Further Characterization of a Secreted Epididymal Glycoprotein in Mice that Binds to Sperm Tails

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Sperm maturation antigen 4 (SMA-4) is a surface component of the mouse sperm tail. Previously, immunofluorescence studies indicated that SMA-4 may be secreted by principal cells of the distal caput epididymidis and bound to spermatozoa as they pass through that region of the duct. In the present study, detergent extracts of spermatozoa from the cauda epididymidis were subjected to polyacrylamide gel electrophoresis under reducing and denaturing conditions, transferred to nitrocellulose, and immunostained with a monoclonal antibody against SMA-4. A band of approximately 54,000 molecular weight was revealed. The band was also stained by the periodic acid-Schiff (PAS) procedure. This glycoprotein was not detected in extracts of spermatozoa from the proximal caput epididymidis or of spermatozoa from the cauda epididymidis that were preincubated for 4 hours in an *in vitro* fertilization environment. Blots of sperm-free fluid from the corpus and cauda epididymidis displayed an immunoreactive and PAS-positive band of about 85,000 molecular weight that was not observed in fluid from the caput epididymidis. The difference in the molecular weights of the antigen in the fluid and that in extracts of cauda spermatozoa suggests that SMA-4 may be modified chemically upon association with the sperm surface.

Key words: spermatozoa, surface antigen, biochemistry, monoclonal antibody, mouse.

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Mammalian spermatozoa from the testis and proximal epididymis are incapable of fertilization but gain this ability as they pass into the distal epididymis (Pavlok, 1974; Bedford, 1975; Orgebin-Crist et al, 1975). Coincident with these functional changes, the sperm surface is modified (Cooper and Bedford, 1971; Olson and Hamilton, 1978; Dravland and Joshi, 1981). These surface changes may be important for spermatozoa to acquire fertilizing ability, the potential for effective flagellar motion (Acott et al, 1983), and the capacity to bind to the zona pellucida (Saling, 1982; Orgebin-Crist and Fournier-Delpech, 1982).

We have used monoclonal antibodies generated from syngeneic immunization of male mice with whole spermatozoa to identify sperm maturation antigens that appear in spatially restricted domains on the surfaces of mouse spermatozoa as they pass through the epididymis (Feuchter et al, 1981). One of these, sperm maturation antigen-4 (SMA-4), has some particularly interesting properties. It is apparently secreted by principal cells of the epididymis, binds specifically to the surface of sperm tails (Vernon et al, 1982), and is lost from spermatozoa maintained *in vitro* under conditions that allow fertilization (Vernon et al, 1985).

Prior studies have used immunolabeling and immunoassay methods to examine the distribution of SMA-4 on intact spermatozoa, in tissue sections, in epididymal fluids, and in detergent extracts of sper-

matozoa. In the present study, we have used the Western blot method to identify SMA-4 in both epididymal fluids and detergent extracts of spermatozoa. Sample components were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and then immunostained with a monoclonal antibody to SMA-4.

Materials and Methods

Animals

Swiss-Webster and C57BL/6 strain male mice, 8 to 20 weeks of age, were housed in a controlled environment with a 12 hour daylight/night cycle and provided with Purina Lab Chow and water *ad libitum*.

Sample Preparation for Gel Electrophoresis

Spermatozoa were isolated from the proximal caput epididymidis (region A defined by Pavlok, 1974) and the cauda epididymidis and washed by a 5-minute centrifugation at 350 × g through 0.5 ml of room temperature Dulbecco's phosphate-buffered saline, pH 7.4. They then were extracted for 30 minutes at room temperature in a pH 7.4 solution of 10 mM HEPES, 0.15 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5% Nonidet P-40 (NP-40). Spermatozoa were removed from the extract by three centrifugations of 5 minutes each at 350 × g, 750 × g, and 2000 × g. Cell pellets were discarded between centrifuging steps. More than 90% of the spermatozoa were removed by the first step. After centrifugation, the extract supernatant was diluted tenfold by dropwise addition of ice-cold acetone, and after 30 minutes, the precipitate was collected by centrifugation for 5 minutes at 2000 × g and dissolved in disaggregation buffer (Herr and Eddy, 1980).

