Characterization of Maturation-dependent Extrinsic Proteins of the Rat Sperm Surface

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ABSTRACT Mammalian spermatozoa must mature in the epididymis before they can fertilize an egg. It is known that modification of the protein composition of the sperm surface is an important part of the maturation process. In this paper, we present data on two related glycoproteins that can be extracted from mature but not immature spermatozoa. Cell surface radioiodination has shown that these proteins are on the sperm surface, and immunofluorescence microscopy, by use of monospecific antibodies to the proteins, has indicated that their localization is restricted to the periacrosomal region of the sperm head. We have also shown that in vitro, these proteins will bind to the identical region of immature sperm. Immunohistochemical localization of the proteins in the epididymis shows that they are produced and secreted by the cauda region. The significance of the addition of these proteins to the sperm surface in both maturation and fertilization is discussed.

As they pass from the caput to the cauda of the epididymis, spermatozoa undergo a group of cellular modifications termed maturation. These modifications are dependent on the epididymal environment and must occur before the spermatozoa can fertilize an egg. Several general classes of changes have been described, which include the development of progressive forward motility, changes in the morphology of the acrosome, an increase in the disulfide cross-linking of several sperm organelles, and alterations in the properties of the plasma membrane (for reviews see references 3, 26, and 37).

Maturation-dependent changes of the sperm plasma membrane are of particular interest because of this membrane's contact with the epididymal environment and the role it plays in fertilization (for review see reference 25). Examples of membrane changes are an increase in the net negative surface charge (9, 19), changes in lectin binding patterns (21, 24), and differences in the proteins and glycoproteins that are available to various nonpenetrating probes (23, 33). Some of these surface changes result from interactions with proteins produced by the epididymis (7, 16, 20, 34) that may either bind to the plasma membrane, or otherwise modify it in some way which may be important in making the spermatozoon capable of fertilizing an egg. There have been reports of several proteins found in epididymal fluid that are thought to have a functional role in sperm maturation. These include forward motility protein (1), acrosome stabilizing factor (ASF) (11), and an epididymal glycoprotein thought to contribute to the development of the ability of spermatozoa to bind to the zona pellucida (27).

To select specific proteins from the great number present in the epididymal environment, we have chosen to look for extrinsic membrane proteins that appear on the sperm surface during maturation. In this paper, we present data on the identification, characterization, and localization of two related polypeptides that fit these criteria.

MATERIALS AND METHODS

Animals: Adult male Sprague-Dawley rats were kept on a 12-h light, 12-h dark cycle with free access to food and water.

Sperm Extractions: Rats were anesthetized with Nembutal and perfused via the testicular artery with phosphate-buffered saline (PBS; 0.145 M NaCl, 0.01 M sodium phosphate, pH 7.2) to remove blood from the reproductive tract. The epididymides were then removed and trimmed of excess fat, and the caput and cauda segments were separated from the remainder of the organ. These segments were then minced in PBS at 37°C, and the sperm suspensions were filtered through cheesecloth, divided into equal parts, and then centrifuged at 400 *g* for 10 min. The cells were washed once more in the same manner and then resuspended in either 2 ml PBS or 2 ml 0.4 M NaCl, 0.01 M sodium phosphate buffer at pH 7.2 (HIS).[1] The spermatozoa were then incubated for 4 min at 37°C and the suspensions centrifuged at 400 *g* for 10 min. The resulting supernatants were then centrifuged at 1,700 *g* for 10 min to remove any remaining spermatozoa. These supernatants were then cooled on ice, made 1 mM in phenylmethylsulfonyl fluoride, and then centrifuged at 100,000 *g* for

[1] *Abbreviations used in this paper:* FT, freeze-thawed; HIS, high ionic strength buffer.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 MAY 1985 1582–1591

45 min at 4°C. An additional 1 mM phenylmethylsulfonyl fluoride was added to these final supernatants, and they were dialyzed against deionized water that contained 0.1 mM phenylmethylsulfonyl fluoride for 15–18 h at 4°C and then concentrated by lyophilization. These samples are referred to as HIS or PBS sperm extracts.

In certain experiments, SDS or freeze-thaw (FT) extracts were made of PBS-washed spermatozoa. In these cases, the cells were washed twice and either suspended in 0.1% SDS or freeze-thawed twice in PBS, then centrifuged at 1700 *g* for 10 min. The supernatants were then prepared in the same manner as the HIS extracts. These samples are referred to as SDS or FT sperm extracts.

Cell Surface Radioiodination: The sperm surface was ^{125}I-labeled by the solid phase method of Fraker and Speck (14) and Markwell and Fox (17). Briefly, the cells were washed twice and the suspensions placed in scintillation vials which had been plated with 100 μg Iodogen (Pierce Chemical Co., Rockford, IL). 100 μCi [^{125}I]NaI were added, and the reaction was allowed to proceed with agitation for 10 min at room temperature. The cells were then pelleted at 400 *g* for 10 min and washed once with PBS. Sperm extracts prepared according to the above procedure were analyzed by SDS PAGE, as described below, and autoradiographed for 16 h on Kodak XAR-5 X-ray film.

Polyacrylamide Gel Electrophoresis: Lyophilized samples were solubilized in sample buffer (62 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) and then heated in boiling water for 1 min. Lanes were loaded with extracts from equal numbers of spermatozoa. Electrophoresis was done by use of the buffer system of Laemmli (15), on a 1.5-mm thick gel with a 10–15% linear gradient of acrylamide at a constant power of 18 W. Proteins were visualized by the silver staining method of Wray et al. (36).

Preparation of Antisera: Monospecific antisera to each of the salt-extractable proteins were prepared as follows. Pooled cauda epididymal spermatozoa from three rats were extracted with HIS as described above. This procedure was repeated five times, and all extracts were then pooled, which yielded 1.8 mg of total extracted protein as determined by the Bradford assay (5). This was prepared for electrophoresis as described above and was electrophoresed on a 7.5–12% gradient SDS gel. The gels were stained with Coomassie Blue (12), and the appropriate bands were cut from the gel. The strips were divided so that 60% of each was used for the initial injection and 20% for each of two booster injections. Before injection, the strips were homogenized with complete Freund's adjuvant for the initial injection or incomplete Freund's for the booster injections. One half of each mixture was then given at multiple subcutaneous and intramuscular sites to each of two rabbits, and booster injections were given at 2-wk intervals after the primary injection. Blood was collected by cardiac puncture 10 d after the final injection, and serum was prepared and stored at −60°C.

Western Blotting: Western blotting was done by modification of the method of Towbin et al. (32). Proteins were electrophoresed as described previously, except a 7.5–12% gradient, 0.75-mm thick running gel was used. The proteins were then transferred from the gel to a nitrocellulose membrane using 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3, transfer buffer, in a Bio-Rad Transblot cell with the cooling coil kept at 4°C. Transfers were done for 2 h at 30 V, then 2 h at 60 V. The blots were then stored at −20°C.

