Evidence That Proteolysis of the Surface Is an Initial Step in the Mechanism of Formation of Sperm Cell Surface Domains

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Abstract. On terminally differentiated sperm cells, surface proteins are segregated into distinct surface domains that include the anterior and posterior head domains. We have analyzed the formation of the anterior and posterior head domains of guinea pig sperm in terms of both the timing of protein localization and the mechanism(s) responsible. On testicular sperm, the surface proteins PH-20, PH-30 and AH-50 were found to be present on the whole cell (PH-20) or whole head surface (PH-30, AH-50). On sperm that have completed differentiation (cauda epididymal sperm), PH-20 and PH-30 proteins were restricted to the posterior head domain and AH-50 was restricted to the anterior head domain. Thus these proteins become restricted in their distribution late in sperm differentiation, after sperm leave the testis. We discovered that the differentiation process that localizes these proteins can be mimicked in vitro by treating testicular sperm with trypsin. After testicular sperm were treated with 20 μg/ml trypsin for 5 min at room temperature, PH-20, PH-30, and AH-50 were found localized to the same domains to which they are restricted during in vivo differentiation. The in vitro trypsin-induced localization of PH-20 to the posterior head mimicked the in vivo differentiation process quantitatively as well as qualitatively. The quantitative analysis showed the process of PH-20 localization involves the migration of surface PH-20 from other regions to the posterior head domain. Immunoprecipitation experiments confirmed that there is protease action in vivo on the sperm surface during the late stages of sperm differentiation. Both the PH-20 and PH-30 proteins were shown to be proteolytically cleaved late in sperm differentiation. These findings strongly implicate proteolysis of surface molecules as an initial step in the mechanism of formation of sperm head surface domains.

How membrane proteins are sorted into specific plasma membrane domains is a basic question in cell biology. In some cases membrane domains encompass major regions of the cell surface. For example, surface proteins may be localized to the anterior head, posterior head, or tail domains of mammalian sperm (Friend, 1982; Primakoff and Myles, 1983; Eddy, 1988) and to either the apical or basolateral regions of epithelial cells (Rodriguez-Boulan and Nelson, 1989). On other cell types, the regions of protein localization may be limited to small patches in the membrane such as the distribution of the acetylcholine receptor on muscle cells (Anderson and Cohen, 1977).

In terminally differentiated cells, localized plasma membrane proteins can be delivered into a specific surface domain by being sorted either in an intracellular membrane or in the plasma membrane. An example of sorting in an intracellular membrane is the segregation of membrane proteins in the *trans*-Golgi into specific transport vesicles directed to either the apical or basolateral domains of epithelial cells (Nelson, 1989). An example of sorting in the plasma membrane also occurs in epithelial cells. Apical domain proteins, initially delivered to the basolateral domain, are segregated to the upper regions of the basolateral region and into vesicles for transcytosis to the apical region (Hubbard and Stieger, 1989; Matter et al., 1990). Both pathways can operate within a single cell (Matter et al., 1990). The localized plasma membrane proteins being sorted through these pathways contain structural signals that address them to specific domains (Lisanti and Rodriguez-Boulan, 1990).

Far less is known about how plasma membrane domains are initially created during the differentiation of polarized cells. The establishment of surface polarity occurs throughout embryogenesis and can also be studied in vitro using freshly seeded cultures of epithelial cells (Ekblom et al., 1986; Rodriguez-Boulan and Nelson, 1989). The initial establishment of cell surface domains appears to require an external signal (cell–cell or cell–substrate contact) and in part involves the redistribution of surface molecules to the apical and basolateral domains (Rodriguez-Boulan and Nelson, 1989).

During sperm differentiation, the localization of plasma membrane proteins is an ongoing process. Postmeiotic differentiation in the testis (spermiogenesis) results in the

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transformation of round spermatid cells to highly polarized testicular sperm. Some patterns of protein localization are established during spermiogenesis, for example, the localization of the PT-1 protein to the posterior tail region (Myles and Primakoff, 1983). The subsequent, late stage of sperm differentiation occurs in the epididymis, a long coiled tube in which sperm are transported from the testis to the vas deferens. By the time sperm arrive at the end of the epididymis (cauda epididymis) they have acquired motility and fertilization competence (Eddy, 1988). The formation of surface domains continues in this late stage of sperm differentiation, when there is no evidence for protein synthesis or new insertion/transcytosis of membrane proteins via vesicles since the cell components necessary for these processes have already been eliminated.

In this study we have investigated proteins whose localization occurs late in guinea pig sperm differentiation. We present evidence that proteolytic cleavage of surface proteins is a step in the mechanism for localizing these proteins. Remarkably, brief treatment of testicular sperm in vitro with trypsin triggers the localization of these proteins and induces the identical localizations that occur during in vivo differentiation.

Materials and Methods

Isolation of Sperm

Sperm were isolated from the testes by centrifugation of minced testicular tissue through a Percoll gradient (Pharmacia Fine Chemicals, Piscataway, NJ) for 10 min at 27,000 g_{av}, 10°C (Phelps et al., 1988). Testicular sperm were washed twice and resuspended in Mg^{2+}-Hepes buffer (Green, 1978). Epididymal sperm were gently expressed from the cauda as previously described (Phelps and Myles, 1987), and washed once with Mg^{2+}-Hepes buffer. All sperm samples were isolated from male Hartley guinea pigs (Buckberg Lab Animals, Inc., Tompkins Cove, NY).