Epididymal fluids were obtained from the proximal caput, mid-corpus, and cauda epididymidis. Epididymal segments from five mice were placed in 100 μl of room temperature Dulbecco's PBS containing 0.2 mM PMSF, pierced in a few places with 00 insect pins and the contents expressed gently into the medium. Fluid obtained in this fashion is derived mainly from the lumen of the epididymidal duct, although a certain amount of peritubular interstitial fluid is likely to be present. Spermatozoa were removed from the diluted epididymal fluids by centrifuging for 5 minutes at 400 × g, 3000 × g, and 10,000 × g, with the pellets discarded after each centrifugation. The fluids were then combined with equal volumes of 2 X-concentrated disaggregation buffer. Normal mouse sera from unimmunized C57BL/6 and Swiss-Webster males and also a lyophilized preparation (Cappel Laboratories, Cochranville, PA) were diluted five-fold in disaggregation buffer.

Polyacrylamide Gel Electrophoresis

Sperm extracts, normal mouse sera, and epididymal fluids were heated at 90 C for 5 minutes in disaggregation buffer containing 1% 2-mercaptoethanol. Samples were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 5% stacking gel and a 10 or 12% separating gel and the Laemmli buffer system (Laemmli, 1970). For all gel samples, 15 μg (total protein) were applied per gel lane.

To reveal proteins, gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 5 parts methanol: 1 part acetic acid: 5 parts water (5:1:5, v/v/v). To reveal glycoconjugates, gels were fixed for 2 hours in 5:1:5 v/v/v methanol:acetic acid:water and then stained using a periodic acid-Schiff procedure described in the booklet Polyacrylamide Gel Electrophoresis, Laboratory Techniques (1980, Pharmacia Fine Chemicals AB, Uppsala, Sweden). Gels were incubated for 2 hours in the Schiff reagent. The R_f values of bands were determined by measuring their migration from the origin of the separating gel relative to the tracking dye front. The tracking dye was Bromophenol Blue.

Immunostaining of Western Blots

Following PAGE, samples were transferred to nitrocellulose paper (BA 85, Schleicher and Schuell, Keene, NH) by lateral electrophoresis (Towbin et al, 1979). Some blots were stained for protein with 0.1% Amido Black 10B in 5:1:5 methanol:acidic acid:water and destained in 90 parts methanol: 2 parts acetic acid: 8 parts water (Schaffner and Weissmann, 1973). To immunostain blots, nonspecific binding sites were blocked by a 3-hour immersion in 50 mM Tris-buffered saline, pH 7.4, supplemented with 1% (w/v) bovine serum albumin and 5% (v/v) normal goat serum. The blots were then incubated for 3 hours in monoclonal antibody to SMA-4 (Feuchter et al, 1981), washed 2 hours in six changes of Tris-buffered saline and incubated 2 hours in peroxidase-conjugated goat antibodies to mouse IgG (Cappel Laboratories) diluted 1:1000 in blocking solution. The blots were then washed for 2 hours in seven changes of Tris-buffered saline. Bound peroxidase was visualized with 4-chloro-1-naphthol (Hawkes et al, 1982).

Results

Western blots of sperm extracts from the cauda epididymidis immunostained with a monoclonal antibody to SMA-4 displayed a major band of R_f 0.47 and, in some preparations, a minor band of R_f 0.56 (Fig. 1). These values are the averages of 10 trials. The bands were assigned average molecular weights of 54,000 (54 K, range of 53 K to 56 K) and 42 K (range of 40 K to 48 K), respectively, based on their positions in a semi-log plot of R_f versus molecular weight for six marker proteins. Bands did not appear on Western blots when the monoclonal antibody was omitted or when concentrated supernatant from NS-1 myeloma cells (Feuchter et al, 1981) was substituted for the monoclonal antibody. To test for binding of the monoclonal antibody to blood serum contaminants present in sperm and epididymal fluid preparations, blots of normal mouse serum were immunostained. No bands were observed.

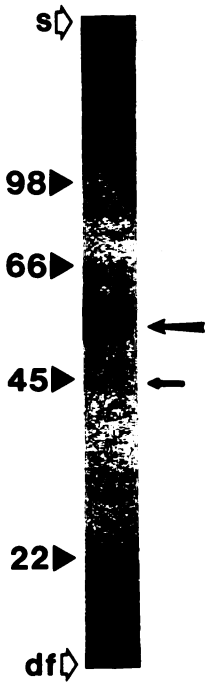

Fig. 1. Western blot of an extract of spermatozoa from the cauda epididymidis labeled with a monoclonal antibody to SMA-4. A single, large band of R_f 0.47 (large arrow) and a faint, narrow band of R_f 0.56 (small arrow) are observed. Open arrows s and df indicate the start of the separating gel and the position of the dye front, respectively. Positions of marker proteins in Figs. 1 through 4 are indicated by arrowheads. Molecular weight values are $\times 10^{-3}$.