Proteins that specifically bound lectins were visualized in the following manner. The blots were thawed, rinsed with PBS, and then incubated for 1 h in 2.5% IgG-free bovine serum albumin (BSA; #7030, Sigma Chemical Co., St. Louis, MO) at 37°C with agitation. The blots were washed in PBS and then incubated at room temperature with either biotinylated-Concanavalin A (1 μg/ml) or biotinylated-wheat germ agglutinin (1 μg/ml) for 1 h. The blots were washed and incubated with an avidin/biotinylated-horseradish peroxidase complex as prepared from a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The blots were washed again, and the proteins that bound lectins were detected by incubation in a solution that contained 0.5 mg/ml diaminobenzidine, 0.01% H_2O_2 in 50 mM Tris-HCl, pH 7.5. Specificity of the binding was checked by the mixing of the lectins with specific competitive inhibitors before incubation. 100 mM α-Methyl-D-mannoside was used to inhibit Concanavalin A, and 10 mg/ml poly-*N*-acetylglucosamine was used for wheat germ agglutinin.

Detection of antibody binding on blots was done in a similar manner except immune or preimmune serum (1:500 dilution) was substituted for lectins, and the blots were incubated with an affinity-purified, biotinylated goat antibody directed against rabbit IgG (as prepared from the Vectastain kit) for 1 h, before incubation with the avidin/biotinylated-horseradish peroxidase complex.

Peptide Mapping: Peptide mapping was done by the method of Cleveland et al. (8), by use of *S. aureus* V8 protease. The bands for peptide mapping were cut from a Coomassie Blue–stained SDS PAGE gel and incubated for 30 min in 0.125 M Tris, 0.1% SDS, and 1 mM EDTA. These gel slices were placed in the wells of a 10–20% SDS PAGE gel, and the proteins they contained were electrophoresed into the stacking gel along with 5 ng of *S. aureus* V8 protease (Miles Laboratories, Elkhardt, IN). Electrophoresis was then stopped for 30 min to allow proteolysis and then completed in a normal manner. After electrophoresis, the polypeptides were visualized by silver staining.

Immunofluorescence: Spermatozoa were expressed from the caput and cauda epididymides, washed once with PBS, and then fixed in PBS that contained 3% formaldehyde. After fixation, the spermatozoa were pelleted and the fixed cells washed with PBS. A dilute sperm suspension was then incubated with either immune or preimmune serum at a dilution of 1:50 for 10 min at 32°C. The suspensions were then centrifuged at 750 *g* for 2 min, resuspended in PBS that contained 4% heat-inactivated normal goat serum, and incubated for 10 min at 32°C. This suspension was then centrifuged as above, washed once with PBS, resuspended in PBS with 1% fluorescein-conjugated, affinity-purified goat anti-rabbit IgG, and incubated at 32°C for 15 min. The cells were then pelleted and washed once with PBS. Matched fluorescence and phase-contrast pictures were then taken on Tri-X at E.I. 1,000 by use of a Zeiss 63 × 1.4 numerical aperture objective (Carl Zeiss, Inc., Thornwood, NY).

In Vitro Binding of HIS Proteins: Cauda epididymal spermatozoa were extracted with HIS as described, and the extracts made 0.1 mM in phenylmethylsulfonyl fluoride were dialyzed overnight against PBS at 4°C. The protein obtained from two cauda epididymides was then divided into two equal aliquots, 2.5–3 ml each, and used in a sperm-binding assay.

For in vitro binding, rats were sacrificed with Nembutal, the epididymides were removed and trimmed of fat, and divided into caput, corpus, and cauda regions. The caput and corpus regions were minced in 32°C PBS, the sperm suspensions filtered through cheesecloth, and diluted to 2.5–3 ml total volume with PBS. Each of these suspensions was then mixed with an aliquot of HIS

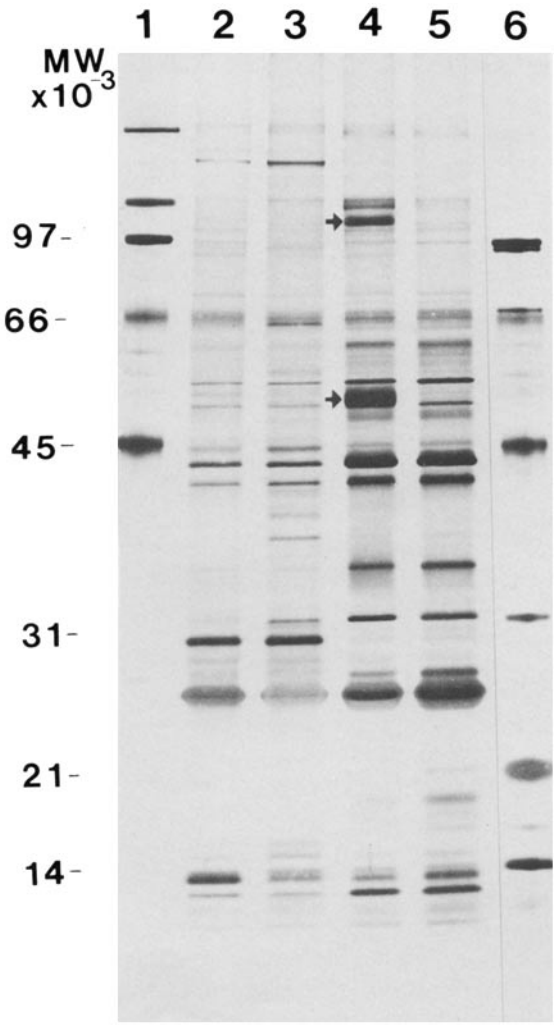

FIGURE 1 Silver-stained gel which shows HIS and PBS extracts of spermatozoa from the caput and cauda of the epididymis. Lanes are as follows: *1*, high molecular weight markers; *2*, caput PBS extract; *3*, caput HIS extract; *4*, cauda HIS extract; *5*, cauda PBS extract; and *6*, low molecular weight markers. HIS-100 and HIS-50 are indicated by arrows.

proteins or an equal volume of PBS, and incubated for 1 h at 32°C. The cells were then pelleted for 10 min at 400 *g*, fixed in PBS that contained 3% formaldehyde, and prepared for immunofluorescence microscopy as described above.

Immunocytochemical Localization: Rats were anesthetized with Nembutal and perfused via the testicular artery with PBS followed by Zamboni's fixative (28). Epididymides were removed, trimmed of fat, divided into caput, proximal, and distal corpus and cauda segments, and immersion fixed for 4 h followed by routine paraffin embedding and sectioning.

