BIOLOGY OF REPRODUCTION **54**, 1009–1017 (1996)

Surface Localization of P34H, an Epididymal Protein, during Maturation, Capacitation, and Acrosome Reaction of Human Spermatozoa[1]

Franck Boué, Johanne Blais, and Robert Sullivan[2]

CHUL Research Center and the Department of Obstetrics and Gynecology, Faculty of Medicine Laval University, Ste-Foy, Quebec, Canada G1V 4G2

ABSTRACT

During epididymal transit, spermatozoa acquire new surface antigens that are involved in the acquisition of their fertilizing ability. We have previously described a 34-kDa (P34H) human epididymal sperm protein that shows antigenic and functional homologies with the hamster P26h. P34H is localized on the acrosomal cap of human spermatozoa and has been proposed to be involved in the interaction with the zona pellucida. The aim of this study was to document the expression of P34H on the sperm surface during transit along the male and female genital tracts. Immunohistochemical techniques were performed on human testes and epididymides by means of an antiserum specific for P34H. No labeling was detected on those spermatozoa found within the seminiferous tubules or in the vasa efferentia. P34H first appeared in the caput epididymidis and was restricted to the acrosomal cap. Signal intensity then increased considerably from the proximal corpus to the cauda region of the epididymis. After ejaculation, the same pattern of P34H distribution was observed, but the intensity was much lower than that characterizing the cauda epididymal spermatozoa. Strong labeling was restored after incubation in B2 medium and was maximal after 5 h of capacitation. After acrosomal exocytosis induced by a Ca^{2+} ionophore, the percentage of P34H-labeled spermatozoa decreased proportionally to the number of acrosome-reacted spermatozoa as determined by *Pisum sativum*-fluorescein isothiocyanate (FITC) labeling. P34H appeared to be strongly anchored to the sperm plasma membrane during epididymal transit as indicated by the requirement for detergent to extract this surface antigen from ejaculated spermatozoa. This confirms the importance of P34H binding to the sperm plasma membrane during epididymal maturation. We have previously proposed that P34H is involved in sperm-zona pellucida interaction. The appearance and accumulation of P34H on the sperm plasma membrane during epididymal maturation, followed by its inaccessibility associated with ejaculation, its unmasking during capacitation, and finally its elimination after the acrosome reaction, are in agreement with the proposed function of this sperm antigen.

INTRODUCTION

Mammalian sperm-egg interactions are mediated by specific complementarity of proteins, glycoproteins, and carbohydrates present at the surface of both gametes [1, 2]. Before spermatozoa can interact with the zona pellucida, which is an extracellular matrix surrounding the egg, they are subjected to posttesticular modifications yielding male gametes that exhibit forward motility and oocyte-binding ability [3]. Epididymal secretory products are essential for mammalian sperm maturation, and current evidence indicates that specific proteins become associated with the spermatozoa during epididymal transit [4–8]. Although many epididymal proteins have been described, the function of the majority of them in sperm physiology remains to be clarified, and this is particularly true in the human [9].

After epididymal transit, male gametes undergo other complex surface transformations that occur when they encounter accessory gland secretions during ejaculation as well as within the female genital tract. Sperm modifications that occur during ejaculation have been poorly defined, and their physiological significance appears to vary from one species to another as well as between different males of the same species [10]. In the human, it appears that the seminal plasma contains one or more so-called decapacitation factors that decrease the fertilizing ability of spermatozoa [11, 12]. Physiological modifications of the spermatozoa within the female reproductive tract have been extensively studied and have been collectively defined as capacitation [13]. This phenomenon is an absolute prerequisite that spermatozoa must undergo in order to interact efficiently with the zona pellucida and to accomplish the acrosome reaction. Although the biochemical modifications involved in this process are not fully understood, it is generally believed that the removal or alteration of coating stabilizers from the sperm surface is an important step in the preparation of sperm to encounter the eggs and their investments. In the vicinity of oocytes, spermatozoa will undergo one of the last steps leading to fertilization, namely the acrosome reaction. This reaction involves the fusion of the sperm plasma membrane with the outer acrosomal membrane, thereby eliminating various surface antigens that are normally exposed on the acrosomal cap of spermatozoa and allowing zona pellucida penetration [13].

Using the hamster as a model, we have previously identified a protein, P26h, that is added to spermatozoa during their epididymal transit [14, 15]. P26h exhibits a species-specific affinity for the glycoproteins of homologous zonae pellucidae [16] and has been proposed to be involved in the

Accepted December 18, 1995.
Received September 11, 1995.
[1]This work was supported by a Medical Research Council of Canada grant to R.S. who is a fellow of the "Fonds de la Recherche en Santé du Québec" (Montréal, Qc, Canada).
[2]Correspondence: Robert Sullivan, Ontogeny/Reproduction Unit, CHUL Research Center, 2705 Blvd. Laurier, Ste-Foy, Qc, Canada, G1V 4G2. FAX: (418) 654-2714; e-mail: robert.sullivan@crchul.ulaval.ca

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binding of the male gamete to the zona pellucida [17]. More recently, we have shown that human ejaculated spermatozoa are characterized by a protein, P34H, that shows antigenic and functional homologies with the hamster P26h [18]. P34H appears on the acrosomal cap of spermatozoa within the caput region of the human epididymis and has been shown to be involved in one of the prerequisites of human fertilization, i.e., sperm-zona pellucida binding [18, 19].

