Proteolytic Processing of a Protein Involved in Sperm–Egg Fusion Correlates with Acquisition of Fertilization Competence

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Abstract. A protein located on the surface of guinea pig sperm (PH-30) has been implicated in the process of sperm–egg fusion (Primakoff, P., H. Hyatt, and J. Tredick-Kline. 1987. *J. Cell Biol.* 104:141–149). In this paper we have assessed basic biochemical properties of PH-30 and have analyzed the molecular forms of PH-30 present at different stages of sperm maturation. We show the following: (*a*) PH-30 is an integral membrane glycoprotein; (*b*) it is composed of two tightly associated and immunologically distinct subunits; (*c*) both subunits are made as larger precursors; (*d*) processing of the two subunits occurs at different developmental stages; (*e*) the final processing step occurs in the region of the epididymis where sperm become fertilization competent; (*f*) processing can be mimicked in vitro; (*g*) processing exposes at least two new epitopes on PH-30—one of the newly exposed epitopes is recognized by a fusion-inhibitory monoclonal antibody. These results are discussed in terms of the possible role of PH-30 in mediating fusion with the egg plasma membrane.

MEMBRANE fusion is ubiquitous in nature. Endoplasmic fusion reactions include fusion of endocytic vesicles, exocytic vesicles, and vesicles mediating other aspects of interorganelle traffic. Exoplasmic fusion events include viral fusion, myoblast fusion, and fertilization. The fusion events that are best understood at a molecular level are those of enveloped viruses. Viral fusion reactions are mediated by specific virally-encoded membrane fusion proteins. These are integral membrane glycoproteins that exist as oligomers on the virion surface. A large number of viral fusion proteins are made as precursors and then cleaved into two subunits that remain associated through disulfide bonds and/or noncovalent interactions. For fusion proteins consisting of two subunits derived from a common precursor, cleavage is essential for fusion activity. Most, but not all, viral fusion proteins possess a "fusion peptide," a stretch of apolar amino acids that is thought to initiate fusion by interacting with lipid components of the target bilayer. For recent reviews on membrane fusion and membrane fusion proteins see references 15, 18–20.

Although it has been hypothesized that cellular fusion events are protein mediated, the identities of cellular membrane fusion proteins and their modes of action remain elusive. Several proteins involved in steps leading to endoplasmic fusion events have been identified and partially characterized (2, 17, 20, 21). Less, however, is known about proteins that mediate exoplasmic fusion events occurring between cellular membranes (19).

We have set out to study the molecular mechanism of gamete fusion, an exoplasmic fusion reaction common to all eukaryotic organisms. A protein that has been strongly associated with the process of gamete fusion is PH-30, a protein found in the posterior head of mature guinea pig sperm. Of two monoclonal antibodies that bind to PH-30, one inhibits fusion whereas the second does not. Neither antibody prevents sperm from binding to the egg plasma membrane (14). Due to its potential role in the overall fusion process we have characterized biochemical features of PH-30. In the course of this work we established that PH-30 is made as a larger precursor and that processing of PH-30 during sperm maturation correlates with the acquisition of fertilization competence.

Materials and Methods

Antibodies

The primary antibodies used in this study were monoclonal antibodies, mAb PH-30 and mAb PH-1 (14), and a rabbit polyclonal antiserum raised against nondenatured PH-30 affinity purified on a mAb PH-30-Sepharose column (14). IgG from the polyclonal antiserum was purified on a column of protein A-Sepharose (Sigma Chemical Co., St. Louis, MO). For immunoblot analyses, primary antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG (Promega Biotec, Madison, WI). For immunofluorescence analyses, primary antibodies were detected with rhodamine-conjugated goat anti-rabbit or goat anti-mouse IgG (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN).

Preparation of Gametes

Sperm from male Hartley guinea pigs (retired breeders) were collected (12) from three regions of the epididymis: the distal corpus epididymis, the proximal cauda epididymis, and the distal cauda epididymis. These regions correspond to region II, region IV, and region VI/VII, respectively, of the guinea pig epididymis as described by Hoffer (9). Testicular cells and testicular sperm were collected from the testis and separated on a 52% isotonic Percoll (Sigma Chemical Co.) gradient in Mg^{++}-Hepes buffer by centrifugation for 10 min at 27,000 *g*, 10°C. Isolated gametes were then washed twice in Mg^{++}-Hepes buffer (0.14 M NaCl, 4 mM KCl, 4 mM Hepes, pH 7.4, 10 mM glucose, 2 mM $MgCl_2$ [8]).

Preparation of Gel Samples, Electrophoresis, and Immunoblot Analysis

Cells were lysed in a nonionic detergent containing cell lysis buffer (1% NP-40, 50 mM Tris, 2 mM EDTA, pH 7.4). Protease inhibitors (1 mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, 4 μg/ml aprotinin, 10 μg/ml antipain, 50 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, and 100 μg/ml iodoacetamide) were added to the cell lysis buffer just before use. All protease inhibitors were from Sigma Chemical Co. Typically ~4 × 10^6 cells were lysed in 100 μl cell lysis buffer and 25 μl of the extract was analyzed per gel lane. Samples were either boiled in sample buffer containing 2% SDS or incubated at room temperature (RT)[1] in sample buffer containing 0.5% SDS as indicated in the text. Samples were not reduced before electrophoresis. Electrophoresis was conducted according to Laemmli (10), with minor modifications, on 10% resolving gels with 5% stacking gels. Prestained molecular mass standards (Sigma Chemical Co.) were: α2-macroglobulin (180,000 D), β-galactosidase (116,000 D), fructose-6-phosphokinase (84,000 D), pyruvate kinase (58,000 D), fumerase (48,000 D), lactic dehydrogenase (36,000 D), and triose phosphate isomerase (26,600 D). For immunoblot analyses (16), proteins were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH), and the nitrocellulose sheets were blocked with 5% dry milk reconstituted in distilled water. Incubation with primary antibodies, alkaline phosphatase-conjugated secondary antibodies (Promega Biotec), and color development with 5-bromo, 5-chloro indolyl phosphate and nitroblue tetrazolium (BCIP/NBT; Promega Biotec) were performed following the Promega Biotec protocols and applications guide. Apparent molecular masses were calculated with reference to the prestained molecular mass markers.