Immunohistochemical staining was done using primary antiserum and preimmune serum which had been affinity purified by the method of Talian et al. (30). Staining of the sections was accomplished using a Vectastain ABC kit (Vector Laboratories). The protocol supplied with the kit was followed without significant modification. In summary, the sections were deparaffinized and rehydrated, endogenous peroxidase activity was blocked by incubation with 0.5% H_2O_2 in methanol, sections were washed and incubated in PBS that contained 4% normal goat serum, then affinity-purified antiserum or preimmune serum. Sections were washed and then incubated with affinity-purified biotinylated anti-rabbit IgG, washed again, and incubated with avidin/biotinylated-horseradish peroxidase complex. Sections were washed again, and color was developed by incubation with 0.05% diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-HCl (pH 7.5). Sections were then washed, and in some cases, counterstained with hematoxylin.

RESULTS

Extractions

Spermatozoa from the caput and cauda epididymides were extracted with HIS buffer to remove extrinsic proteins from the sperm surface. The extracts were then compared using SDS PAGE to identify components that appear during maturation. Two major polypeptides with molecular masses of 50 and 100 kD were specifically extracted from mature spermatozoa by HIS (Fig. 1). We have designated these proteins HIS-50 and HIS-100, respectively. In addition, there was a minor HIS-specific band present at 112 kD in the extracts of caudal spermatozoa. Even in very heavily loaded gels, there were no indications of these proteins in either HIS or PBS extracts of spermatozoa from the caput of the epididymis or the PBS control extract of caudal spermatozoa. The PBS extracts did, however, contain a number of components that corresponded to proteins found in epididymal fluid. Three minor bands at 38, 36, and 30 kD were specifically extracted from spermatozoa of the caput epididymides by HIS.

Surface Radioiodination

Intact cells were surface radioiodinated before HIS extraction to determine if HIS-50 and HIS-100 are exposed to the external environment. Autoradiograms of gels of iodinated extracts show (Fig. 2*b*, lane 1) that HIS-50 and HIS-100 were labeled. The other proteins present in the HIS and PBS extracts were not labeled, which indicated that either these proteins are not located on the sperm surface or that they do

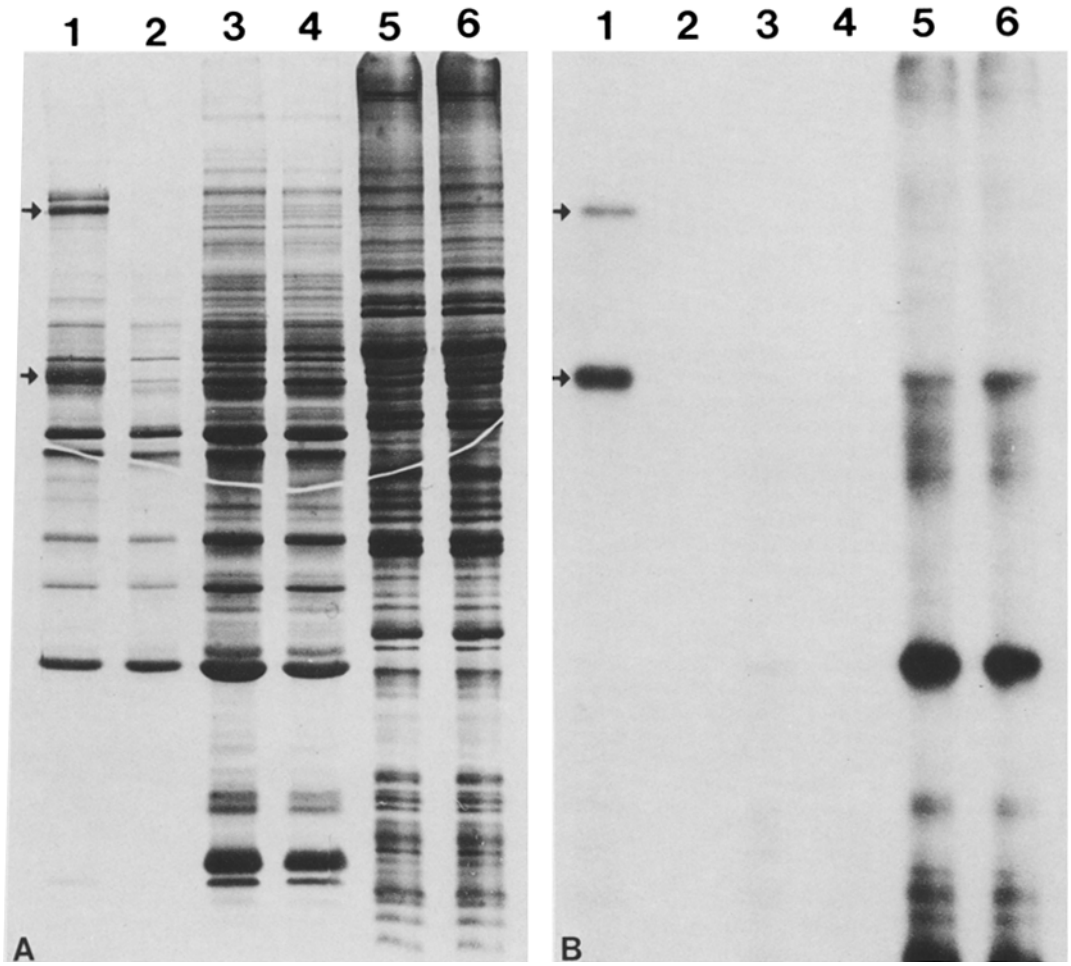

FIGURE 2 Silver-stained gel (*A*) and autoradiogram (*B*) of HIS, FT, and SDS extracts of ^{125}I-labeled caudal spermatozoa. Lanes are as follows: *1*, HIS extract; *2*, PBS extract; *3*, FT extract of spermatozoa after HIS extraction; *4*, FT extract of sperm after PBS extraction; *5*, SDS extract of spermatozoa after HIS and FT extractions; and *6*, SDS extract of spermatozoa after PBS and FT extractions.

not have tyrosine residues available to the probe. Cytoplasmic proteins were released by freeze-thawing after HIS extraction to check the surface specificity of the labeling, and these proteins were analyzed by SDS PAGE and autoradiography (Fig. 2, *a* and *b*; lanes *3* and *4*). Silver staining reveals that many proteins were released by freeze-thawing, but the matching autoradiogram shows that none of these proteins have been ^{125}I-labeled. In addition, the freeze-thawed cells were extracted with 0.25% SDS to solubilize membrane proteins, and these extracts were then analyzed as above. The silver-stained lanes (Fig. 2*a*, lanes *5* and *6*) of these extracts show that many proteins are present in the detergent extracts, but autoradiography (Fig. 2*b*, lanes *5* and *6*) indicates that only a specific subset of these proteins has been labeled, which demonstrates that not all of the detergent-soluble proteins were available to the probe.