Protease Treatment of the Cell Surface

Trypsin (6502; Calbiochem-Behring Corp., La Jolla, CA), at 1 mg/ml in Mg^{2+}-Hepes was made up just before use, and was added to 1.0 ml of 5-10 $\times 10^6$ live sperm at a final concentration of 20 μg/ml. Sperm were incubated for 5 min at room temperature and the reaction was stopped by the addition of an equal or greater concentration of soybean trypsin inhibitor (SBTI)[1] (Sigma Chemical Co., St. Louis, MO). The cells were then washed through 3% BSA and stained by indirect immunofluorescence.

Antibodies and Immunofluorescence

All mAbs were isolated as described (Primakoff and Myles, 1983). The mAbs used recognize distinct surface proteins and include AH-50, WH-1, PH-20, PH-22, and PH-30. Nomenclature (AH, PH, and WH) indicates the pattern of antibody binding on cauda epididymal sperm. Polyclonal antibody to the PH-30 antigen was made against purified PH-30 protein (Primakoff et al., 1987) in female rabbits or female guinea pigs. The two mAbs, PH-20 and PH-22, were both previously shown to recognize the PH-20 protein (Primakoff et al., 1985), and were used interchangeably. Second antibodies used were a FITC goat Fab anti-mouse IgG (Cappel Laboratories, Malvern, PA) and FITC goat IgG anti-guinea pig IgG (Sigma Chemical Co.). Indirect immunofluorescence was carried out on live sperm as previously described (Phelps and Myles, 1987).

Video Microscopy

Sperm were stained live with a Fab fragment (Cowan et al., 1986) of the PH-22 mAb followed by a rhodamine goat Fab anti-mouse $F(ab')_2$ second antibody (Cowan et al., 1987), and fixed in 3% formaldehyde. An uncorrected fluorescent image $[F_u(r)]$ was generated using an argon laser and DAGE-MTI silicon intensified target camera. 256 video frames were averaged to form an image that was computer digitized, processed, and analyzed using a RTI Station II imaging system (Recognition Technology, Inc., Westborough, MA) with an IBM/AT as host computer and software written especially for this purpose. For correction purposes, three auxiliary images were recorded: a background image of a cell-free area on the same slide $[F_b(r)]$, an image of fluorescence from a thin uniform layer of fluorescent dye solution $[F_d(r)]$, and a black-level image taken without illumination $[F_o(r)]$. The corrected fluorescence images $[F(r)]$ were calculated as in Koppel et al. (1989):

$$F(r) = A\left[\frac{F_u(r) - F_b(r)}{F_d(r) - F_o(r)}\right] + B.$$

B is a constant offset (typically set to 25) added to eliminate possible negative numbers in background areas, where they otherwise would show up on the video monitor as large positive values. Parameter A is a multiplicative constant set to make use of the full dynamic range of the digital framestores. All subsequent quantitative analysis of the corrected images takes constants A and B into account. A phase image of the same cell was also recorded and used to determine the boundaries of the regions of the head to be analyzed. The regions were outlined using a mouse and the fluorescent images were analyzed for total and average fluorescence within that region. Measurements on cauda epididymal sperm and testicular sperm, treated with trypsin, were normalized to the average fluorescence on control testicular sperm, not treated with trypsin. Comparisons were made only within individual experiments where the staining conditions were the same. In some cases testicular sperm and cauda sperm, which can be distinguished on a morphological basis, were stained in the same tube to insure that staining conditions did not vary. For each sample used in the measurements, a parallel sample of sperm treated identically, but not incubated with first antibody, was measured and subtracted from each measurement.

Total fluorescence intensity within a region was measured by using a mouse to outline the entire region. In addition, the average fluorescence intensity per pixel was measured within a standard-sized box drawn near the center of the region.

Iodination of Sperm Surface Proteins

Aliquots of 10^8 testicular sperm and 5×10^7 distal cauda epididymal sperm were surface iodinated with either 2.5 or 1.25 mCi $Na^{125}I$ using Iodogen (Primakoff and Myles, 1983). Half of the testicular sperm were then exposed to 20 μg/ml of trypsin for 5 min at room temperature, followed by the addition of 100 μg/ml SBTI to both trypsin-treated and untreated testicular sperm samples. All three sperm samples were pelleted at 12,000 g_{av} and were detergent extracted with 1% NP-40 in the presence of protease inhibitors (0.5 mM PMSF, 10 μM leupeptin, 3.3 μM antipain, 1.7 μM chymostatin, 1.5 μM pepstatin A, and 64 μM benzamidine, all purchased from Sigma Chemical Co.).

Immunoprecipitation of PH-20 and PH-30

The PH-20 protein was immunoprecipitated from the detergent extracts using the PH-22 mAb bound to goat anti-mouse IgG Sepharose beads (E. Y. Laboratories, Inc., San Mateo, CA). The PH-30 protein was immunoprecipitated with a rabbit polyclonal antibody against affinity purified PH-30 protein plus protein A-Sepharose beads (Sigma Chemical Co.). Protein was removed from the beads by boiling in SDS sample buffer, the beads were pelleted, and supernatants were run either nonreduced or reduced on 10% SDS-PAGE. Gels were dried and autoradiographed as described (Primakoff and Myles, 1983; Primakoff et al., 1985).