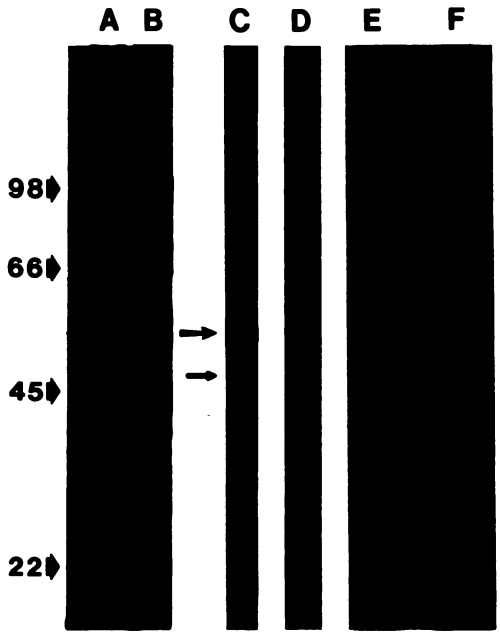

Fig. 2. NP-40-extracted material from cauda spermatozoa is compared with similarly prepared material from caput spermatozoa. Lanes A and B: Western blots of cauda (A) and caput (B) sperm extracts stained with Amido Black 10B. Band patterns show differences. Lanes C and D: Western blots of epididymal sperm extracts labeled with a monoclonal antibody to SMA-4. In the cauda sperm extract (C), major and minor bands (large and small arrows) are immunostained. In the caput sperm extract (D), no bands are visible. Lanes E and F: gel of sperm extracts from the cauda and caput epididymis stained for saccharides by the PAS method. The cauda sperm extract (E) shows a prominent, broad band (arrow) that corresponds in R_f to the broad, SMA-4-positive band of lane C. In the caput sperm extract (F), this band is not present.

Immunohistochemical studies on sections of epididymis and on intact spermatozoa indicate that SMA-4 is expressed only on spermatozoa obtained distal to the caput epididymidis (Vernon et al, 1982). This was confirmed by comparing extracts of spermatozoa from the caput and cauda epididymidis. Results are shown in Fig. 2. Blots of cauda and caput spermatozoa had different band patterns when stained for protein with Amido Black (lanes A and B). On blots immunostained for SMA-4 (seven trials), caput sperm extracts had no bands (lane D), while the cauda sperm extract had the major and minor bands (lane C). In gels stained for saccharides by the PAS procedure, a wide, strongly stained band that was very similar in appearance to the immunolabeled major band was observed in the 54 K region in cauda sperm extracts (lane E) but was not present in sperm extracts from the caput epididymidis. A faint, diffuse PAS-stained area, spanning roughly 45 K to 52 K, was present in three of six caput sperm preparations (lane F).

Although both the monoclonal antibody and PAS stained a 54-K band in cauda sperm extracts, the band was not readily apparent on similar blots stained with Amido Black or in gels stained with Coomassie Blue. Under these conditions, the 54-K band coincided with a very lightly stained or unstained region located just below a heavily stained band (Fig. 4, lane A). In some preparations, several narrow bands were seen within the region (Fig. 2, lane A, star).

Previous immunofluorescence studies suggest SMA-4 is secreted by the epididymis; therefore, sperm-free fluids from different regions of the duct were analyzed. Results are shown in Fig. 3. Caput, corpus, and cauda fluids had different patterns in gels stained with Coomassie Blue or PAS (lanes A, B, C, and E, F, G). These patterns also differed from those of NP-40 extracts of cauda spermatozoa (lanes D and H). On Western blots immunostained for SMA-4, caput fluid had no bands (lane I) but fluid from the cauda epididymidis (lane K) displayed a broad band that ran considerably slower (at about 85 K) than the corresponding band from the cauda sperm extract (lane L). The 85-K band corresponded in position to a portion of a large PAS-positive band that was present in fluid from the cauda epididymidis (lane G, arrow),