Glycosylation

Western blots of HIS, PBS, and SDS extracts of spermatozoa were prepared and stained for lectin binding. HIS-50 and HIS-100 bind both Concanavalin A which is specific for mannose, and wheat germ agglutinin which is specific for *N*-acetylglucosamine. The binding is blocked by competition with appropriate sugars. The blot stained for Concanavalin A binding also shows that there are many other glycoproteins present in all three extracts, and the SDS extract is particularly rich in glycoproteins. Labeling of the HIS proteins is not obvious in the SDS extracts as compared with the HIS extracts, which indicates that the HIS proteins make up a minor fraction of the total sperm protein.

Antibody Specificity

Western blots were used to determine the specificity of the antisera prepared against the HIS proteins. Fig. 3 illustrates the binding of preimmune serum and antisera prepared against HIS-50 and HIS-100 to HIS, PBS, and SDS extracts of cauda epididymal spermatozoa. These experiments show that both anti-HIS-50 and anti-HIS-100 sera react with both of the HIS proteins, but neither antiserum shows cross reactivity towards any of the other proteins in the PBS or SDS extracts. There is only faint staining of the HIS proteins in the SDS extracts because of the relatively minor abundance of the HIS proteins as compared with total SDS-extractable

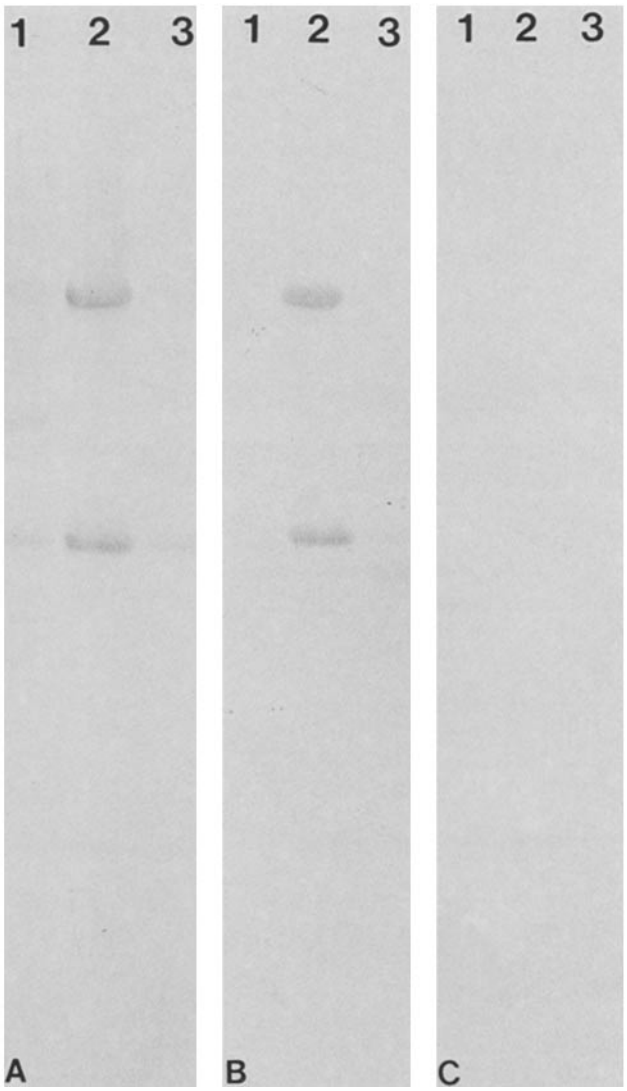

FIGURE 3 Western blots of extracts of caudal spermatozoa stained with: *A*, anti-HIS-100 serum; *B*, anti-HIS-50 serum; and *C*, preimmune serum. Lanes: *1*, PBS extract; *2*, HIS extract; *3*, SDS extract.

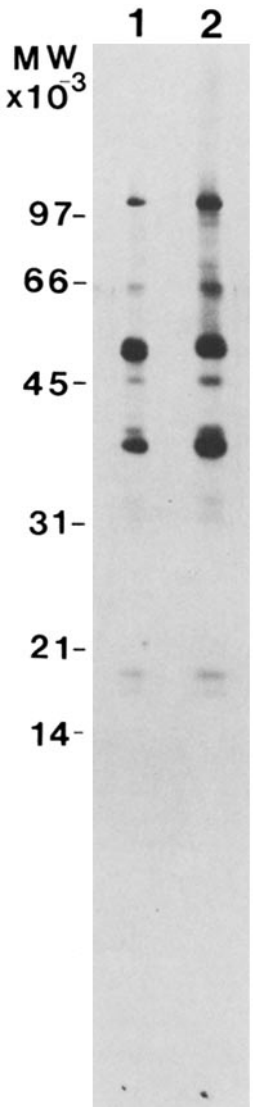

FIGURE 4 Peptide map of HIS-50 (lane *1*) and HIS-100 (lane *2*).

protein. The preimmune serum shows no binding to any of the extract proteins.

Peptide Mapping

To determine if the antigenic similarity of the HIS proteins had a structural basis, we compared the proteins by using the peptide mapping technique of Cleveland et al. (8). The map (Fig. 4) shows that HIS-50 and HIS-100 have very similar polypeptide compositions, both having components at 100, 66, 50, 46, 39, and 38 kD. The components with molecular masses >50 kD are, however, present in relatively smaller amounts in the HIS-50 map than are in the HIS-100 map.

Immunofluorescence

Immunofluorescence microscopy was done to determine the distribution of HIS-50-100 on the surface of mature spermatozoa. The micrographs shown used anti-HIS-100 serum, but identical results were obtained using anti-HIS-50. It can be seen in Figs. 5 and 6 that the antigens are present only on the periacrosomal region of the sperm head, with no significant fluorescence occurring on any other region of the spermatozoa. Fluorescence was observed in ~70% of the cells. Spermatozoa from the caput of the epididymis (Fig. 7) and spermatozoa treated with preimmune serum showed no fluorescence (Figs. 5 and 7).

