Immunogold Localization of the Regulatory Subunit of a Type II cAMP-dependent Protein Kinase Tightly Associated with Mammalian Sperm Flagella

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Abstract. We have shown previously that the regulatory subunit (RII) of a type II cAMP-dependent protein kinase is an integral component of the mammalian sperm flagellum (Horowitz, J. A., H. Toeg, and G. A. Orr. 1984. J. Biol. Chem. 259:832–838; Horowitz, J. A., W. Wasco, M. Leiser, and G. A. Orr. 1988. J. Biol. Chem. 263:2098–2104). The subcellular localization of this flagellum-associated RII in bovine caudal epididymal sperm was analyzed at electron microscope resolution with gold-conjugated secondary antibody labeling techniques using anti-RII monoclonal antibodies. By immunoblot analysis, the flagellum-associated RII was shown to interact with mAb 622 which cross reacts with both neural and nonneural isoforms of RII. In contrast, a neural specific monoclonal antibody (mAb 526) failed to interact with flagellar RII. In the midpiece of the demembranated sperm tail, gold label after mAb 622 incubation was primarily associated with the outer mitochondrial membrane. Although almost all specific labeling in the midpiece can be assigned to the mitochondria, in the principal piece, there is some labeling of the fibrous sheath. Labeling of the outer dense fibers and the axoneme was sparse. Specific labeling was virtually absent in the sperm head. Sections of sperm tails incubated in the absence of primary antisera or with mAb 526 showed little labeling. A β-tubulin monoclonal antibody localized only to the 9 + 2 axoneme. These results raise the possibility that a type II cAMP-dependent protein kinase located at the outer mitochondrial membrane plays a role in the direct cAMP stimulation of mitochondrial respiration during sperm activation.

Cyclic AMP plays a key role in the initiation and regulation of motility in mammalian sperm (Brokaw, 1987). Since cAMP-dependent protein kinases are the sole mediators of all cAMP-regulated processes in eukaryotic cells (Flockhart and Corbin, 1982), we have been interested in characterizing these regulatory enzymes in mammalian sperm. We have shown previously that a type II cAMP-dependent protein kinase is an integral component of demembranated mammalian sperm tails (Horowitz et al., 1984, 1988). The purpose of the present study was to identify by electron microscopic immunocytochemistry the location(s) of the regulatory subunits of type II cAMP-dependent protein kinase (RII)1 in the demembranated bovine sperm tail.

Mammalian sperm tails or flagella are surrounded by the cell membrane, which is removed by detergent in this study. Soluble enzymes, cell membrane proteins, and constituents loosely bound to tail structures are lost after this procedure. In addition to the 9 + 2 axoneme, the fundamental cytoskeletal complex responsible for sperm motility, the demembranated sperm tail, contains other structurally well-defined elements, including nine outer dense fibers that closely parallel the axonal doublet microtubules in the midpiece and through much of the principal piece (Linck, 1979). In the midpiece, the fibers are surrounded by mitochondria that are physically attached to the complex and resistant to detergent solubilization. Presumably, the mitochondria are the major source of ATP for the dynein ATPases of the axoneme, and therefore for motility. Mitochondrial function may also be involved in the onset of motility during sperm capacitation, but this is more speculative. In the principal piece, a fibrous sheath replaces the mitochondria as the element encircling the outer dense fibers.