The aim of the present study was to determine the appearance of P34H on human spermatozoa along the epididymal duct and to establish whether ejaculation, capacitation, or the acrosome reaction affects the distribution of this sperm antigen. Furthermore, we have used different protein extraction procedures as well as high-pressure nitrogen cavitation of the plasma membrane to document the kind of protein-plasma membrane interaction involved in the anchoring of P34H to the surface of human spermatozoa.

MATERIALS AND METHODS

Immunostaining of Human Testis and Epididymis

Human testes and epididymides were surgically removed from subjects registered in an organ transplantation program. Tissues were collected from donors 20 to 33 yr of age, following accidental death, with no medical antecedent that could affect reproductive function. Reproductive tissues were collected while artificial circulation was assured to preserve different organs assigned for transplantation. Tissues from vasectomized men were discarded. Testicular fragments as well as epididymal caput, corpus, and cauda regions were immediately dissected and rapidly frozen in liquid nitrogen. They were embedded in Tissue Teck II medium (Labtek, Nunc Inc., Naperville, IL) at $-30°C$. Sections approximately 5 μm thick were placed on microscopic slides, air-dried, fixed with methanol at $-20°C$ for 10 min, and stored at $-20°C$ until used.

Antigen localization was performed by the avidin-biotin peroxidase staining procedure using the Vectastain ABC kit (Vector Labs, Burlingame, CA). Nonspecific staining was avoided by a 1-h preincubation of the tissue sections with 10% (v:v) goat serum in PBS (pH 7.4) followed by three washes in PBS. Tissue sections were incubated for 2 h with a specific anti-P34H rabbit antiserum or with the corresponding preimmune serum. The anti-P34H antiserum was previously described by Boué et al. [18] and shown to be highly specific for this human sperm antigen. Before use, immune as well as preimmune sera were absorbed for 30 min on an acetone powder of rabbit liver proteins [20]. Sera were used at a dilution of 1:200 in PBS. After three washes with PBS-Tween (0.05% [v:v] Tween 20 in PBS), tissue sections were treated for 30 min with goat biotinylated anti-rabbit IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:300 in PBS, washed three times in PBS-Tween, and then incubated for 30 min with Vectastain ABC reagent. After three washes in PBS-Tween, sections were revealed with peroxidase substrate (0.118 M aminoethyl carbazol [Sigma] in 100 mM sodium acetate buffer, pH 5.2) in the presence of 0.002% H_2O_2 for exactly 10 min. Slides were washed in H_2O and counterstained with Harris hematoxylin (Sigma), fixed with a 5% acetic acid solution, washed extensively in H_2O, and finally mounted with glycergel solution (Dako, Carpinteria, CA) under coverslips.

Immunostaining of Human Spermatozoa

Ejaculated spermatozoa from fertile volunteers were washed by centrifugation (800 × *g*) in Dulbecco's PBS (D-PBS; Gibco, Grand Island, NY) after a liquefaction period of 30–60 min at room temperature. Spermatozoa were separated on the basis of their buoyant density by centrifugation on a discontinuous Percoll gradient according to the method of de Lamirande and Gagnon [21]. Spermatozoa pelleted under the 95% Percoll layer were recovered, washed in D-PBS, and resuspended in B2 medium (Bio Mérieux, Marcy-l'étoile, France) and allowed to capacitate at 37°C under a 5% CO_2 atmosphere. At 3, 5, or 20 h later, aliquots of sperm suspensions were taken, and the acrosome reaction was induced with the calcium ionophore A23187 at a final concentration of 1 μM during the last 30 min of capacitation. At each time period, spermatozoa incubated or not incubated with the ionophore were fixed in ice-cold ethanol for 30 min and smeared onto microscope slides as described by Cross et al. [22]. After this treatment, the percentage of spermatozoa with an acrosomal cap coated with P34H was determined by incubation with the anti-P34H antiserum followed by indirect immunostaining using avidin-biotin peroxidase as described above for the tissue cryosections. The percentage of spermatozoa with P34H was correlated with the percentage of acrosome-reacted spermatozoa as determined by labeling with fluorescein-conjugated lectin from *Pisum sativum* (PSA-FITC; Sigma) as described by Cross et al. [22]. Acrosome reaction was defined as the loss by vesiculation of the plasmalemma and the outer acrosomal membrane.

During the procedures used to immunodetect P34H on cryosections of reproductive tissues as well as on spermatozoa smears, great care was taken to treat all specimens in the same manner. Different samples from one individual were all processed in parallel in order to avoid technical variability and to allow comparison. Results were consistent from one series of samples from one individual to another, and the results set forth here represent typical data.

Sperm Plasma Membrane Preparation

Spermatozoa of higher buoyant density were selected by Percoll gradient centrifugation, resuspended in PBS, and placed on ice. They were submitted to nitrogen cavitation at 750 psi for 20 min in a Parr bomb as described by Noland

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et al. [23] for bovine spermatozoa and modified by P. Leclerc (personal communication) for human spermatozoa. Cavitated cells were centrifuged at 15 000 × *g* for 10 min. The supernatant was recovered, and the pellet was resuspended in PBS and recentrifuged for 10 min. The two supernatants were pooled and centrifuged at 120 000 × *g* for 60 min at 4°C. The membrane pellet was resuspended in a small volume of PBS, and the proteins were precipitated with ice-cold acetone, air-dried, and resuspended in SDS-PAGE sample buffer. Pellets of demembranated spermatozoa were resuspended directly in sample buffer. These samples were then Western blotted and probed with the anti-P34H antiserum.