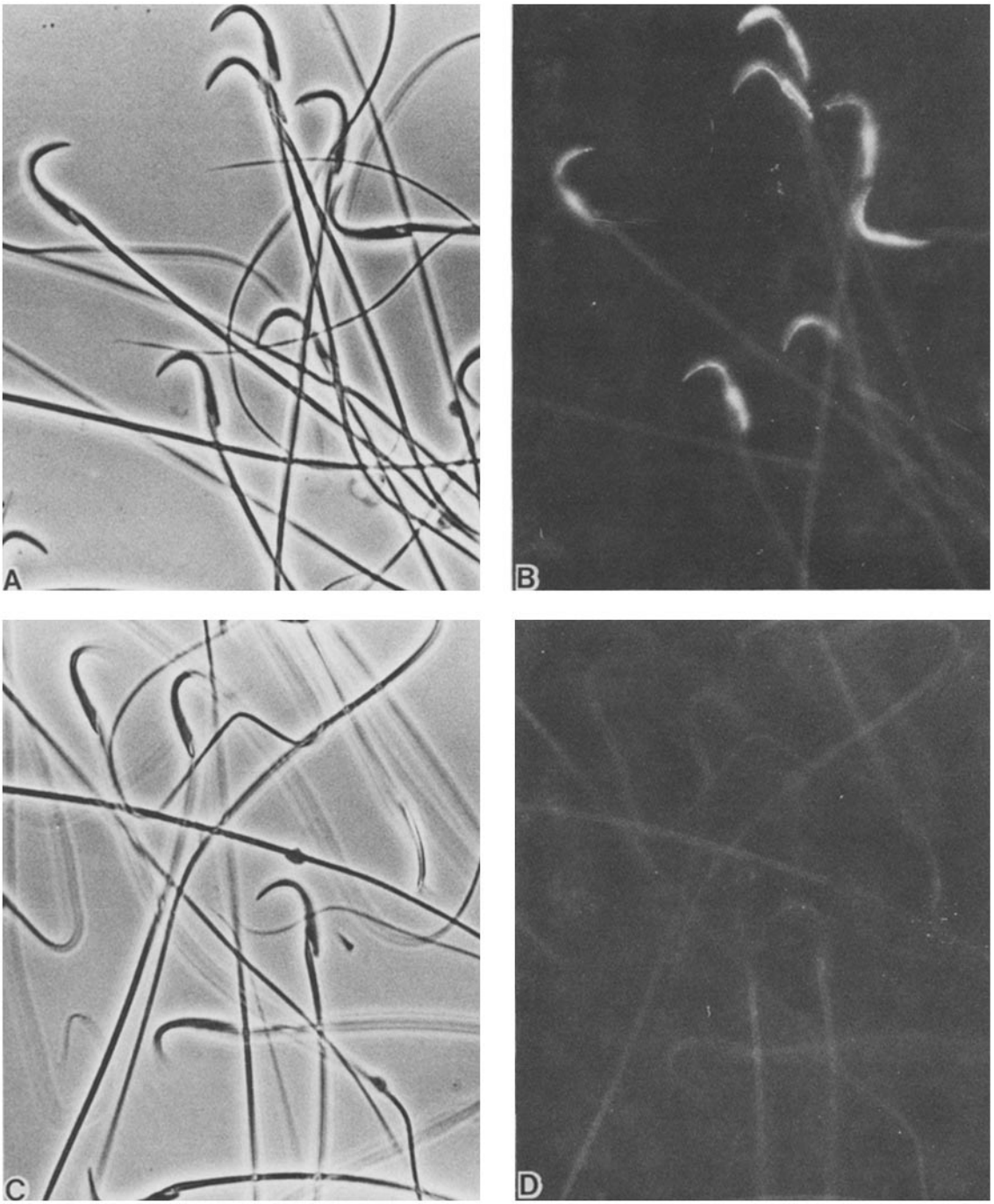

FIGURE 5 Matched phase-contrast (*A* and *C*) and immunofluorescence (*B* and *D*) micrographs of spermatozoa from the cauda of the epididymis treated with anti-HIS (*A* and *B*) and preimmune (*C* and *D*) sera. ×1,100.

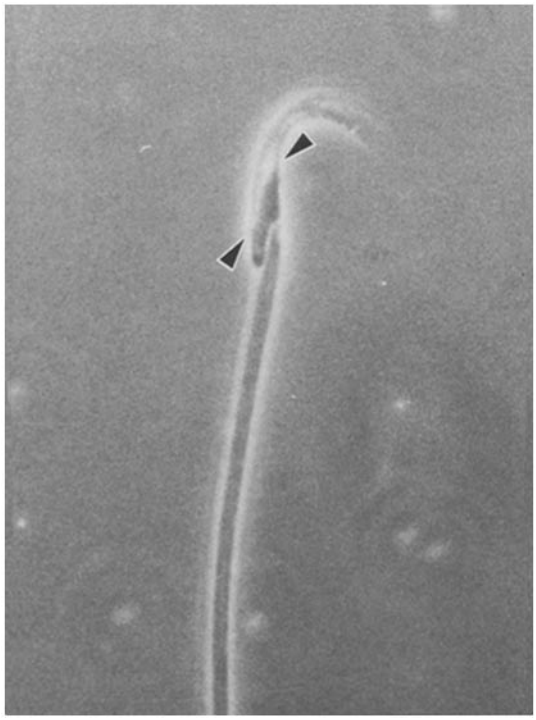

FIGURE 6 Phase-contrast and immunofluorescent double exposure of a spermatozoon from the cauda of the epididymis treated with anti-HIS serum, which shows fluorescence of the acrosomal region of the sperm head. Arrowheads indicate the posterior border of the fluorescent region. ×1,900.

In Vitro Binding of HIS Proteins

To determine if immature spermatozoa would bind the HIS proteins, we incubated caput and corpus epididymal spermatozoa with the HIS proteins as described in Materials and Methods. Immunofluorescence microscopy of corpus spermatozoa which had been treated in this manner demonstrated binding of the proteins to the acrosomal region of the sperm head (Fig. 8, *a–d*). Approximately 40% of the corpus spermatozoa exhibited fluorescence over this region, and there was no fluorescence visible on any other region of the cell. This pattern of binding is identical to that found in caudal spermatozoa. In contrast, none of the caput spermatozoa incubated with HIS proteins showed any indication of fluorescence (Fig. 8, *e* and *f*). Control incubations of caput and corpus spermatozoa incubated in PBS did not exhibit any fluorescence.

Immunohistochemical Localization of HIS-50-100

Immunohistochemical localization of the HIS proteins in the epididymis was done to determine if the proteins are synthesized by the epididymal epithelium and, if so, to identify the region of the epithelium that produces the protein. The proteins were not detectable in any region of the epididymis proximal to the proximal cauda region where they first appear in the cytoplasm of the principal cells (Fig. 9). Staining was restricted to the supranuclear area of these cells, with no indication of staining of the nucleus or in the basal region of the cells (Fig. 10). Clear cells, basal cells, peritubular cells, and connective tissue were negative in all regions of the epididymis.

The HIS proteins are first detectable in the epididymal lumen slightly distal to the region where the proteins first appear in the epithelium. As shown in Fig. 9, there is both specific staining of the spermatozoa and the appearance of diffuse patches of reaction product beginning in this area. The epithelium of this region is more intensely stained than where the proteins first appear, but the pattern of staining is unchanged. In the most distal tubules of the cauda region, staining is no longer present in the principal cell cytoplasm, but their stereocilia and the contents of the tubule lumen remain heavily stained. There are also light deposits of reaction product at the base of the epithelium (Fig. 10*c*).