A type II cAMP dependent protein kinase is tightly associated with the demembranated flagellum via the regulatory subunit (RII), the catalytic subunit being released after incubation with cAMP (Horowitz et al., 1984). cAMP-dependent protein kinase activity is regained in the demembranated sperm tails depleted of all endogenous catalytic subunits when the sperm tails are incubated with bovine heart catalytic subunits, indicating that the bound flagellar protein...
RII is functional (Horowitz et al., 1984). [3H]cAMP binding studies demonstrated that the flagellar RII is not a minor component of the tail but is present in amounts stoichiometric with other structures such as dynein arms and radial spokes (Horowitz et al., 1988). RII is a 57-kD polypeptide that interacts specifically with polypeptides of subunit $M_r$ of 120 and 80 kD in both rat and bovine flagella (Horowitz et al., 1988). Dithiothreitol (DTT) was shown to be effective in causing the release of RII from the flagellum, suggesting that disulfide bonding is involved in the RII-flagellar interaction (Horowitz et al., 1988). Association of RII with the flagellum via a direct disulfide bond with its binding proteins was ruled out on the basis that 8-azido-[32P]cAMP-labeled RII had the same electrophoretic mobility on NaDodSO4-PAGE irrespective of the presence or absence of thiol reducing agents. NaDodSO4-PAGE analysis of the DTT-released material showed that only a limited number of flagellar polypeptides were released. Neither tubulin, dynein ATPase, nor any of the RII-binding proteins cosolubilized with RII. Despite this intensive biochemical characterization, the structural documentation of RII tightly associated with the sperm tail has not been undertaken previously. The localization of RII to specific sperm tail components will help to define the functional role of type II cAMP-dependent protein kinase in the sperm tail.

Materials and Methods

Isolation of Caudal Epididymal Sperm Flagella

Bovine or rat sperm were isolated as described by Horowitz et al. (1988) and demembranated at 4°C for 15 min in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 0.1 mM cAMP, and 10 µg/ml each of leupeptin, apotinin, and pepstatin. Purified flagella were obtained by sonication followed by discontinuous sucrose gradient centrifugation as described by Calvin (1979). Flagellar preparations were checked for the presence of RII by the [3H]cAMP binding assay, 8-azido-[32P]cAMP photoaffinity labeling and immunoblot analysis (Horowitz et al., 1984, 1988).

Electrophoresis and Immunoblotting

The polypeptide composition of the demembranated bovine flagella was analyzed by one-dimensional NaDodSO4-PAGE (Laemmli, 1970). Typically 2–10 x 10^6 flagella were added to each lane. The resolved proteins were transferred electrophoretically to nitrocellulose sheets (0.45 µm) as described by Burnette (1981). Nonspecific binding sites were blocked with 5% non-fat dry milk in 10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Triton X-100, 10 µg/ml each of leupeptin, aprotinin, and pepstatin. Purified flagella were obtained by sonication followed by discontinuous sucrose gradient centrifugation as described by Calvin (1979). Flagellar preparations were checked for the presence of RII by the [3H]cAMP binding assay, 8-azido-[32P]cAMP photoaffinity labeling and immunoblot analysis (Horowitz et al., 1984, 1988).

Preparation of mAbs against RII

Hybridomas, secreting mAbs against RII, were injected into pristane-primed, BALB/c mice (Paupard et al., 1988). The hybridomas were a generous gift of Dr. C. S. Rubin (Albert Einstein College of Medicine). Ascites fluid was collected between days 9 and 16, pooled, centrifuged, and stored at ~70°C until used. IgG was obtained from the ascites fluid by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Ey et al. (1978).

Fixation and Embedding in LR White

Sperm flagella pellets were fixed with either 0.5% or 1.0% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, for 1 h at 4–6°C. The pellets were dehydrated by a series of washes in buffer, 50% and then 70% ethanol, and infiltrated with a 2:1 ratio of LR White (medium grade; London Resin Company, Ltd., Woking, Surrey, England); 70% ethanol mixture for 30 min, followed by pure LR White resin for 1 h, and then overnight incubation in fresh resin at room temperature. The LR White was then changed a final time before polymerization in gelatin capsules at 60°C for 24 h. Thin sections were cut with a diamond knife and collected on 300 mesh nickel grids.

Immunocytochemical Procedures

Grids containing sections were incubated on 15–30 ul drops of appropriate solutions at room temperature. Nonspecific binding sites were blocked by incubation in 4% BSA in TBS for 15 min; before use, blocking solution was centrifuged at 14,000 g for 5 min as per Weinmann et al. (1986). Sections were labeled with a 1:1 dilution of anti-RII monoclonal antibody (mAb 622 or mAb 526) or a 1:100 dilution of anti-β-tubulin monoclonal antibody (kindly provided by Dr. S. Horwitz) for 1 h. All antibodies were made up in 1% BSA in TBS. Grids were then washed extensively in 1% BSA in TBS. Immunogold labeling was performed for 15 min with a 1:20 dilution of a rabbit anti-mouse IgG antibody conjugated to 15-nm gold particles (E. Y. Laboratories, Inc., San Mateo, CA) in 1% BSA in TBS. Grids were washed extensively in TBS (without BSA) and then in distilled water, air dried, and stained with 1% uranyl acetate (aqueous) and lead citrate. Sections were then examined in a JEOL 100 CX electron microscope.