Results

Surface Proteins Are Recruited to the Anterior and Posterior Head Domains Late in Sperm Differentiation

To determine when surface proteins become restricted to the posterior and anterior head sperm surface domains, we observed live guinea pig sperm at different developmental stages by indirect immunofluorescence. We found that dis-

1. *Abbreviations used in this paper*: GPI, glycosyl phosphatidylinositol; SBTI, soybean trypsin inhibitor.

tinct surface proteins become localized to the posterior and anterior head surface domains late in sperm differentiation, when sperm pass through the epididymis. The two proteins known to be localized in the posterior head domain are PH-20 (required in sperm–zona binding, Primakoff et al., 1985) and PH-30 (required in sperm–egg fusion, Primakoff et al., 1987). PH-20 protein is distributed over the whole surface of round spermatids (Phelps and Myles, 1987) and testicular sperm (Fig. 1 *a*), but is restricted to the posterior head of sperm from the cauda epididymis (Fig. 1 *b*). Therefore, PH-20 protein becomes localized to the posterior head domain during sperm differentiation in the epididymis. PH-30 protein is distributed over the whole head of testicular sperm (Fig. 1 *e*) but is found only in the posterior head domain of cauda epididymal sperm (Fig. 1 *f*). A third protein antigen of unknown function, AH-50, also becomes localized during sperm differentiation in the epididymis. AH-50 is distributed over the whole head of testicular sperm (Fig. 1 *m*), but is restricted to the anterior head of sperm from the cauda epididymis (Fig. 1 *n*).

The exact patterns of antibody binding at different developmental stages for the PH-30 protein were found to depend upon which anti-PH-30 antibody was used. Polyclonal antisera (raised in rabbits or guinea pigs against affinity-purified PH-30 protein), as well as one mouse anti-PH-30 monoclonal antibody (Cowan, A. E., and D. G. Myles, unpublished observations), recognized PH-30 protein on the whole head of testicular sperm (Fig. 1 *e*). However, the PH-30 protein was not detected on testicular sperm by a different anti-PH-30 monoclonal (PH-30 mAb) (Fig. 1 *i*). This indicates that the epitope recognized by the PH-30 mAb does not exist, or is inaccessible, on testicular sperm and this epitope is created or revealed during sperm development in the epididymis. The antibodies that recognized PH-30 protein on testicular sperm were found on cauda sperm to be largely restricted in their binding pattern to the posterior head domain, but trace amounts of staining were detectable on the anterior head (Fig. 1 *f*). However, the PH-30 mAb stained only the posterior head of cauda sperm (Fig. 1 *j*). This suggests there may be a small fraction of PH-30 protein, retained on cauda epididymal sperm, on which the PH-30 mAb-recognized epitope is not created or revealed. This small fraction of PH-30 protein may remain on the anterior head of cauda sperm where it can be detected only by the polyclonal antisera and the mAb that recognize the PH-30 protein on testicular sperm.

Although certain sperm surface proteins (PH-20, PH-30, AH-50), with a broad distribution (whole cell, whole head) at the testicular sperm stage, ultimately become restricted to a specific head domain, this is not the case for all head surface proteins. For example, we observed that during sperm differentiation in the epididymis, the WH-1 protein distribution did not change; WH-1 was seen on the whole head of testicular sperm (Fig. 1 *q*) and also the whole head of cauda epididymal sperm (Fig. 1 *r*).

Trypsin Treatment of Testicular Sperm In Vitro Is Sufficient to Localize Sperm Surface Proteins to Their Correct Domains