P34H Sperm Protein Extraction

As described for immunolocalization of P34H on ejaculated spermatozoa, only semen samples from fertile volunteers were used in this study. After centrifugation on a discontinuous Percoll gradient, the more dense spermatozoa were recovered, washed in PBS, and resuspended at a constant concentration in PBS (150 mM NaCl) or in different extraction buffers: 1 M NaCl in phosphate buffer (PB), 30 mM octylglucoside in PBS, 0.05% (v:v) Nonidet P-40 in PBS, or 0.1% (v:v) Triton X-114 in PBS. Spermatozoa resuspended in all these buffers, except Triton X-114, were kept for 15 min at room temperature and then pelleted by centrifugation at 1400 × *g* for 10 min. Supernatants were recovered, and the extracted proteins were precipitated overnight with acetone at −20°C, air-dried, and resuspended in SDS-PAGE sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, and 5% β-mercaptoethanol) according to Laemmli [24]. Pellets of spermatozoa were dissolved directly in SDS-PAGE sample buffer. All samples were heat-denatured at 100°C for 3 min. Samples extracted with Triton X-114 were treated differently, i.e., according to the procedure described by Bordier [25]. After Percoll gradient centrifugation, spermatozoa were resuspended for 15 min in 0.1% Triton X-114 in PBS at 4°C. At this temperature, the detergent forms a miscellaneous phase. After the insoluble material was discarded by centrifugation, those solutions containing the extracted proteins were incubated at 37°C and centrifuged to allow partition of the solution into two phases: aqueous and detergent. The extracted proteins in each phase were then precipitated with cold acetone and processed as described above.

The various protein samples were submitted to SDS-PAGE according to Laemmli [24] on a 12% polyacrylamide gel. Gels were stained with silver nitrate [26] or electrotransferred onto a nitrocellulose membrane as described by Towbin et al. [27]. These Western blots were saturated for 2 h in PBS containing 10% (w:v) defatted milk and 2% normal goat serum and then incubated overnight with the anti-P34H antiserum diluted 1:1000 in PBS supplemented with 1% (v:v) goat serum. The anti-P34H polyclonal antiserum was previously adsorbed on human keratin as described by Bérubé et al. [28]. After three washes with 0.1% (v:v) Tween 20 in PBS, the nitrocellulose membranes were incubated for 1 h with a peroxidase-conjugated anti-rabbit IgG (1:3000 in PBS) and washed three times with PBS-Tween. The immune complexes were detected with a BioMax film (Eastman Kodak, Rochester, NY) after incubation with a chemiluminescent substrate of peroxidase (ECL kit; Amersham, Buckinghamshire, UK) according to the supplier's instructions.

Statistical Analysis

The percentages of acrosome-reacted and P34H-labeled spermatozoa were determined three times, and a minimum of two hundred cells were counted each time. Results are presented as the mean ± SD of the mean and were compared through use of the chi-square test.

RESULTS

Detection of P34H Protein in the Male Genital Tract

Immunoperoxidase staining of frozen sections of human testis and epididymis, probed with the anti-P34H antiserum, is shown in Figure 1. The anatomical regions of the human epididymis corresponding to the cryosections are illustrated in Figure 2. All these samples were treated in parallel in order to allow comparison. The spermatozoa within the seminiferous tubules (Fig. 1a), as well as within the vasa efferentia (Fig. 1b), did not stain after incubation with the antiserum specific for P34H. Those spermatozoa found within the lumen of the caput epididymal tubules showed a very weak staining that was restricted to the acrosomal cap (Fig. 1c). Sperm labeling in the lumen of the proximal corpus increased considerably compared to that of the more proximal epididymal spermatozoa, and tissues bordering the lumen of this epididymal region exhibited some positive staining (Fig. 1d). The P34H staining continued to increase from the proximal corpus segment (Fig. 1d) to the distal corpus (Fig. 1e) and then to the cauda epididymidis, where the majority of spermatozoa were stained (Fig. 1f). All along the epididymis, the staining was restricted to the acrosomal cap, and no change in the distribution of the antigen on the spermatozoa was observed. This staining was specific, as shown by the absence of labeling when preimmune serum was used on testicular (data not shown) as well as on epididymal (Fig. 1g) sections. This pattern of labeling was consistent from one individual to another.

Detection of P34H on Ejaculated Spermatozoa

When submitted to exactly the same immunohistochemical procedures, ejaculated spermatozoa obtained from fertile donors showed the same patterns of P34H distribution as found with cauda epididymal sperm (Fig. 3a). The intensity of labeling was, however, much lower than that char-