Results

Characterization of the mAbs Used in this Study

The anti-RII monoclonal antibodies used in this study were mAb 526 which recognizes the neural specific isofrom of RII and mAb 622 which recognizes both the neural and nonneural isoforms of RII (Stein, 1985; Fig. 1 B, lane 1). Both of these monoclonals are of the IgG2 sub-class and, after affinity purification on immobilized protein A, were shown to contain a single heavy and light chain by NaDodSO4-PAGE (data not shown). Immunoblot analysis of NaDodSO4-PAGE-resolved demembranated bovine flagella (Fig. 1 A) reveals that mAb 526, the neural specific antibody, does not cross react with any flagellum-associated polypeptide (Fig. 1 B, lane 2). In contrast, mAb 622 interacts strongly with a single band of 57,000 $M_r$ (Fig. 1 B, lane 3). A flagellar protein of similar $M_r$ has been shown previously to be labeled by the photoaffinity analogue of cAMP, 8-azido-
Figure 2. (A) Low power overview of a typical field of the sperm tail preparation. Note minor contamination with sperm heads (H). Fields are originally selected at magnifications too low to visualize label. (B) High power detail of typical field showing label distribution after immunolocalization procedures (1° antibody mAb 622, 1:15 dilution; 2° antibody: anti-mouse IgG conjugated to 15 nm gold [E.Y. Laboratories, Inc.]). Note specific localization to sperm structures, especially midpiece mitochondria (arrowheads). Electron-opaque gold particles are found within 35 nm of exposed antigenic sites on the surface of the section. Bars: (A) 1.0 μm; (B) 0.25 μm.
Figure 3. Primary antibody: mAb 622 (mouse anti-neural and nonneural RII), 1:15 dilution. (A–C) Representative longitudinal sections of sperm tail midpieces. (D) Transition between midpiece and principal piece. Label is prominent along the outer membrane of mitochondria (arrowheads). Label in the swollen (artifactual) space between the mitochondria and outer dense fibers is at background levels. Specific labeling is found along the fibrous sheath in the principal piece (arrows). Labeling of the outer dense fibers (x) and the axoneme is sparse. Bar, 0.5 μm.

\[^{32}P\]cAMP (Horowitz et al., 1984, 1988). An anti-β-tubulin mAb cross reacts with a single flagellar polypeptide of 52,000 M, (Fig. 1 B, lane 4).

**General Remarks Regarding Immunolocalization**

For analysis, random regions containing numerous sperm tail profiles were chosen at low power where gold localization was not resolved. Such regions show minor contamination with sperm heads and a variety of sections, generally through mid- and principal pieces, of the tails (Fig. 2 A). A typical high power field showing labeling distribution after immunolocalization procedures is shown in Fig. 2 B. Localization in different regions of one preparation and in replicate preparations proved highly consistent and reproducible. With appropriate dilutions of the antibodies, nonspecific labeling in the background and in artifactual spaces was minimal.

**Localization of mAb 622 to the Mitochondrial Outer Membrane**

mAb 622 immunoreactivity, as identified by the electron-dense gold label of the secondary antibody, was found on the mitochondria at levels that greatly exceeded background (Fig. 2 B, Fig. 3, A–D, Fig. 4 A, Fig. 5 A). Where the sperm tail was sectioned transversely or the mitochondrial gyres themselves were cross-sectioned, label was most often near the edges of the mitochondrion (Figs. 3 B, 4 A, and 5 A), which suggests that the label is predominantly found on the outer mitochondrial membrane. No label above background was found in the swollen space between the mitochondria and the outer dense fibers (Fig. 3 A). Although in the mid-piece almost all specific labeling can be assigned to the mitochondria, in the principal piece there is some specific mAb 622 immunoreactivity along the fibrous sheath (Figs. 3 D and 4 B). However, labeling of the fibrous sheath seems sparser than labeling of the mitochondria. Some immunogold label is occasionally seen on the outer dense fibers or in the axoneme, but labeling of these regions of the sperm tail is too sparse to be considered significant.