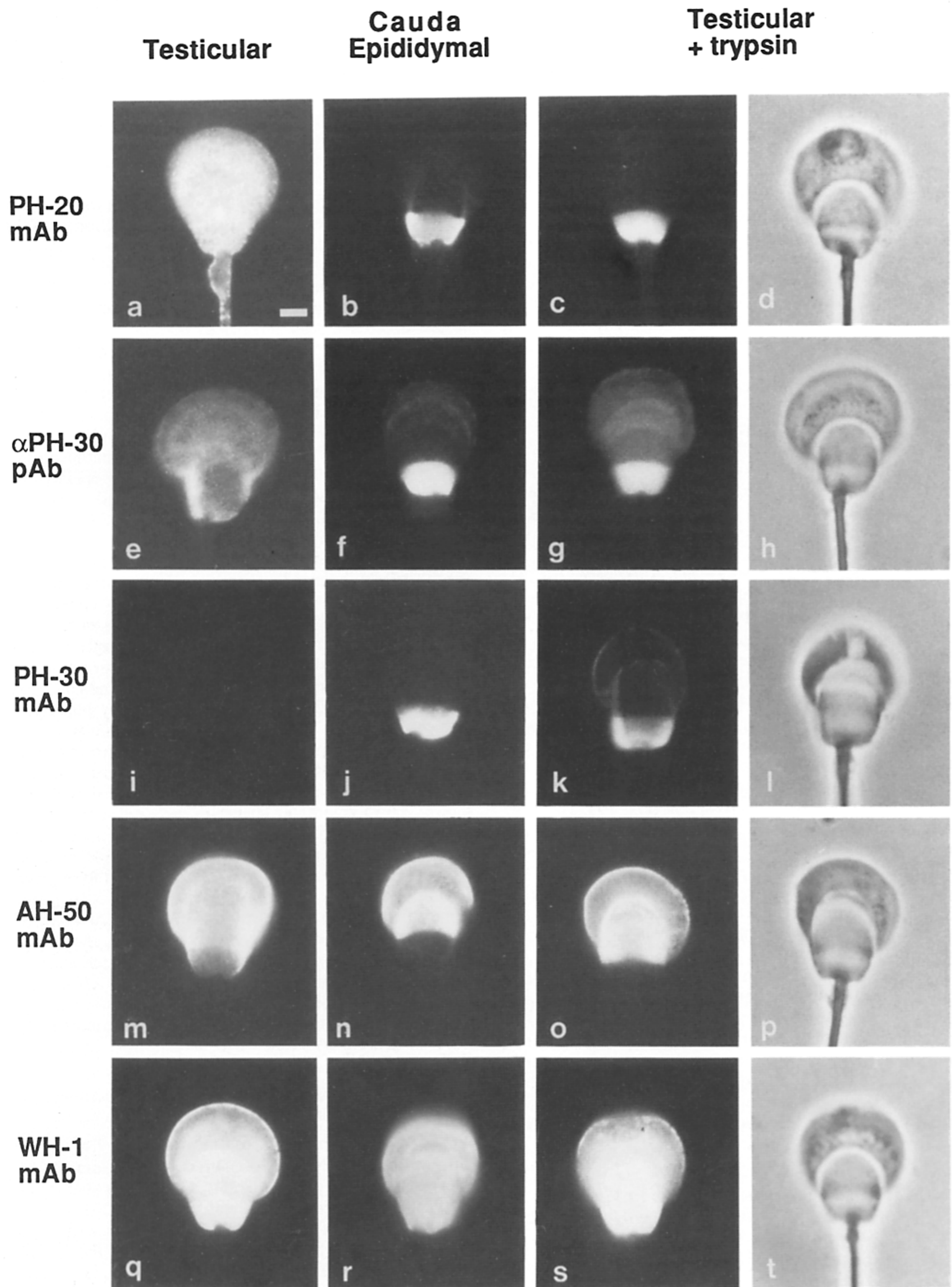
We found that the localization of surface proteins into the posterior and anterior head domains, normally occurring during sperm differentiation in the epididymis, could be mimicked by treating testicular sperm in vitro with trypsin. Sperm were isolated from the testis and treated with 20 μg/ml trypsin for 5 min at room temperature. The trypsin-treated testicular sperm were then stained with antibodies to the individual surface proteins. The staining revealed that trypsin treatment was sufficient to establish the posterior head localization of the PH-20 and PH-30 proteins (Fig. 1, *c*, *g*, and *k*). After trypsin treatment, the detected patterns of PH-30 protein distribution differed between the anti-PH-30 polyclonal antisera and the PH-30 mAb. With anti-PH-30 polyclonal staining, residual PH-30 remaining on the anterior head surface could be seen (Fig. 1 *g*), whereas with mAb PH-30 staining, virtually all the PH-30 protein detected was on the posterior head (Fig. 1 *k*). This was similar to the difference seen for PH-30 protein distribution on cauda epididymal sperm when the anti-PH-30 polyclonal antisera and mAb PH-30 were compared (see above, Fig. 1, *f* and *j*). Trypsin treatment of testicular sperm also resulted in the AH-50 antigen becoming localized to the anterior head (Fig. 1 *o*) and did not alter the pattern of the WH-1 antigen (Fig. 1 *s*), thus also mimicking the domain formation occurring during late sperm differentiation in the epididymis. Trypsin treatment of testicular sperm consistently produced the patterns shown in Fig. 1 on 70–100% of the testicular sperm population.

Binding to a divalent antibody is apparently not required for the trypsin-induced localizations. For example, the PH-20 protein was localized to the posterior head of epididymal and trypsin-treated testicular sperm regardless of whether the staining was carried out with a divalent first antibody and a monovalent second antibody (Fig. 1 *c*) or if both antibodies were monovalent Fab fragments (Table I). This indicates that localization of PH-20 protein is not induced by binding of divalent antibody.

The effect of trypsin appears to be specific to testicular sperm: trypsin treatment did not induce a change in the antibody staining patterns on cauda epididymal sperm (data not shown). Furthermore, the change in surface distribution after trypsin treatment is not the result of differential loss of membrane. If the membrane were being removed or damaged in selected areas to produce a new pattern for a given protein, those areas would be expected to lose other membrane proteins as well. The various antibodies tested showed this was not the case. After trypsin treatment, the PH-20 mAb, for example, showed loss of staining in the anterior head region, but stained the posterior head; the AH-50 pattern was reversed, with loss of staining in the posterior head, but not in the anterior head. The WH-1 mAb continued to stain the entire head surface after trypsin treatment.