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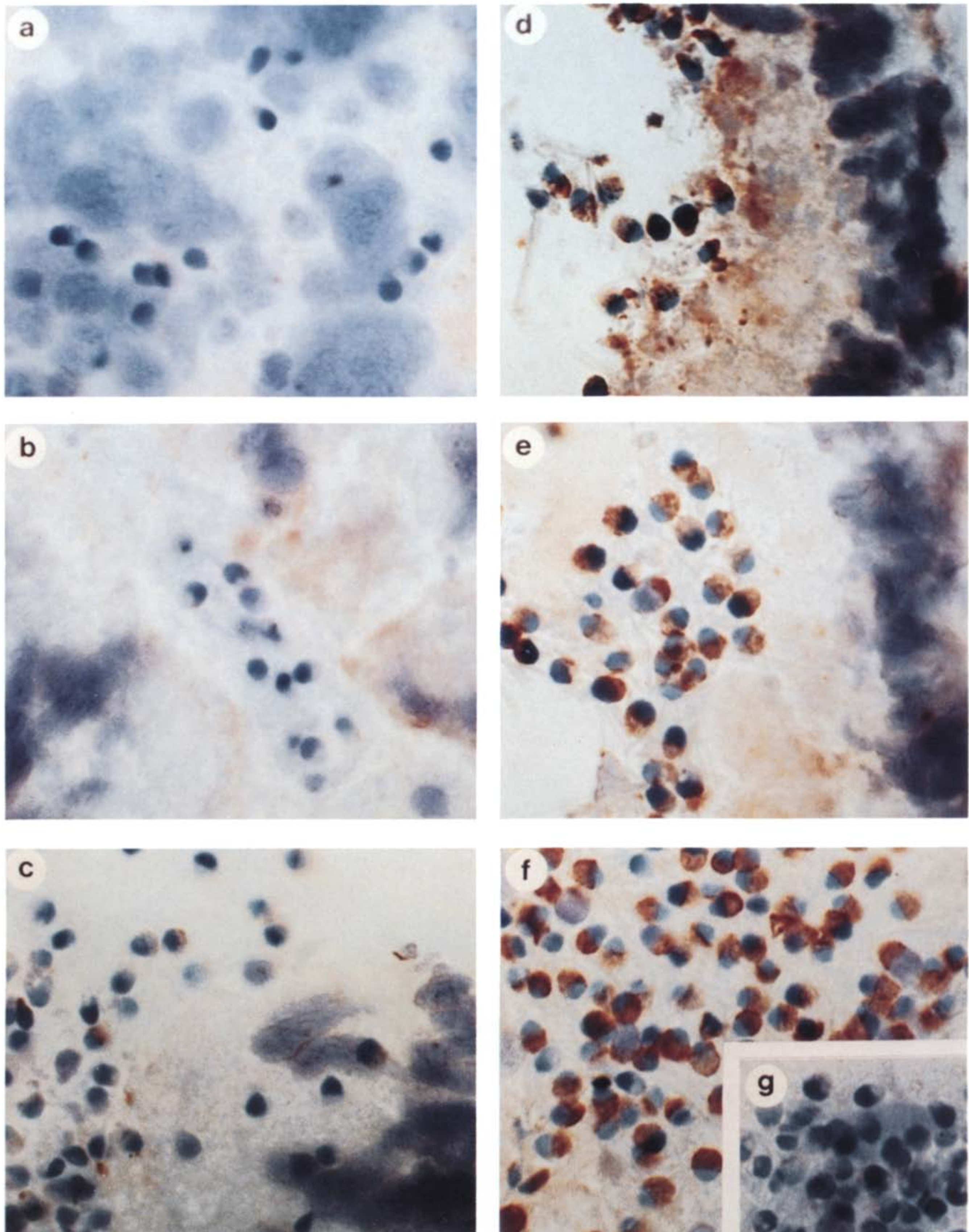

FIG. 1. Localization of P34H on cryosection of human seminiferous tubules (**a**), vasa efferentia (**b**), caput (**c**), and proximal (**d**) and distal (**e**) corpus and cauda (**f, g**) segments of the epididymis. Indirect avidin-biotin peroxidase staining using an anti-P34H antiserum (**a-f**) or a control preimmune serum (**g**). ×900.

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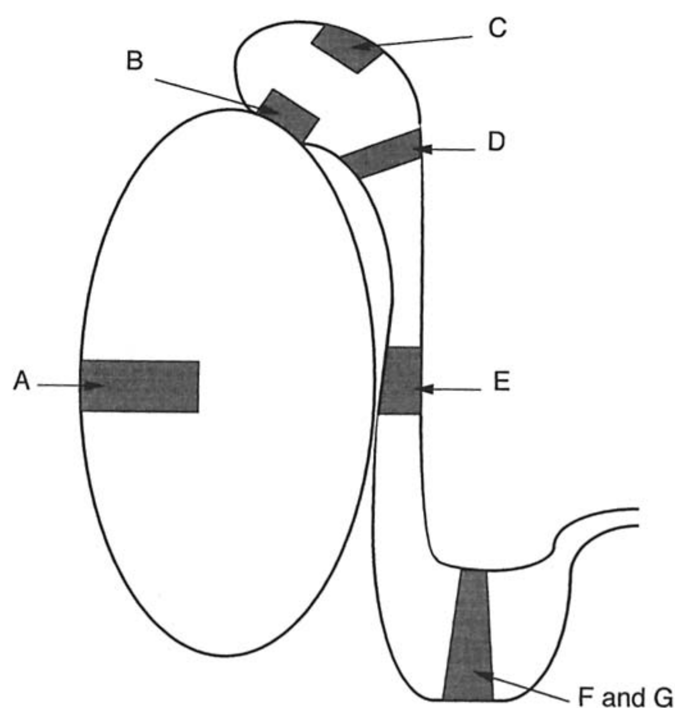

FIG. 2. Schematic representation of the anatomical regions of the human epididymis corresponding to the cryosections presented in Figure 1.

acterizing the male gamete within the distal region of the epididymis (compare Fig. 3a with Fig. 1f). The ability of the anti-P34h antiserum to bind to the surface of spermatozoa was reestablished after an incubation period in B2 medium, culminating in an intense labeling of the acrosomal cap after 5 h of incubation in capacitating medium (Fig. 3b). Whereas the level of labeling appeared to vary from one freshly ejaculated spermatozoon to another, the intensity of labeling was much more uniform in a population of spermatozoa capacitated for 5 h. These spermatozoa had been previously selected by centrifugation on a Percoll gradient.