**Controls for Immunospecificity**

The RII associated with rat sperm flagella does not react with mAb 622 (Horowitz et al., 1988). To exclude the possibility that RII was nonspecifically absorbed to mitochondria or other flagellar components, demembranated rat sperm flagella preparations were incubated with bovine heart RII for 60 min on ice and centrifuged through a sucrose cushion. When these preparations were analyzed after electrophoresis, no RII band was identified by immunoblotting with mAb 622 (data not shown), indicating that nonspecific absorption of RII was minimal.
Several controls were used to insure the immunospecificity of the results with mAb 622. A β-tubulin mAb was used as a positive control to check the reliability of the methods of fixation, dehydration, embedding in LR White, and labeling of thin sections. The β-tubulin control, as expected, specifically labeled only axonemal microtubules, including the central pair, along the length of the flagellum (Figs. 4 C, 5 B, and 6, A and B). In areas other than those containing microtubules, virtually no nonspecific label was observed. In a control where mAb 622 was omitted from the procedure, virtually no gold label was found on the sperm flagellum (Fig. 6 D). When mAb 526 was substituted for mAb 622, the low level of background observed was the same as with the control omitting the primary antibody (Figs. 4 D and 6 C), regardless of whether mAb 526 was used at the same dilution as mAb 622 (1:15) or if it was used virtually undiluted (data not shown).

It was previously shown by biochemical methods that upon separation of sperm head and tailpieces, RII was almost exclusively located on the tailpiece (Horowitz et al., 1984). Therefore, an additional control was to determine if any mAb 622 immunospecificity was present in bovine sperm heads. After postembedding labeling procedures, no mAb 622 immunoreactivity was found on the sperm head contaminants above background levels (Fig. 6 E).

**Discussion**

We have used an immunogold procedure to map the location of RII immunoreactivity in a demembranated mammalian sperm flagellum, demonstrating a major localization to the outer mitochondrial membrane and a minor localization to the fibrous sheath. We have shown previously that a type II cAMP-dependent protein kinase is an integral structural component of the mammalian sperm flagellum with association mediated via the regulatory subunit, RII (Horowitz et al., 1984, 1988). It is known that type II protein kinases can form complexes with specific proteins (e.g., microtubule-
Figure 5. High power detail of midpiece cross sections showing localization of RII vs. localization of a known protein (tubulin). (A) Localization of RII. Labeling is primarily to the outer mitochondrial membrane (arrowheads). Axonemal microtubules are unlabeled in this section. (1° antibody: mAb 622; 2° antibody: anti-mouse IgG, 15-nm gold). (B) Localization of β-tubulin. As expected, β-tubulin localizes only to axonemal microtubules. (1° antibody: mouse anti-β-tubulin; 2° antibody: anti-mouse IgG, 15-nm gold). Bar, 0.125 μm.

We have shown previously that the amount of RII associated with demembranated intact sperm, prepared by either Triton X-100 treatment or by freeze thawing, was almost the same as that associated with demembranated flagella prepared by Triton X-100 demembranation followed by sonication/discontinuous sucrose gradient centrifugation (Horowitz et al., 1984). We concluded from this data that mam-
Figure 6. (A and B) Primary antibody: mouse anti-β-tubulin, 1:100 dilution. (A) Transition between midpiece and principal piece. (B) Midpiece. Localization is specific to axonemal microtubules. (C-E) Labeling found only at background level in the following controls: (C) primary antibody mAb 526, 1:15 dilution, midpiece; (D) primary antibody omitted, midpiece; and (E) primary antibody mAb 622, 1:15 dilution, sperm head. Bars: (A–D) 0.5 μm; (E) 0.25 μm.

Malian sperm contains a subpopulation of RII that is tightly associated with the flagellum. We subsequently showed that RII is present on the flagellum in similar molar amounts as components of the axoneme; e.g., dynein arms and radial spokes (Horowitz et al., 1988). However, we stressed that this data should not be construed as suggesting an axonemal location of RII, but only that it demonstrated that RII was not a minor flagellar component. The situation appears to be different in sea urchin sperm where >83% of the cAMP-dependent protein kinase was released upon sonication (Gray et al., 1976).