Quantitation of PH-20 Fluorescence and the Migration of PH-20 Protein During Domain Formation

To quantitatively compare protein localization in vivo to protein localization induced by in vitro trypsin treatment, we analyzed the PH-20 protein in more detail. After staining of live cells with Fab fragments of both an anti-PH-20 mAb and a rhodamine-labeled second antibody, fluorescence intensity was measured on three samples: (*a*) control testicular sperm, incubated without trypsin, (*b*) testicular sperm, incubated with trypsin, and (*c*) cauda epididymal sperm. Fluorescence


Testicular
Cauda Epididymal
Testicular + trypsin
PH-20 mAb
αPH-30 pAb
PH-30 mAb
AH-50 mAb
WH-1 mAb
a
b
c
d
e
f
g
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s
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Table I. Comparative Measurements of Antibody Bound and Surface Area

Region	Sperm type		
	Testicular	Cauda epididymal	Testicular + trypsin
Fluorescence intensity			
Posterior head; total fluorescence	1.00 (±.18)	2.06 (±.45)	1.80 (±.41)
Posterior head; average fluorescence	1.00 (±.25)	1.99 (±.64)	2.22 (±.39)
Relative surface area in pixels (area × 10^{-3})			
Posterior head	1.00 (±0.1) $n = 20$	1.00 (±.09) $n = 21$	0.99 (±0.1) $n = 7$

(*Top*) Sperm were incubated with or without trypsin, then stained with a Fab fragment of the PH-22 mAb and a rhodamine-conjugated Fab goat anti-mouse $F(ab)_2$. Fluorescence intensity was measured within the posterior head region as described. For each experiment the mean of testicular sperm fluorescence was determined and other data were normalized to it. Normalized data from all experiments were pooled to determine overall mean (± SEM). A two-tailed Student's *t*-test with α set at 0.01 indicated that there was no significant difference between cauda epididymal sperm and testicular + trypsin sperm. Sample size ranged from 21 to 50 sperm. (*Bottom*) Relative surface area for the posterior head was determined by recording the number of pixels included in an outline of the posterior head region, demonstrating that the increase in posterior head fluorescence did not occur because of an increase in posterior head surface area. *n*, number of sperm.

Table II. Measurement of Antibody Bound to Posterior Head Region of Sperm Stained before Trypsin Treatment

Treatment after staining	Fluorescence intensity
None (control)	1.00 ± 0.26 ($n = 10$)
5 min trypsin + 1-h incubation	2.39 ± 0.68 ($n = 9$)
5 min trypsin + 1-h incubation (in presence of excess Fab PH-20 mAb)	2.12 ± 0.78 ($n = 13$)

Testicular sperm were first stained with a Fab fragment of an anti-PH-20 mAb and a rhodamine-conjugated Fab second antibody. One aliquot of stained sperm was then treated with trypsin for 5 min at room temperature, SBTI was added, cells were washed and incubated in Mg^{2+}-Hepes buffer for an additional 60 min. Note that fluorescence intensity in the posterior head of trypsin-treated sperm, relative to untreated, was the same, i.e., twofold greater, if sperm were labeled with antibodies before trypsin treatment (Table II) or after trypsin treatment (Table I). This indicated that the brief trypsin treatment did not result in a loss of the antibodies used for prelabeling. Fluorescence intensity was measured as described in Materials and Methods. For each treatment the mean was normalized to mean intensity on control sperm and (± SEM) was calculated. *n*, number of sperm measured.

intensity from the control testicular sperm was assigned an arbitrary value of 1.00. For both total fluorescence and average fluorescence (see Materials and Methods), cauda epididymal sperm and trypsin-treated testicular sperm had about two times more fluorescence in the posterior head region than control testicular sperm (Table I). The surface area of the posterior head remains constant (Table I). These results show that the trypsin-induced restriction of PH-20 to the posterior head domain mimics the differentiation-induced restriction quantitatively as well as qualitatively.

The increase in fluorescence intensity in the posterior head suggests that PH-20 molecules, present in other domains on testicular sperm, migrate to the posterior head. Alternatively, the measured increase in mAb binding in the posterior head region could reflect new sites being revealed in that region. To test directly if PH-20 protein is able to move into the posterior head from other surface regions, testicular sperm were prelabeled with a Fab fragment of an anti-PH-20 mAb and a rhodamine-Fab second antibody. We then treated the cells with trypsin for 5 min and after stopping the trypsin reaction, incubated the cells in Mg^{2+}-Hepes medium, for 1 h, a time that allows localization to proceed to completion. (We found the brief trypsin treatment, required to achieve PH-20 localization, did not result in significant loss of the labeled Fab fragments; see legend to Table II). After localization had occurred, we measured fluorescence intensity over the posterior head and compared it with the posterior head fluorescence of testicular sperm that had not been trypsin treated. To control for the possibility that antibody was unbinding from one region of the cell and rebinding to newly revealed antigen sites in the posterior head region, we incubated one set of cells in the presence of a 45-fold excess of unlabeled anti-PH-20 Fab that should bind to any newly revealed sites, preventing the binding of any mAb–second Ab complex. The excess unlabeled antibody will also bind to any second antibody that becomes free in the medium. Under both conditions, the fluorescence intensity over the posterior head region after trypsin treatment was more than twofold higher than fluorescence intensity over the posterior head of untreated testicular sperm, indicating that PH-20 protein from other regions of the cell surface migrated into the posterior head region (Table II).

