrambled version of the peptide (S). RII binding to AKAP82 was inhibited in the presence of the 14-amino-acid peptide. However, there was no effect in the presence of the scrambled peptide. The stock peptide solutions contained dimethyl sulfoxide (DMSO) to aid in solubilization. As a control, an equal volume of DMSO was added without the RII-binding peptide and was shown to have no effect (D). This experiment was performed three times and shown are the results of a typical experiment.

total cAMP distributes between cellular and extracellular compartments (Messager *et al.*, 1996; Vyas *et al.*, 1996; Sirotkin *et al.*, 1996; Rosenberg, 1992) and, therefore, measurements of the total amount of cAMP do not necessarily reflect the effective intracellular cAMP concentrations. In addition, although PK-A is the only known downstream effector for cAMP in sperm, the overly simplistic view that PK-A mediates all of the effects of cAMP in eukaryotic cells has been amended with the discovery of new types of cyclic nucleotide receptors. These include cyclic nucleotide-gated channels, cGMP binding cyclic nucleotide phosphodiesterase, and extracellular cAMP receptors (for review see Shabb and Corbin, 1992). Recently, alternative pathways for the regulation of PK-A have also emerged. These include the regulation of PK-A activity by proteolysis of the regulatory subunit (Greenberg *et al.*, 1987; Hegde *et al.*, 1993) and the regulation of PK-A activity by IκB (Zhong *et al.*, 1997). These observations demonstrate that an activation of PK-A activity does not always follow increases in cAMP concentrations and, likewise, increases in cAMP concentrations do not always correlate directly with PK-A activation. In the present report we have approached these issues by measuring the only known downstream effector of cAMP in sperm, that is PK-A activity.

Using this approach, we have shown that capacitation was correlated with a time-dependent increase in PK-A activity. In contrast, sperm did not display an increase in PK-A activity when incubated in a medium that does not support capacitation. Although PK-A activity was found in both the soluble and cytoskeletal fractions of the cell, the increase in activity seen during capacitation was associated specifically with the soluble PK-A. Furthermore, while both type I and II holoenzymes were found in both fractions of the sperm, our data suggest that the activation of PK-A during capacitation was associated with the preferential release of the C subunit from the soluble pool of PK-A. It remains to be demonstrated whether this activation is associated with the activation of the type I, type II, or both holoenzyme forms of PK-A.

The presence of the RII subunit in the flagellum is consistent with previous reports (Lieberman *et al.*, 1988; Pariset and Weinman, 1994; Johnson *et al.*, 1997). However, both the RI and C subunits were found in the head and tail, indicating that the type I holoenzyme was present throughout the sperm. The presence of PK-A in the anterior head of the sperm is consistent with our previous work (Visconti *et al.*, 1995b; Carrera *et al.*, 1996) and work by others (White and Aitken, 1989; Parrish *et al.*, 1994; Leclerc *et al.*, 1996) suggesting the involvement of the PK-A pathway in sperm capacitation. Considering that the activity of PK-A increased under conditions conducive to capacitation, it became important to know whether this increase was due to changes in PK-A activity in specific regions of the sperm, i.e., the heads or tails. Since sperm motility is regulated by a cascade of phosphorylation events mediated by PK-A, some of this increase is probably due to the flagellar-associated PK-A. Unfortunately, efficient methods are not available to fractionate heads from tails while preserving the soluble PK-A in each fraction.

Our experiments suggest that there are at least two clearly differentiated pools of PK-A activity. One is bound to cytoskeletal structures (e.g., RII to AKAP82 in the FS) and the other remains soluble or is bound to the membrane directly or through protein–protein interactions. Although the Triton X-100-insoluble fraction of PK-A activity did not change during capacitation, we cannot discount the involvement of this particular fraction of PK-A activity in the regulation of sperm motility or in some aspects of the capacitation process. PK-A activity is regulated by local cAMP concentrations that are dependent on the synthesis and degradation of cAMP; a rapid increase followed by a rapid decrease in its concentration might result in no observable changes in PK-A activity, but still could result in important physiological changes.