The percentage of anti-P34H-labeled spermatozoa decreased from 87% to 67% over 20 h of capacitation (Fig. 4A). During this period, the percentage of spontaneously acrosome-reacted sperm, as determined by PSA-FITC, increased by a similar proportion, 26% (Fig. 4B). When acrosomal exocytosis was induced with Ca^{2+} ionophore at different times during capacitation, the percentages of P34H-labeled spermatozoa also decreased in proportion to the increase in percentages of acrosome-reacted spermatozoa (Fig. 4, A and B). For each capacitation time, the arithmetic sum of the percentage of acrosome-reacted spermatozoa and the percentage of P34H-labeled spermatozoa was not significantly different from 100%. This was the case for both spontaneously and ionophore-induced acrosome-reacted spermatozoa (Fig. 4C). In all these conditions, no modification of P34H localization was observed on the acrosome-intact spermatozoa (data not shown).

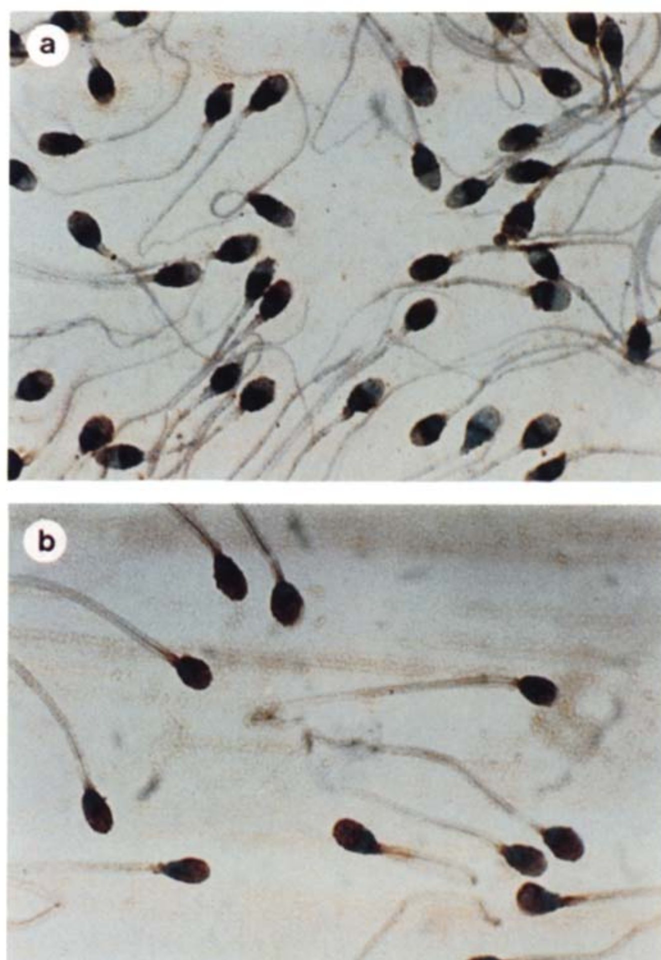

FIG. 3. Localization of P34H on smears of human spermatozoa immediately after Percoll gradient centrifugation (**a**) or after a 5-h incubation in B2 medium (**b**). Indirect avidin-biotin peroxidase staining using an anti-P34H antiserum. ×760.

Membrane Fractionation

After nitrogen cavitation, the spermatozoa were centrifuged and the supernatants were observed under the light microscope to ensure that no sperm cells or fragments remained before high-speed centrifugation (data not shown). Proteins from the plasma membrane fraction as well as from demembranated spermatozoa were separated on SDS-PAGE, Western blotted, and probed with the anti-P34H antiserum. P34H was immunodetectable only in the electrophoretic patterns of proteins from the plasma membrane preparation. The antigen appeared as a band at a relative migration mobility corresponding to 34 kDa together with another band at a lower molecular mass of 30 kDa. No protein could be detected in the demembranated sperm fractions when they were probed with the anti-P34H antiserum (Fig. 5).

Extraction of P34H from Spermatozoa

Washed spermatozoa were treated with different extraction buffers. Silver stains of SDS-PAGE patterns (under re-