Evidence Confirming That There Is Proteolytic Action on Sperm Surface Proteins During Sperm Differentiation in the Epididymis

The finding that trypsin treatment of testicular sperm is sufficient to localize sperm surface proteins to their domains suggests that protease action on the sperm surface in vivo is a step in the mechanism of domain formation. To confirm that there is in vivo protease action on sperm surface proteins in the epididymis, we did a series of biochemical experiments to analyze surface protein structure. Live testicular and cauda sperm were isolated and surface iodinated. After iodination of the testicular sperm, half of the sample was treated with 20 μg/ml trypsin for 5 min at room temperature. Membrane proteins were extracted from the three samples with 1% NP-40.

Figure 1. Indirect immunofluorescence patterns of antibody bound to the surface of testicular, cauda, and trypsin-treated testicular sperm. (*a–c*) PH-20 mAb; (*e–g*) rabbit polyclonal anti-PH-30; (*i–k*) PH-30 mAb; (*m–o*), AH-50 mAb; and (*q–s*) WH-1 mAb. (*a, e, i, m,* and *q*) Testicular sperm; (*b, f, j, n,* and *r*) cauda sperm; and (*c, g, k, o,* and *s*) trypsin-treated testicular sperm. (*d, h, l, p,* and *t*) Phase-contrast image of same sperm shown in *c, g, k, o,* and *s*. Sperm were stained live and then fixed for photography. Bar, 2 μm.

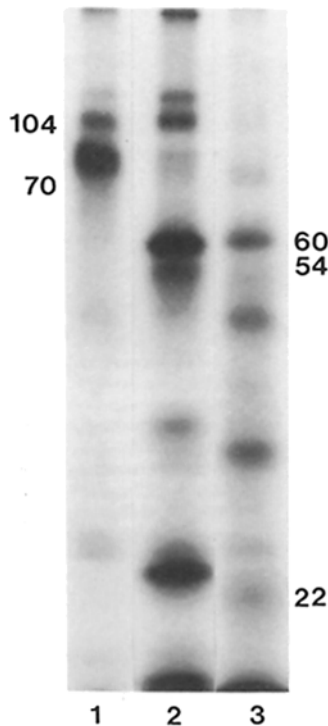

Figure 2. Autoradiographs of nonreducing SDS-PAGE of detergent extracts of ^{125}I surface-labeled sperm. Lane *1*, testicular sperm; lane *2*, trypsin-treated testicular sperm; lane *3*, cauda epididymal sperm. Molecular mass is in kilodaltons.

We compared the SDS-PAGE patterns of the detergent extracts from the control testicular sperm, trypsin-treated testicular sperm and cauda epididymal sperm (Fig. 2). The major ^{125}I surface-labeled proteins from testicular sperm were found clustered at higher relative molecular weights, with the most heavily labeled band at 70–104 kD (Fig. 2, lane *1*). In contrast, sperm taken from the cauda epididymis had the major labeled bands distributed between ~60 and ~22 kD (Fig. 2, lane *3*). The pattern of iodinated surface proteins of trypsin-treated testicular sperm resembled, but did not exactly match, surface proteins of cauda epididymal sperm. After trypsin treatment of testicular sperm, the band at 70–104 was greatly reduced, and new bands appeared at ~60, 54, 43, 36, and 23 kD (Fig. 2, lane *2*).

We next immunoprecipitated from testicular sperm and cauda epididymal sperm two individual antigens that become localized in the epididymis (PH-20 and PH-30). We found that both these surface proteins undergo proteolytic cleavage during sperm development in the epididymis and that trypsin treatment of testicular sperm resulted in similar proteolytic cleavages.

Previous work had shown that PH-20, purified from the whole testis (which includes PH-20 from spermatids as well as testicular sperm), ran at a slightly higher molecular mass on reducing SDS-PAGE (~66 kD) than PH-20 isolated from cauda epididymal sperm (~64 kD) (Phelps and Myles, 1987). In this study, PH-20 was immunoprecipitated from the three detergent extracts of sperm and the precipitates were electrophoresed to determine: (*a*) if there was a difference in molecular mass between PH-20 from purified testicular sperm and cauda epididymal sperm, and (*b*) if there was a difference, could trypsin treatment of testicular sperm alter PH-20 to a form similar to the PH-20 from cauda sperm. A difference in the molecular mass of PH-20 from testicular sperm compared to cauda epididymal sperm was detected; PH-20 precipitated from testicular sperm and run on nonreducing gels, was ~2 kD larger than PH-20 precipitated from cauda epididymal sperm (Fig. 3, lanes *1* and *3*). Trypsin treatment of testicular sperm did not change the relative molecular mass of PH-20 on nonreducing gels (Fig. 3, lane *2*). However, results examining PH-20 immunoprecipitates under reducing conditions did reveal an effect of the trypsin treatment. Previously we had found that PH-20, purified from cauda sperm and run on SDS-PAGE under reducing conditions, showed predominantly two subunits of 41–48 and 27 kD (the 27-kD polypeptide is not labeled by ^{125}I; Primakoff et al., 1988*a*), and a small amount ran at ~64 kD. The experiments showed that most of the PH-20 on cauda sperm had been endoproteolytically cleaved into two disulfide bonded fragments (41–48 and 27 kD) while a small fraction of it remained as the uncleaved 64 kD (Primakoff et al., 1988*a*). PH-20 from control testicular sperm was not endoproteolytically cleaved and ran at M_r ~66 kD under reducing conditions (Fig. 3, lane *4*). PH-20 protein from trypsin-treated testicular sperm ran differently than PH-20 from control testicular sperm, and partially mimicked the pattern seen with cauda epididymal sperm; the major band at ~66 kD was drastically reduced, and a new band appeared at ~48 kD (Fig. 3, lane *5*). PH-20 precipitated from cauda sperm, was found primarily in the 41–48-kD bands with trace amounts detected in the ~64-kD band (Fig. 3, lane *6*).