DISCUSSION

The association of epididymal fluid proteins with spermatozoa has been established in a variety of species, and it is thought that these proteins have a functional role in the development of fertilizing ability by the spermatozoa. In the rat, there have been many studies (6, 13, 16, 35) that have dealt with a group of similar proteins with molecular masses of ~32 kD, and there has been a suggestion that a protein of this group might have a role in the binding of the spermatozoa to the zona pellucida (27). There have also been reports that in other species epididymal proteins have a physiological effect on maturing spermatozoa. These include forward motility protein, which is thought to help induce progressive forward motility in bovine spermatozoa (1), and ASF, which seems to inhibit the acrosome reaction in rabbit spermatozoa. ASF appears to be removed from rabbit spermatozoa by a high ionic strength medium (4, 22), but a medium with approximately the same ionic strength (200 mM NaCl) did not extract the HIS proteins. Current molecular mass estimates for the ASF subunits are 90 and 38 kD (31), but with no data on the effect of the HIS proteins on the acrosome reaction, it is impossible to make further comparisons between these proteins.

The data presented here characterize two proteins secreted by the epididymis that bind to the sperm surface during maturation. These proteins are removed from the plasma membrane by high ionic strength media and therefore can be classed as extrinsic membrane proteins, and although they migrate very differently on SDS PAGE, the proteins exhibit a high degree of immunological and structural similarity. The presence of polypeptides with molecular masses of 100 and 66 kD in the HIS-50 peptide map indicates that the HIS-50 polypeptides can reassociate, and suggests that HIS-100 may be a dimeric form of HIS-50, which is not completely dissociated under the electrophoretic conditions used. At this time, nothing is known about what may be cross-linking the dimer or the native forms of HIS-100 and HIS-50.

Immunofluorescence microscopy shows that the HIS proteins are located on the periacrosomal region of caudal spermatozoa. The lack of fluorescence on 30% of the spermatozoa is probably due to the loss of the plasma membrane from this region, and electron microscopy (unpublished data) confirms that this structure is easily lost. In vitro, spermatozoa from the corpus of the epididymides can bind the HIS proteins specifically to the periacrosomal region, but caput spermatozoa cannot bind the HIS proteins, which suggests that a receptor for HIS-50-100 is unmasked or in some way modified to allow binding during transit through the corpus epididymis. The lack of HIS binding by 60% of the corpus spermatozoa

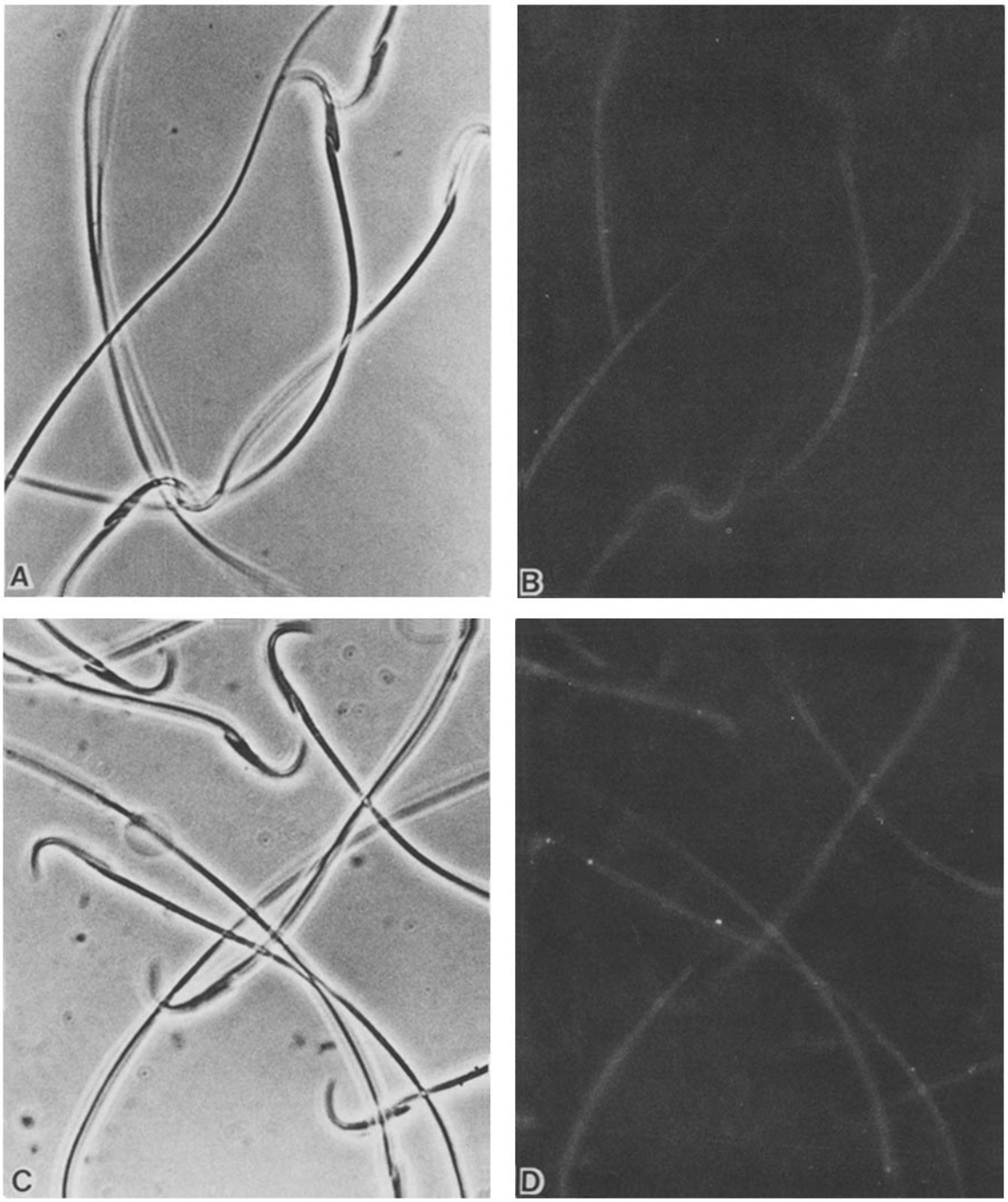

FIGURE 7 Matched phase-contrast (*A* and *C*) and immunofluorescence (*B* and *D*) micrographs of spermatozoa from the caput of the epididymis treated with anti-HIS (*A* and *B*) and preimmune (*C* and *D*) sera. ×1,100.

probably represents both the spermatozoa which have not reached the degree of maturity necessary for binding of the proteins and those which have lost their periacrosomal plasma membrane. The corpus epididymides corresponds to the region of the epididymis where the variable glycocalyx material which coats the periacrosomal region of caput spermatozoa is lost (29), and it seems possible that this loss may be related to the development of the ability to bind the HIS proteins by the spermatozoa. The requirement of elevated ionic strength for the removal of the HIS proteins is also consistent with the idea that their binding to the sperm surface may be receptor mediated.