How could the PK-A be partitioned into an insoluble compartment? One way could be the tethering of the enzyme to AKAP82, the major protein of the mouse FS and a member of the AKAP family (Carrera *et al.*, 1994). The presence of AKAP82 in the sperm FS is in agreement with the well-established role of PK-A in sperm motility. The role of AKAPs in other cell types is to tether PK-A to cytoskeletal structures of the cell, thus increasing the proximity of this enzyme to its substrates. Using recombinant proteins of

GST fused to truncated forms of AKAP82 and synthetic peptides, the RII-binding domain mapped to a 14-amino-acid stretch of the N-terminus of the mature protein. Interestingly, the characteristics of this region are similar to the RII-binding domains of other AKAPs; namely, this stretch is rich in long-chain aliphatic and charged residues and is predicted to form an amphipathic alpha helix with hydrophilic and hydrophobic domains. RII binding to AKAP82 was inhibited in the presence of the 14-amino-acid peptide but when the amino acid sequence was rearranged to disrupt the amphipathic helix, the inhibitory effect was abolished. The role of secondary structure governing the interaction of AKAPs and RII is further supported by experiments where site-directed mutagenesis was used to disrupt the secondary structure of the RII-binding domain of the AKAP Ht31. In this study, substitution of proline for Ala-498 significantly diminished RIIα binding, as did similar mutations of Ile-502 and Ile-507 (Carr *et al.*, 1991).

While it has been shown previously that the type II holoenzyme can be anchored (e.g., via AKAPs) in a number of cell types including sperm, it was somewhat surprising that the RI subunit was found to be associated with the sperm cytoskeleton. Although typically cytoplasmic, the type I holoenzyme has been shown to interact specifically with the antigen-specific T cell receptor–CD3 (TCR–CD3) complex during T cell activation and capping (Skalhegg *et al.*, 1994). While the type of association(s) that RI forms in sperm is not known, an AKAP (D-AKAP1) has been described recently which has specificity for both the RI and RII subunits (Huang *et al.*, 1997). While expressed in a number of different cell types, D-AKAP1 is highly transcribed in testis. In addition, a splice variant of D-AKAP1 is homologous to S-AKAP84, an AKAP that localizes to the mitochondrial sheath during spermiogenesis (Lin *et al.*, 1995). S-AKAP84 is not detected in mature sperm either by immunofluorescence or immunoblotting analysis, suggesting that RI does not bind S-AKAP84 in these cells. However, S-AKAP84 may be responsible for transporting RI to the flagellum from the cytoplasm during spermiogenesis as has been suggested for RII (see Johnson *et al.*, 1997). We already know that AKAP82 is not responsible for the translocation of RII to the principal piece during spermiogenesis since pro-AKAP82 is detected in this region of the flagellum prior to the appearance of RII (see Johnson *et al.*, 1997). In fact, it is possible that pro-AKAP82 (the precursor of AKAP82) cannot bind RII *in vivo*, although it does so *in vitro* (Carrera *et al.*, 1994). In this regard, it is interesting to note that the RII-binding domain is present at the amino-terminal end of AKAP82, extremely close to the cleavage site. In sperm it is possible that other proteins (e.g., other splice variants of D-AKAP1, AKAP82?) could anchor RI to specific locations in the cell.

These results address the unique system of cross talk between PK-A and protein tyrosine phosphorylation in mammalian spermatozoa (Visconti *et al.*, 1995a,b; Leclerc *et al.*, 1996). Further evidence for this intracellular signaling pathway is our recent finding that the prominent M_r 82,000 and M_r 97,000 phosphotyrosine-containing proteins in human sperm are the human homologues of mouse AKAP82 and pro-AKAP82 (the AKAP82 precursor polypeptide), respectively (Carrera *et al.*, 1996). Moreover, the increase in the tyrosine phosphorylation of AKAP82 and pro-AKAP82 in human spermatozoa is capacitation-dependent. Although the tyrosine phosphorylation of AKAP82 in mouse sperm has not been observed, it is phosphorylated on serine/threonine residues and thus may be a substrate for PK-A (Johnson *et al.*, 1997). The results presented in this paper indicate that the cAMP-dependent up-regulation of protein tyrosine phosphorylation accompanying capacitation occurs via the activation of soluble PK-A and that AKAP82 may be involved in partitioning a fraction of the PK-A into a cytoskeleton-associated compartment.

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