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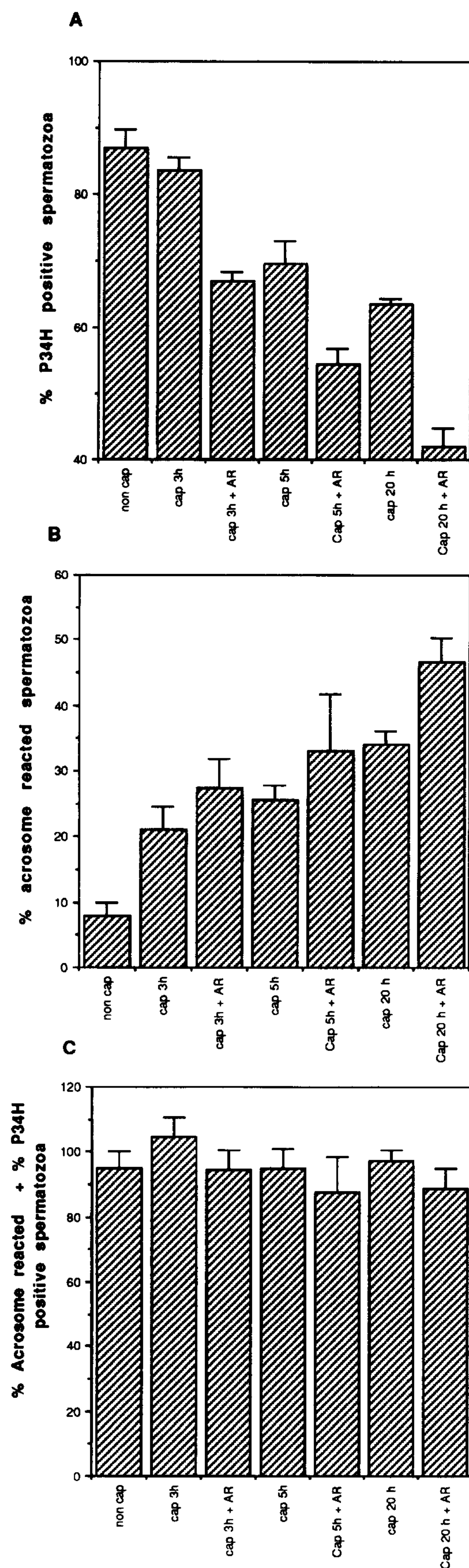

ducing conditions) of those proteins present in the different supernatants, as well as of those that remained associated with the sperm pellets after these treatments, are presented in Figure 6A. Corresponding Western blots probed with an anti-P34H antiserum are shown in Figure 6B. When spermatozoa were treated with an iso- or hypertonic sodium chloride-buffered solution, all of the P34H was associated with the sperm pellets and was immunodetected as a single band. In contrast, different detergent treatments resulted in a release of P34H from the sperm surface (Fig. 6). After detergent extractions of sperm, all of the antigen detected by the anti-P34H antiserum appeared in the supernatants as one band with an Rf corresponding to a molecular mass of 30 kDa. As observed with octylglucoside and Nonidet P-40 extraction, treatment with Triton X-114 resulted in the release of P34H from the sperm surface. After phase-partitioning of the Triton X-114 extract, silver-stained gels revealed different patterns of proteins present in the aqueous and detergent phases. P34H was localized in one band of 30 kDa and was immunodetectable in the aqueous phase only.

DISCUSSION

Through the use of different animal models, the physiological functions of the epididymis have been defined and shown to promote forward motility and fertilizing ability of the spermatozoa [3]. Whereas epididymal protein synthesis and secretion have been shown to be necessary in order for these sperm modifications to occur, the translational products involved in the latter are unclear. This is particularly true for the human, where the role played by the epididymal proteins remains unknown. This has fostered controversies concerning the involvement of the epididymis in human sperm physiology [9, 29–31], due in part to the paucity and poor quality of human tissues available for research. In fact, the majority of the epididymal tissues available come from pathological organs or from older patients suffering from prostatic carcinoma who are often under antiandrogen therapy. All tissues used in the present study were from men under 33 yr of age with no reported pathology that could have affected their reproductive function. The fact that these men were donors for organ transplantation allowed tissue recovery under optimal conditions. Thus, our data represent physiologically normal human epididymides.

P34H, while absent in seminiferous tubules and vas efferentia, appeared faintly within the caput and more significantly into the corpus epididymidis. Labeling on the sper-

FIG. 4. Percentages P34H-positive (**A**) and acrosome-reacted (**B**) spermatozoa as well as the arithmetic sum of **A** and **B** (**C**) as determined immediately after resuspension in B_2 medium (non cap) or after 3, 5, or 20 h of capacitation (cap). After these different periods of capacitation, the acrosome reaction was induced (AR) with ionophore A23187 or was not induced. Means of three determinations. Vertical bars represent SD of the mean.

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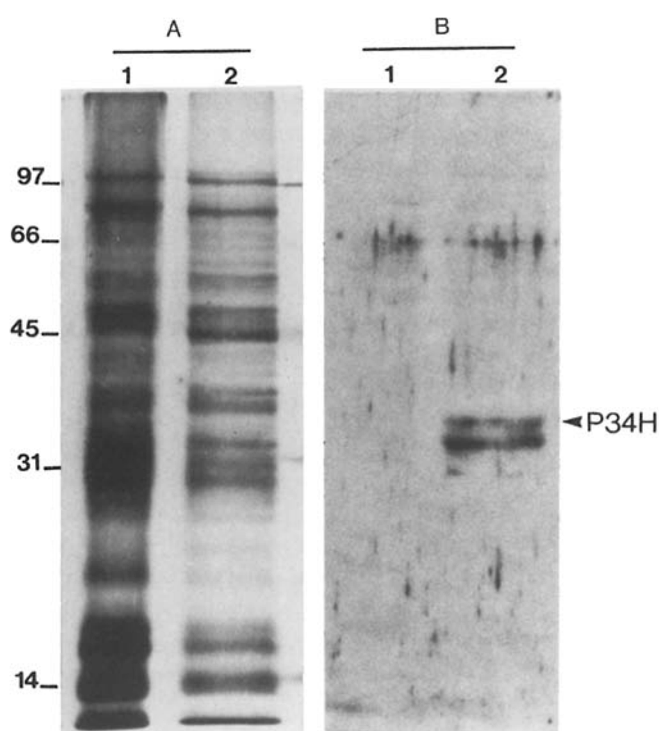

FIG. 5. Silver-stained SDS-PAGE (**A**) and corresponding Western blots probed with an anti-P34H antiserum (**B**) of proteins from demembranated spermatozoa (1) or from the sperm membrane fraction (2) prepared by high-pressure nitrogen cavitation. Molecular masses of standard proteins are indicated to the left of the figure.