Other attempts to bind epididymal proteins to spermatozoa in vitro have used radiolabeled proteins to detect binding. In the rat, Wong and Tsang (35) have claimed specific binding of a 32-kD protein isolated from cauda epididymal fluid to both immature and mature spermatozoa. Also in the rat, Brooks and Tiver (7) have shown that specific proteins secreted by the caput epididymides will bind to both testicular and caudal spermatozoa; however, the same set of proteins will also bind to erythrocytes which makes it difficult to interpret the specificity of the binding. The binding of the HIS proteins would not have been detected by these experiments because they would not have been included in the

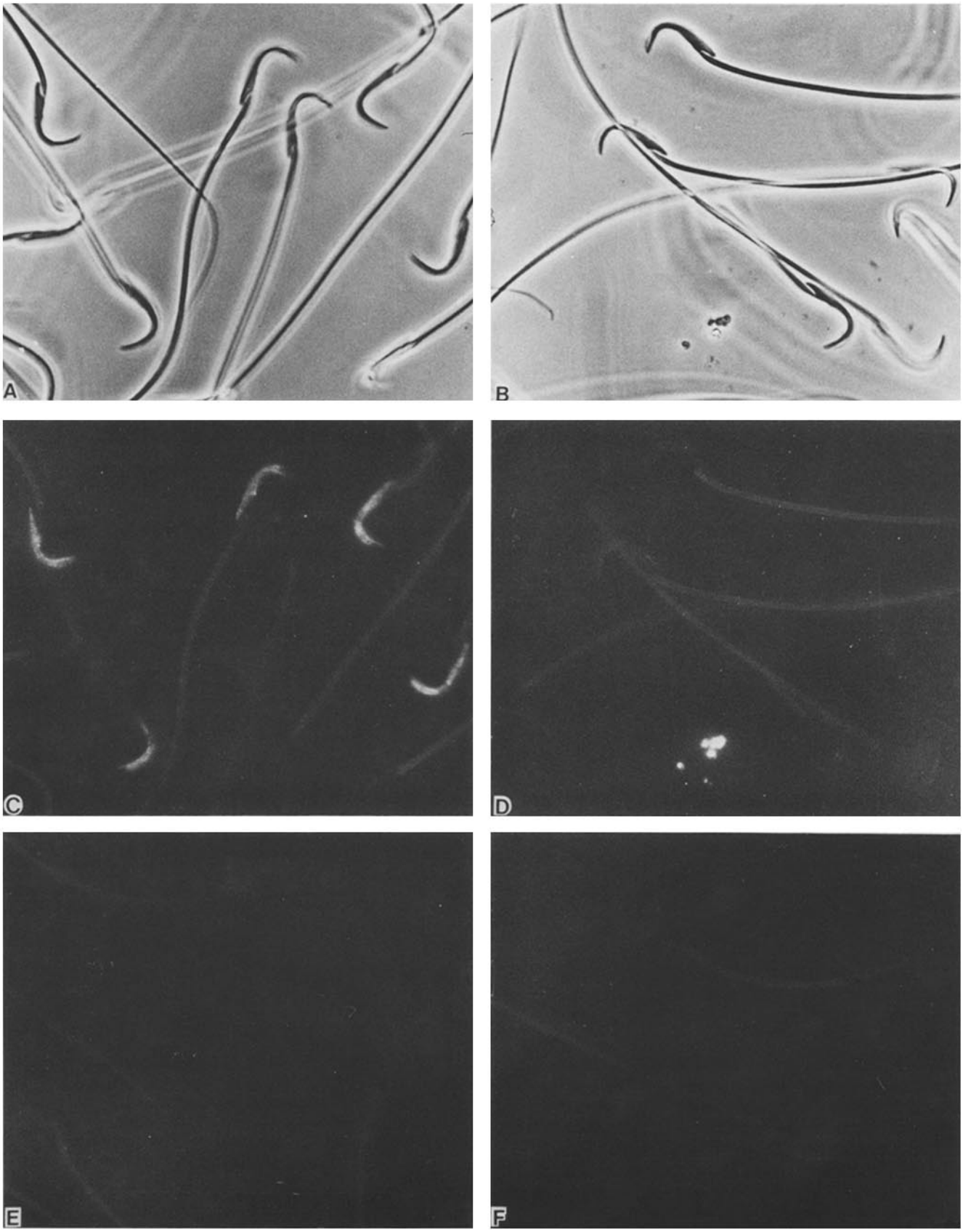

FIGURE 8 Immunofluorescent localization of the HIS proteins on caput and corpus spermatozoa after in vitro binding of the proteins. (*A–D*) Matched phase-contrast (*A–B*) and immunofluorescence (*C–D*) micrographs of corpus spermatozoa after in vitro binding (*A* and *C*) and PBS control (*B* and *D*). (*E–F*) Immunofluorescence micrographs of caput spermatozoa after in vitro binding (*E*) and PBS control (*F*). ×1,100.