The immunogold localization observed in this study reflects the distribution of RII in the demembranated bovine caudal epididymal sperm flagellum. The mAb (mAb 622) used for localization has been shown to recognize the non-neural as well as the neural form of RII in various tissues and cells (Stein, 1985). Immunoblot analysis of NaDodSO4-PAGE–resolved flagella reveals that mAb 622 recognizes a single 57,000-Mᵦ polypeptide, within the expected Mᵦ range for RII. A flagellar protein of similar Mᵦ, is specifically labeled with 8-azido-[³²P]cAMP, the photoaffinity analogue of cAMP (Horowitz et al., 1984, 1988). Photoaffinity labeling of this protein is inhibited by cAMP but not by 5'-AMP or adenosine (Horowitz et al., 1984). Moreover, no specific immunogold labeling of sperm flagella is observed if the primary antibody is omitted or, more importantly, if an anti-RII monoclonal (mAb 526) that does not recognize the flagellar-associated RII is used in place of mAb 622. As an additional control, a β-tubulin mAb was shown to decorate the 9 + 2 axoneme of the flagellum. No specific gold labeling of the mitochondria or fibrous sheath was observed with this antibody.

Using immunofluorescence techniques on intact dog sperm flagella, Tash and Means (1982) found that localization of the catalytic subunit of cAMP-dependent protein kinase was in the principal piece behind the midpiece. Our localization of RII to the fibrous sheath is consistent with this...
data. Our localization of RII to the mitochondrial outer membrane in bull sperm would suggest that cAMP-dependent protein kinase should also be localized to the midpiece. A possible explanation for the discrepancy between the results is that the mitochondrial catalytic subunit may be inaccessible to antibody because of penetration difficulties in pre-embedding localization procedures.

What role cAMP-dependent phosphorylation plays in flagellar function remains to be established definitely, although it is known that the addition of either dibutylryl cAMP or of phosphodiesterase inhibitors to bovine or porcine caudal epididymal sperm leads to an increase both in motility and in respiratory activity (Garbers et al., 1971; Frenkel et al., 1973; Milkowski et al., 1976; Cascieri et al., 1976). Further, sperm flagellar motion is initiated via the cAMP-dependent phosphorylation of a cytosolic protein present in detergent extracts of mammalian sperm (Tash et al., 1984). The identity of this polypeptide(s) and the mechanism by which it initiates movement is currently controversial (Noland et al., 1987; Paupard et al., 1988). Respiratory stimulation could be achieved in one of two, not mutually exclusive, pathways. First, stimulation could be the result of an increased energy demand via a primary effect of cAMP on sperm motility; i.e., stimulation of respiration could be indirect. Second, cAMP could act directly on respiratory enzymes, presumably via a phosphorylation mechanism. The unanticipated localization of the major RII immunoreactivity on the flagellum to the outer mitochondrial membrane suggests that cAMP may be affecting respiration via a direct effect on mitochondrial enzyme or transport activities. Type II cAMP-dependent protein kinase could also play a role in the special differentiation and placement of mitochondria in the midpiece. Preliminary results suggest that RII immunoreactivity in caput sperm is also localized in the mitochondria, but bovine heart mitochondria have negligible immunoreactivity.

The possible localization of RII immunoreactivity to the fibrous sheath in caudal epididymal sperm suggests a role of cAMP-dependent phosphorylation in the mechanical activities thought to be mediated by this structure. The relative lack of RII immunoreactivity directly associated with the other dense fibers and the axoneme suggests that direct regulation of these structures by cAMP is unlikely, the kinase mediating changes in motility is not tightly bound to tail structure, or possibly that a much smaller amount of RII cAMP-dependent kinase is sufficient for motility regulation as opposed to regulation of respiration. The identification of the mitochondria as the specific structures involved in binding the RII that remains tightly associated with the sperm tail after demembranation may permit further dissection of the molecular events associated with the increase in respiration or onset of motile behavior in these preparations.

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