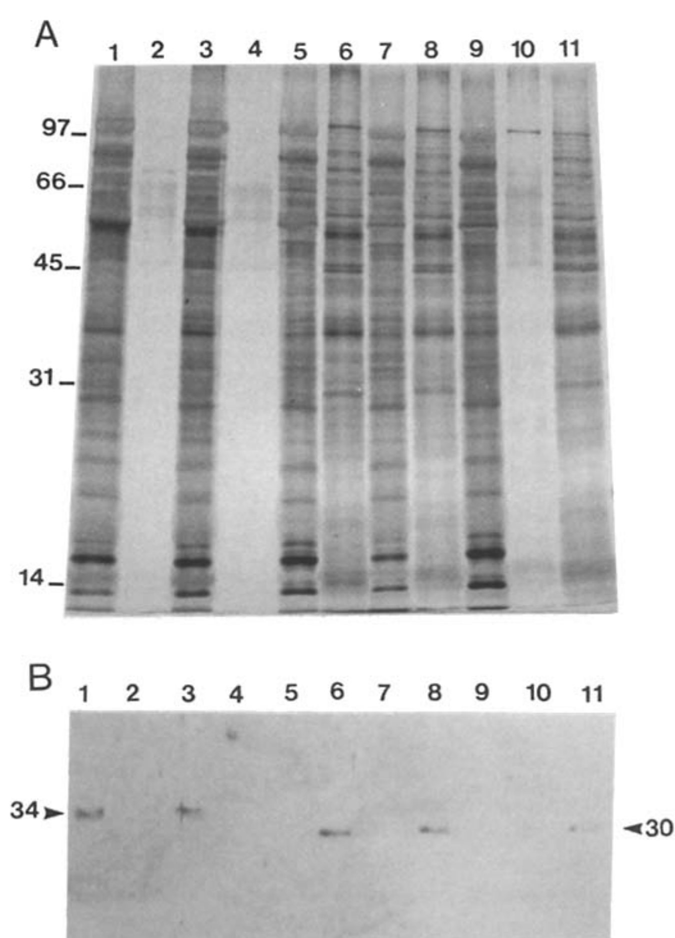

FIG. 6. Silver-stained SDS-PAGE (**A**) and corresponding Western blots probed with an anti-P34H antiserum (**B**) of sperm pellet (1, 3, 5, 7, and 9) and supernatant (2, 4, 6, 8, 10, and 11) after extraction with PBS (1, 2), 1 M NaCl (3, 4), 30 mM octylglucoside (5, 6), Nonidet P-40 (7, 8), and Triton X-114 (9, 10, 11). Detergent (10) and aqueous (11) solution following Triton X-114 phase partition. Only the region of the Western blots that showed some staining is illustrated. Molecular masses of standard proteins are indicated to the left of the figure.

matozoa then increased progressively during transit through the more distal part of the epididymis (Fig. 1). We have recently shown that the human sperm antigen P34H is involved in those processes implicated in binding to the zona pellucida [18]. Furthermore, we have shown that certain men presenting with idiopathic infertility, whose spermatozoa failed to bind to the zona pellucida in vitro, were also characterized by low levels of P34H [19]. Considering its proposed function, the site of appearance of P34H along the male reproductive tract is in agreement with the fact that zona pellucida-binding ability as well as mature motility patterns of human spermatozoa first appears within the caput region of the epididymis [30].

While the intensity of P34H labeling increased considerably as spermatozoa moved from the proximal corpus (Fig. 1d) to the distal cauda (Fig. 1f), the distribution of the label remained the same. In fact, P34H was restricted to the acrosomal cap during maturation as well as during capacitation (Figs. 1 and 3). Thus, P34H differs from other sperm antigens that have been shown to change their location on the sperm surface during epididymal maturation [32], capacitation [33], or acrosome reaction [34, 35]. In view of the fact that acrosome-intact human spermatozoa interact with the zona pellucida [36], P34H is added to the domain of the sperm surface that is involved in binding to the egg's extracellular coat. It is reasonable to postulate that similarly to P26h in the hamster [14, 15], P34H is primarily synthesized by the epididymal epithelium and secreted into the luminal space before being taken up by human spermatozoa. Thus, P34H is a sperm antigen that could be considered a marker of epididymal maturation.

Human seminal plasma contains so-called decapacitation factors that bind firmly to the sperm surface. The removal of these stabilizing coating molecules is one of the steps of capacitation [37]. As spermatozoa progress through capacitation, dramatic changes occur in the sperm membrane and in surface protein distribution [37, 38]. P34H was easily detected on the acrosomal cap of epididymal spermatozoa, whereas the intensity of labeling was much lower on fresh ejaculated sperm (Fig. 3a). However, after 5 h of incubation in B2 medium, the P34h labeling was reestablished (Fig. 3b) and was comparable to the staining characterizing the cauda epididymal spermatozoa. The mole-

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cules that coat the spermatozoa at the time of ejaculation may mask the P34H. This essential sperm antigen is not accessible when the male gamete initiates its transit within the female reproductive tract. P34H becomes accessible after capacitation, when fertilizing sperm are ready to interact with the zona pellucida [39]. The initial appearance of P34H in the caput epididymidis, followed by its accumulation along the epididymis, its subsequent masking at ejaculation, and its surface reappearance after capacitation, is in agreement with the proposed function of this human sperm antigen [18, 19].