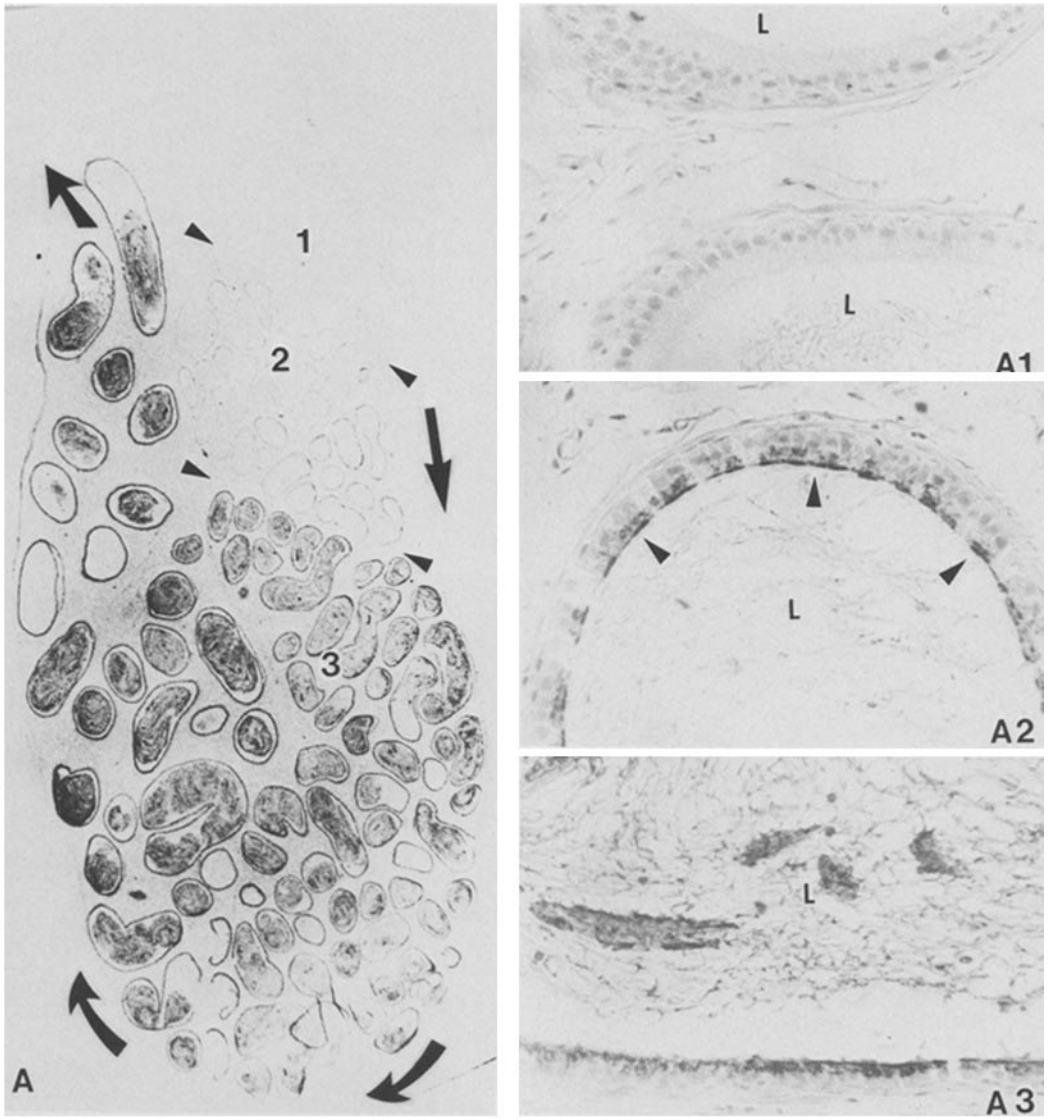

FIGURE 9 Immunohistochemical localization of HIS proteins in sagittal section of the cauda epididymides. (*A*) Low magnification view of the entire cauda region. The arrows surrounding the tissue indicate the direction of flow through the epididymis. Arrowheads indicate connective tissue septae which divide the lobules of the epididymis. (*A1*, *A2*, and *A3*) Higher magnification views corresponding to regions 1, 2, and 3 indicated in *A*. *L*, lumen of the epididymal tubule. There is no indication of the proteins proximal to region 2, where they first appear in the epithelium (*A2*, arrowheads). In region 3, the proteins are detectable both in the epithelium and the lumen of the tubule. *A*, not counterstained; *A1*, *A2* and *A3*, counterstained with hematoxylin. (*A*) ×12; (*A1*, *A2*, and *A3*) ×280.

proteins secreted by the caput epididymides. Voglmayr et al. (34) have shown that in the ram, there is a 24-kD protein which specifically binds to testicular spermatozoa when they are incubated with radioiodinated cauda epididymal fluid. The present study is the only one we know of where the in vitro binding of an epididymal protein to a specific region of immature spermatozoa has been demonstrated.

The secretion of the HIS proteins by a restricted region of the epididymal epithelium is similar to the pattern of secretion of other sperm-associated proteins such as acidic epididymal glycoprotein (16) and ASF (31), although the HIS proteins appear to be secreted lower in the epididymis than these proteins. This helps to confirm the idea that different regions of the epididymis make distinct contributions to sperm maturation.

The periacrosomal plasma membrane, where the HIS proteins are localized, is a region of the sperm surface that is very active during fertilization, being involved in the acrosome reaction (2) and possibly in the binding of the spermatozoa to the zona pellucida (27, 37). It also has been suggested that the plasma membrane that overlies the equatorial segment is involved in the initial fusion of spermatozoa with the egg plasma membrane (18). In addition, the HIS proteins first appear in the proximal cauda of the epididymis, which is the region where rat spermatozoa show a great increase in fertilizing ability (10). These factors make it a possibility that the appearance of the HIS proteins on the sperm surface could be a significant event in the development of fertilizing ability by the spermatozoa during epididymal maturation. This, combined with the ability to specifically bind the HIS proteins

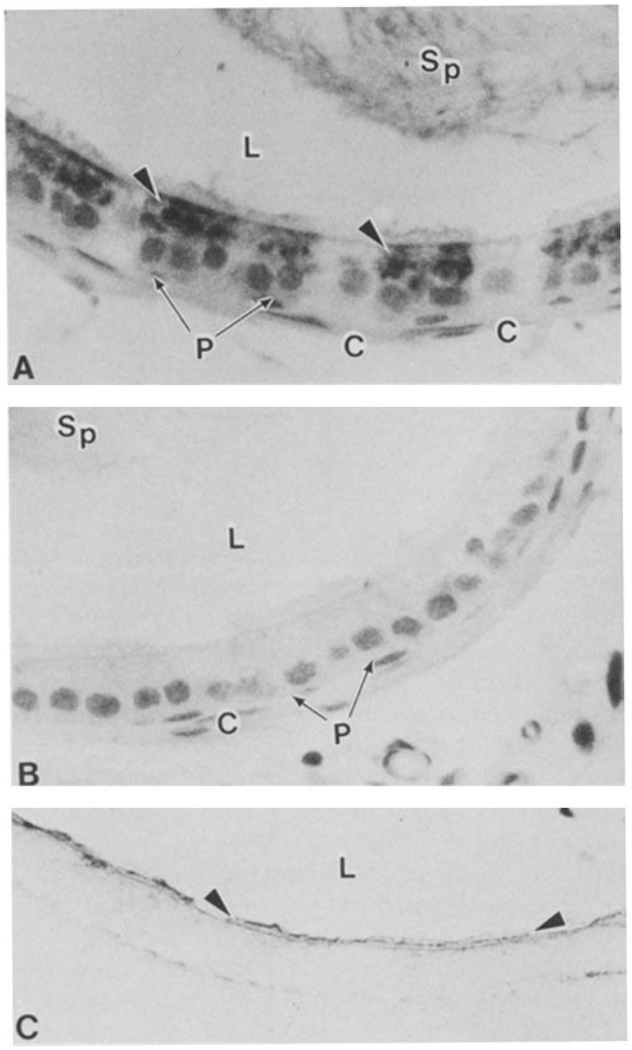

FIGURE 10 Immunohistochemical localization of the HIS proteins in the cauda epididymides. (*A*) Immunoperoxidase staining with anti-HIS serum, counterstained with hematoxylin. Reaction product (arrowheads) is located in the apical part of the principal cells (*P*) and on the spermatozoa (*Sp*). (*B*) Immunoperoxidase staining with preimmune serum, counterstained with hematoxylin. (*C*) Immunoperoxidase staining of the distal cauda epididymides with anti-HIS serum, not counterstained. Reaction product (arrowheads) is located mainly on the stereocilia of the principal cells. *P*, principal cells; *C*, clear cells; *L*, tubule lumen; *Sp*, spermatozoa. ×580.

to immature spermatozoa, makes these proteins interesting candidates for further studies on the molecular mechanisms of maturation and fertilization.

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