The rabbit anti-PH-30 polyclonal antibody, which recognizes PH-30 on testicular sperm as well as cauda epididymal sperm (Fig. 1), was used to immunoprecipitate PH-30. We asked the same questions: does PH-30 undergo proteolysis during sperm differentiation in the epididymis, and if so, can this proteolysis be mimicked in vitro by trypsin treatment of testicular sperm? Autoradiographs of the PH-30 immunoprecipitates showed that this protein also underwent a change in molecular mass during differentiation in vivo, and that this change was partially mimicked on testicular sperm that had been treated with trypsin in vitro (Fig. 4). From cauda epididymal sperm (Fig. 4, lane *3*), the anti-PH-30 polyclonal precipitated two polypeptides, one at 49 kD, termed α, the other at 33 kD, termed β. From other work it is known that

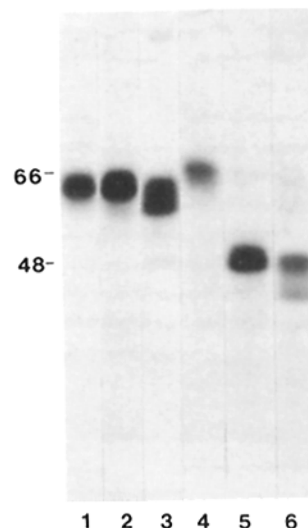

Figure 3. Autoradiographs of SDS-PAGE of PH-20 immunoprecipitated from ^{125}I surface-labeled sperm. Lanes *1–3*, nonreducing conditions; lanes *4–6*, reducing conditions. Lanes *1* and *4*, testicular sperm; lanes *2* and *5*, trypsin-treated testicular sperm; lanes *3* and *6*, cauda epididymal sperm. Molecular mass is in kilodaltons.

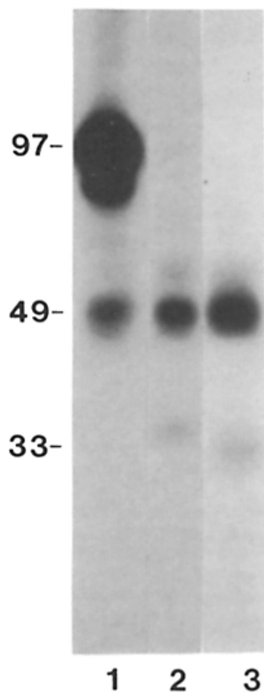

Figure 4. Autoradiographs of nonreducing SD-PAGE of PH-30 protein immunoprecipitated from ^{125}I surface-labeled sperm. A rabbit polyclonal antibody to the PH-30 protein was used for immunoprecipitation. Lane *1*, testicular sperm; lane *2*, trypsin-treated testicular sperm; lane *3*, cauda epididymal sperm. Molecular mass is in kilodaltons.

the α and β polypeptides copurify on PH-30 mAb affinity columns and are two distinct subunits of the PH-30 protein. α and β have different peptide maps (Primakoff et al., 1987) and affinity-purified polyclonal antibodies from α bind only to α or from β bind only to β (Blobel et al., 1990). From testicular sperm, we found that the anti-PH-30 polyclonal precipitated the α band at 49 kD, and no β band but instead showed an additional heavily labeled band(s) at 69–112 kD (Fig. 4, lane *1*). Polyclonal antibody, affinity purified from the 33-kD β band of cauda epididymal sperm, immunoblots a band centered at ~85 kD on testicular sperm, indicating that this higher molecular mass polypeptide is a precursor of β (Blobel et al., 1990). PH-30, immunoprecipitated from trypsin-treated testicular sperm, shows that the β precursor is lost and a band of molecular mass very close to mature β (33 kD) is produced (Fig. 4, lane *2*).

Discussion

Our results strongly implicate proteolysis of the surface as one step in the mechanism for protein localization to the posterior and anterior head domains. We found that brief in vitro trypsin treatment of testicular sperm is sufficient to produce the identical localizations that occur in vivo late in sperm differentiation. In the case of the PH-30 protein, trypsin treatment not only mimicked the change in localization of PH-30 protein but also resulted in the appearance of a new PH-30 protein epitope, recognized by the PH-30 mAb. This epitope also appears in vivo late in sperm differentiation. In addition, trypsin treatment of testicular sperm produced the same quantitative increase in PH-20 protein on the posterior head as was observed on in vivo–differentiated cauda sperm. These findings suggest that there may be protease action on sperm surface molecules during in vivo differentiation and that this proteolysis results in the formation of the head surface domains.

Immunoprecipitation experiments confirmed that there is protease action on sperm surface proteins during in vivo differentiation. Both the PH-20 and PH-30 proteins are proteolytically cleaved on sperm in vivo. PH-20 protein undergoes an endoproteolytic cleavage into two disulfide-linked fragments. The cleavage of the β chain precursor of PH-30 protein (M_r = ~85 kD, testicular sperm) to the mature β (M_r = ~33 kD, cauda epididymal sperm) could be endoproteolytic, exoproteolytic, or both.