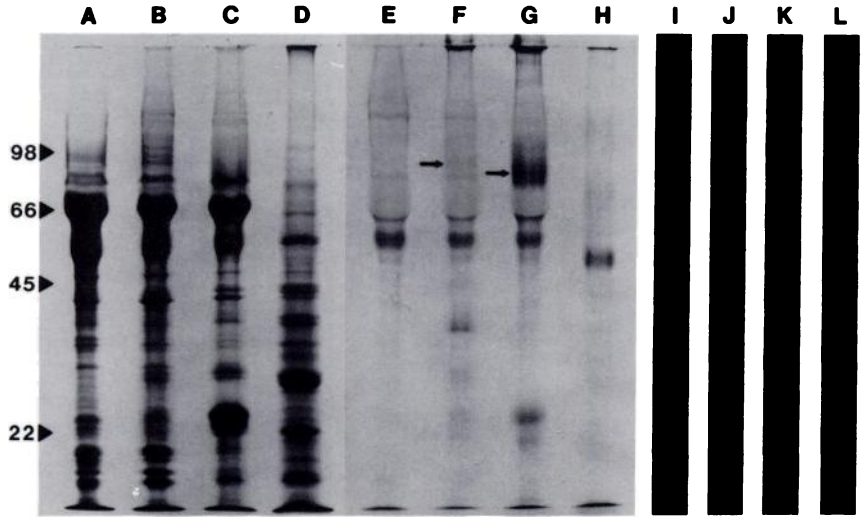

Fig. 3. Components of sperm-free fluids isolated from the caput, corpus, and cauda epididymidis are compared with NP-40-extractable material from cauda spermatozoa. Lanes A through D: gel stained with Coomassie Blue. Lanes E through H: gel stained with the PAS method. Lanes I through L: Western blot labeled with a monoclonal antibody to SMA-4. Lanes A, E, I: caput fluid. Lanes B, F, J: corpus fluid. Lanes C, G, K: cauda fluid. Lanes D, H, L: cauda sperm extract. SMA-4 monoclonal antibody binds to an 85-K component in corpus and cauda fluids. This band is also PAS-positive (arrows).

but absent from caput fluid (lane E). A narrow, immunostained band in the 85-K area was seen in corpus fluid (lane J) and corresponded to a PAS-positive band (lane F, arrow).

Studies using immunofluorescence indicate that SMA-4 is not expressed on 90% of cauda spermatozoa after a 3-or-more-hour incubation in an *in vitro* fertilization medium (Vernon et al, 1985). No differences were noted in the Coomassie Blue band patterns (Fig. 4, lanes A and B) of NP-40 extracts made from spermatozoa incubated for 4 hours (4-hour samples), compared with extracts from nonincubated spermatozoa (0-hour samples). However, the PAS-positive, 54-K band present in the 0-hour samples (lane C) was not seen in the 4-hour samples (lane D). Results obtained with a monoclonal antibody to SMA-4 paralleled the PAS findings; 0-hour samples displayed a 54-K band (lane E), while 4-hour samples did not (lane F).

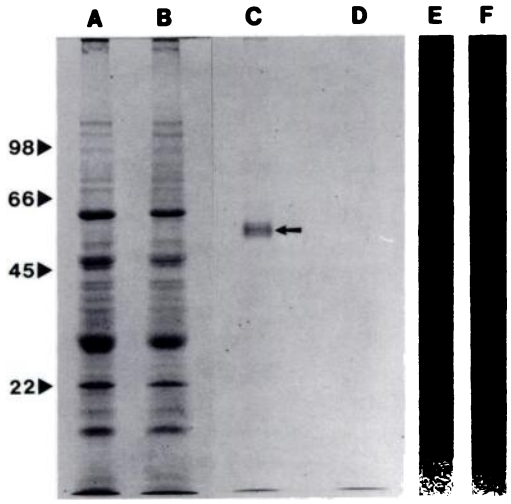

Fig. 4. NP-40-extractable material obtained from cauda spermatozoa that were incubated for 4 hours under *in vitro* fertilization conditions (4-hour sample) is compared with material obtained from cauda spermatozoa washed and immediately extracted with NP-40 (0-hour sample). Lanes A and B: gel stained with Coomassie Blue; lane A, 0-hour sample; lane B, 4-hour sample. Band patterns of the two samples appear identical. Lanes C and D: gel stained with the PAS procedure. The 54-K PAS-positive band of the 0-hour sample (lane C, arrow) is not present in the 4-hour sample (D). Lanes E and F: Western blot labeled with a monoclonal antibody to SMA-4. As in the PAS stained gel, the 54-K band present in the 0-hour sample (E) is absent from the 4-hour sample (F).