After binding to the zona pellucida, human spermatozoa must undergo the acrosome reaction, and this exocytotic process is necessary for penetration of the egg's extracellular coat [40, 41]. The acrosome reaction consists of fusion of the overlying plasma membrane with the outer acrosomal membrane, resulting in vesicles that allow the release of acrosomal enzymes [13]. Under different experimental conditions, the percentage of spontaneously or calcium ionophore-induced acrosome-reacted spermatozoa is inversely proportional to the percentage of P34H-labeled spermatozoa (Fig. 4). In each case, the arithmetic sum of the percentage of acrosome-reacted spermatozoa and the corresponding percentage of P34H-labeled sperm does not vary from 100% (Fig. 4C). This strongly suggests that P34H is lost when spermatozoa undergo the acrosome reaction. This is in agreement with the surface localization of P34H on the acrosomal cap of intact spermatozoa, the sperm region involved in the interaction with the zona pellucida. In order to allow comparison of P34H surface localization on spermatozoa in different physiological conditions, immunohistological staining was performed on methanol-fixed cells. This fixation procedure was particularly important to preserve the integrity of the epididymal cryosections. This fixation can permeabilize plasmalemma and raise some concerns about the surface localization of P34H. However, immunostaining performed on unfixed spermatozoa preincubated in capacitating medium revealed the same pattern of P34H labeling as that seen in methanol-fixed cells (data not shown). Furthermore, the fact that preincubation of acrosome-intact spermatozoa with an anti-P34H antiserum inhibits their ability to bind to the homologous zona pellucida [18] is another strong indication of the surface localization of P34H.

Plasma membranes were prepared from human ejaculated spermatozoa using a Parr bomb. Nitrogen cavitation has been shown to be very efficient in removing the plasma membrane from spermatozoa without contamination from nuclear, mitochondrial, or acrosomal membranes, the latter remaining intact and attached to spermatozoa. Membranes from cytoplasmic droplets could, however, contaminate plasma membrane preparations [23]. Plasma membrane cavitation has been performed on spermatozoa without cytoplasmic droplets obtained by Percoll gradient centrifugation [42]. This eliminates the possibility of contamination by other types of membrane. P34H appears to be associated with the plasma membrane as shown by Western blot analysis of those proteins obtained from the sperm plasma membrane preparation and demembranated cells (Fig. 5).

The P34H antigen appeared within the epididymis (Fig. 1), where it became associated with the sperm plasma membrane (Fig. 5). In order to document the type of interaction between this protein and the sperm surface, we submitted ejaculated spermatozoa to different extraction buffers. Physiological saline and hyperosmotic solutions were inefficient in removing P34H from the sperm surface, suggesting that this antigen is not simply an external coat. Destabilization of the sperm membrane with detergent was necessary to extract P34H from the sperm surface. Whereas Western blot analysis of those proteins associated with sperm treated with buffered saline revealed a band at 34 kDa, the detergent-extracted proteins probed with anti-P34H revealed a band at an Rf corresponding to 30 kDa. Proteolytic activity released after detergent treatment may explain the immunodetection of a protein band at a lower molecular mass than that associated with membrane-intact spermatozoa. When spermatozoa were extracted with detergent in a large volume of buffer, the band at 34 kDa together with the band at 30 kDa was detected on Western blots of extracted proteins (data not shown). Proteolytic cleavage of P34H was probably partially efficient when dilution was performed with a larger volume. A similar partial degradation of surface protein occurs when mouse spermatozoa are extracted with detergent [43] or when membranes are prepared from human spermatozoa by nitrogen cavitation (Fig. 5). Whether or not this endoproteolytic activity that cleaves P34H has any physiological significance, as suggested for the guinea pig sperm PH-20 antigen, remains to be established [44].

One intriguing property of P34H is its hydrophilic nature as demonstrated by its presence in the aqueous phase after phase-partitioning of the Triton X-114 extract (Fig. 6). This was unexpected in view of the requirement for detergent to extract this surface antigen covering the acrosomal cap. On the other hand, the hydrophilic property of P34H is consistent with the fact that this antigen is acquired by spermatozoa while they are passing through the epididymis (Fig. 1). The anchoring mechanism of this epididymis-generated antigen on the sperm surface is unknown; however, P34H is tightly bound to the sperm surface. Moreover, P26h, which is the hamster counterpart of human P34H, behaves in the same way when extracted with Triton X-114 [45].

The subcellular localization of P34H on spermatozoa, its surface appearance during transit within the male reproductive tract and capacitation, and its loss following the acrosome reaction are all in agreement with the proposed function of this human epididymal sperm antigen, i.e., its involvement in those processes leading to binding to the

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homologous zona pellucida. These results strongly suggest that P34H is an epididymal antigen involved in the acquisition of the fertilizing ability of human spermatozoa.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical assistance of Ms. A.-M. Bleau. The authors are also grateful to Dr. Kenneth D. Roberts for critical reading of the manuscript and to Dr. Pierre Leclerc for advice concerning the use of the Parr bomb.

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