Two kinds of models can be envisioned for steps in the mechanism of domain formation after the protease step. In one model, the only event required would be the proteolysis of specific surface molecule, which would allow the localizations to proceed spontaneously. The key event might be proteolysis of some type of network holding PH-20, PH-30, and AH-50 in their broad distributions on testicular sperm or the key event could be proteolysis of PH-20, PH-30, or AH-50 themselves. A glycocalyx or extracellular coat can be seen as part of the surface of mammalian sperm (Suzuki and Nagano, 1980), including guinea pig sperm (Bearer and Friend, 1982), and could be a restraining network holding surface proteins in a broad distribution. In a second model, the proteolysis could trigger a subsequent sequence of biochemical modifications. The sequence of modifications could occur entirely on the surface or proteolytic cleavage of a particular surface protein could act as an extracellular signal which could be transduced across the plasma membrane and initiate cytoplasmic events resulting in localization.

Previous measurements of PH-20 protein diffusion, at different stages of sperm differentiation, show that there is a constraint on its diffusion on testicular sperm that could be partially disrupted by proteolysis in the epididymis. On testicular sperm, diffusion of the PH-20 protein is slow ($D = 1.9 \times 10^{-11}$ cm^2/s; Phelps et al., 1988), but on cauda epididymal sperm it is 10-fold higher ($D = 1.8 \times 10^{-10}$ cm^2/s; Cowan et al., 1987). Restriction to diffusion on testicular sperm would be expected to result from extracellular rather than cytoplasmic interactions, since PH-20 is anchored in the membrane by linkage to glycosyl phosphatidylinositol (GPI) (Phelps et al., 1988). Such an external constraint system could be analogous to the maintenance of surface distribution of the fibronectin receptor by interaction with extracellular fibronectin (Roman et al., 1989) and would be accessible to external proteases. Our results show that one of the steps after proteolysis is the migration of PH-20 protein into the posterior head region, which could occur after a release from constraint.

In epithelial cells, the GPI anchor is a sorting signal for localization of proteins to the apical domain (reviewed in Lisanti and Rodriguez-Boulan, 1990). It is conceivable that the GPI anchor is critical for localization of PH-20 to the posterior head domain and that other structural information in involved in targeting to the posterior head for PH-30, an integral membrane protein that is not GPI anchored (Primakoff et al., 1987; Phelps et al., 1988; Blobel et al., 1990).

The hypothesis that proteases or other enzymes might modify sperm surface proteins in the epididymis has been previously suggested (see Eddy et al., 1985) and proteases have been found in the male reproductive tract (Vanha-Perttula et al., 1985; Huarte et al., 1987). There are at least

four potential cellular or extracellular sites of in vivo protease(s) that could trigger domain formation. The sperm itself may contain surface proteases that are activated or internal proteases that are released (for example, from the cytoplasmic droplet) while sperm are in the epididymis. Alternatively sperm may be acted upon by proteases that are in the epididymal fluid or on the plasma membrane of epithelial cells lining the lumen of the epididymis. Biochemical identification of the in vivo protease(s) will require determining their localization and will also require overcoming the difficulties of working with the small quantities of material available in the early region(s) of the epididymis where proteolytic processing occurs (our unpublished observations). In addition it will be necessary to distinguish the physiological protease(s) from irrelevant proteases that may be present in the fluid or cell preparations.

The localization of surface proteins during sperm differentiation in the epididymis may be essential for sperm fertilizing ability which is acquired as sperm pass through the epididymis (Orgebin-Crist and Olson, 1984; Eddy, 1988). Antibody inhibition studies indicate that PH-20 has a required role in sperm–zona binding (Primakoff et al., 1985; Primakoff et al., 1988*b*) and PH-30 functions in sperm–egg fusion (Primakoff et al., 1987). Given the geometry of sperm–egg interaction, surface localization could be important simply in bringing these proteins into the correct domain for their activity. A second possible function of localization is to increase the surface density of the proteins by restricting them to a small surface area. Indeed, changes in the localization of PH-20 protein act to concentrate it in the region where acrosome-reacted sperm bind to the zona pellucida. Protein concentration is divided into two stages. First, migration of PH-20 protein into the posterior head surface domain results in a twofold increase in the posterior head PH-20 concentration (Table I). After the acrosome reaction a further 2.5-fold increase in surface expression will occur in the region of the inner acrosomal membrane as a second migration moves PH-20 into this newly inserted surface membrane where it joins a previously covert population (Myles and Primakoff, 1984; Cowan et al., 1986). The restriction of PH-30 from the whole head to the posterior head may also act to increase surface density of PH-30 protein. In other systems, cell adhesion and membrane fusion have both previously been shown to require a threshold density of functional surface molecules (Gething and Sambrook, 1981; Hoffman and Edelman, 1983; Gething et al., 1986; Norment et al., 1988; Doherty et al., 1990).

The current findings represent an initial insight into a mechanism for establishing surface domains during polarized cell differentiation. As more information accumulates about surface domain formation in developing sperm and in other differentiating cells, either proteolysis or different initiating events may be discovered and the subsequent steps in the mechanism(s) of protein localization established.

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