Discussion

Mammalian spermatozoa passing through the epididymis are exposed to a fluid environment of steadily changing composition resulting from the secretory and absorptive activity of the duct epithelium. Analyses of fluid obtained from the lumen of the epididymis by micropuncture (Koskimies and Kormano, 1975; Huang and Johnson, 1975; Turner et al, 1979; Olson and Hinton, 1985) and studies on the incorporation of radioactively labeled amino acids into proteins synthesized by isolated duct segments (Brooks and Higgins, 1980; Jones et al, 1980; 1981) indicate that protein secretion is regionalized in the epididymis.

SMA-4 is not detected in extracts of spermatozoa or luminal fluid from the caput epididymidis using Western blot assays, which agrees with immunohistochemical findings, obtained from epididymal sec-

tions, that spermatozoa and epithelial cells of the caput lack the antigen (Vernon et al, 1982). However, SMA-4 is present in extracts of spermatozoa from the cauda epididymidis. Under disulfide bond reducing conditions, the antigen exists predominantly in a 54-K form on a 10 to 12% gel. The sensitivity of SMA-4 to trypsin (Vernon et al, 1985), its PAS-staining characteristics and its affinity for wheat germ agglutinin (C. H. Muller, unpublished observations) suggest that it is a glycoprotein, as are secreted epididymal proteins of other species (Voglmayr et al, 1980). The low affinity of Coomassie Blue or Amido Black stains for the antigen is not unusual; the glycosylated, PAS-positive proteins of the erythrocyte membrane also have this characteristic (Fairbanks et al, 1971). Because SMA-4 is glycosylated, it is unlikely that its SDS-PAGE mobility accurately reflects its true molecular weight. The 54-K molecular weight value should be considered a preliminary estimate until the antigen can be purified and subjected to more rigorous analytic procedures. The minor, immunoreactive band of R_f 0.56 that accompanies the 54-K band is seen infrequently in extracts prepared with an elevated concentration (0.5 mM) of the serine protease inhibitor PMSF. The band may be a degraded form of SMA-4 that results from proteolysis occurring during sample preparation. Peptide bond cleavage induced by a disulfide bond reduction at high temperature (90 C) may contribute to minor band formation, although the band has also been observed in preparations reduced by a 2-hour, 37 C incubation with dithiothreitol.

It is noteworthy that SMA-4 may have been identified in a previous study that used a heterologous antiserum. Saling and O'Rand (1982) produced a rabbit antiserum to mouse epididymal spermatozoa that inhibited *in vitro* fertilization and recognized, in 10% polyacrylamide gels, seven sperm antigens with molecular weights ranging from 33 K to 200 K. Agglutination studies suggested that head and tail surface components were recognized. One antigen (Band C) was very similar to SMA-4 in R_f, calculated molecular weight, quantity relative to other components, and in its low affinity for Coomassie Blue.

SMA-4 detected on blots of fluid from the corpus and the cauda epididymidis is of higher apparent molecular weight than the antigen extracted from spermatozoa. The higher molecular weight form may be reduced in size prior to or during its association with the sperm surface. Such a modification may expose regions of the molecule that bind to specific acceptor sites residing in restricted areas of the sperm surface. The spatial restriction of SMA-4 to the sperm tail, and its relatively tight attachment to the cell surface (Vernon et al, 1985) lend support to this hypothesis. It is possible that the 85-K and 54-K antigens are unrelated; however, this seems unlikely due to the highly restricted specificity of the monoclonal antibody. The monoclonal antibody has been tested, by indirect immunofluorescence and Western blotting, for cross-reactivity with spermatozoa of other species and with a great variety of mouse somatic tissues, early embryonic stages, and cultured cell lines (Feuchter et al, 1981; unpublished observations; Vernon et al, 1982). Results of these assays indicate that the monoclonal antibody binds only to spermatozoa, apical regions of principal cells, and luminal fluids from the corpus and the cauda epididymidis of mice. A definitive determination of homology between the 85-K and 54-K antigens will require comparison of peptide fragments and amino acid sequences of the purified molecules.

Immunoassays of spermatozoa and incubation media indicate that SMA-4 is lost from the surface of spermatozoa in an *in vitro* fertilization environment (Vernon et al, 1985). Western blot and PAS labeling studies reported here confirm the loss at the biochemical level.

The function of SMA-4 is not known, but previous immunofluorescence studies have demonstrated that its secretion is regionalized in the mouse epididymis and that it is affected by fertilization environments. The determination of a molecular identity for SMA-4 reported here should provide a basis for future studies to determine the role of the antigen in reproduction.

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