Immunodepletion of Material Reactive with mAb PH-30

An extract of mature epididymal sperm was immunodepleted of mAb PH-30 reactive material by passage over a column containing a large excess of mAb PH-30 coupled to cyanogen bromide-activated Sepharose CL-4B (14). To verify the immunodepletion, the column flow through was passed a second time over the column. A sample of the material eluted from the second column was analyzed by SDS-PAGE. Silver staining (1) of the gel revealed that all of the mAb PH-30 reactive material had been removed during the first passage over the column.

Digestion with Endoglycosidase F

Distal cauda epididymal sperm were lysed in cell lysis buffer and adjusted to 10 mM sodium acetate (pH 5.5), 0.1% SDS and 1.5 mM PMSF. Samples (10 μl) containing 30 μg of sperm protein and 0.5 U of endoglycosidase F (Boehringer Mannheim Diagnostics, Inc.) were incubated overnight at 37°C. Mock digested samples were prepared and incubated as above in the absence of added enzyme.

Binding to Concanavalin A-Sepharose

Con A-Sepharose CL-4B beads (100 μl; Sigma Chemical Co.) or control Sepharose CL-4B beads (100 μl; Sigma Chemical Co.) were washed twice in cell lysis buffer and incubated on ice for 1 h with an extract of 5 × 10^6 distal cauda epididymal sperm lysed in 100 μl cell lysis buffer. The beads were centrifuged for 10 min at 12,000 *g* in a microfuge. Unbound material was saved and adjusted to 2% SDS in sample buffer. The beads were then washed four times with 500 μl cell lysis buffer and the bound material removed by boiling in sample buffer (2% SDS). After removing the beads by centrifugation (10 min at 12,000 *g*), the samples were analyzed by SDS-PAGE.

1. *Abbreviation used in this paper*: RT, room temperature.

Microaffinity Purification of Antibodies

Microaffinity purification of antibodies was conducted essentially as described by Gluck and Caldwell (7). The proteins in a boiled extract of epididymal sperm were separated by preparative SDS-PAGE and transferred to nitrocellulose. Nitrocellulose sheets were blocked as described above and incubated with a 1:20 dilution of PH-30 rabbit polyclonal antiserum for 1 h at RT. A narrow strip was cut from both sides of the blot, incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega Biotec), and developed with BCIP/NBT (Promega Biotec) as described above. A strip corresponding to the desired band was then cut from the nitrocellulose sheet and placed into a 15 ml Falcon tube. Bound antibodies were eluted with elution buffer (5 mM glycine, 500 mM NaCl, 0.1% BSA, 0.5% Tween 20, pH 2.5) for 1 min, and immediately neutralized with a predetermined amount of saturated $NaHPO_4$. The strip was then washed with PBS, 0.05% Tween 20, followed by two more elution and wash cycles.

Proteolytic Treatment of Testicular Sperm

Testicular sperm were washed and resuspended at a concentration of 10^7 sperm/ml in Mg^{++}-Hepes buffer. For immunoblot analysis, testicular sperm were incubated for 2 h at 37°C with 20 μg/ml proteinase K (Sigma Chemical Co.), chymotrypsin (Boehringer Mannheim Diagnostics, Inc.), or L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma Chemical Co.), or with 100 μg/ml dispase (Boehringer Mannheim Diagnostics, Inc.). At the end of the incubation, the protease inhibitor cocktail described above was added to the cells and the cells were then lysed in cell lysis buffer, and prepared for SDS-PAGE as described above. Approximately 1 × 10^6 testicular sperm were analyzed per lane. Testicular sperm to be analyzed by immunofluorescence microscopy were incubated on ice for 1 h with fivefold higher concentrations of proteases: 100 μg/ml proteinase K, chymotrypsin or trypsin, or 500 μg/ml dispase. Digestion with fivefold higher protease concentrations on ice generated immunoblot patterns identical to those seen when testicular sperm were treated with the lower concentrations of proteases at 37°C (not shown).

Indirect Immunofluorescence Microscopy

Indirect immunofluorescence staining was performed on live sperm as described previously (12). Primary antibodies were detected with rhodamine-conjugated goat anti-mouse or goat anti-rabbit IgG (Boehringer Mannheim Diagnostics, Inc.).

Results

The α and β Subunits of PH-30 Are Immunologically Distinct

A monoclonal antibody, mAb PH-30, blocks fusion of acrosome-reacted guinea pig sperm with zona-free guinea pig eggs. This antibody immunoprecipitates two protein chains of M_r 60 and 44 kD on reducing SDS-PAGE (14). When these polypeptides are boiled, but not reduced, their apparent masses on SDS polyacrylamide gels are ~45 and ~27 kD, respectively. In this report, the larger of the two polypeptides will be referred to as the α chain and the smaller as the β chain.

Differences in peptide fragments released from α and β after digestion with V8 protease have suggested that α and β are not related (14). An immunological approach confirmed this finding. A rabbit polyclonal antiserum was raised against PH-30 protein that had been purified, in a nonreduced and nondenatured form, on a column containing mAb PH-30. Antibodies against the individual α and β chains were microaffinity purified as described in Materials and Methods. The complete antiserum and the affinity-purified α and β antibodies were then used to probe immuno-

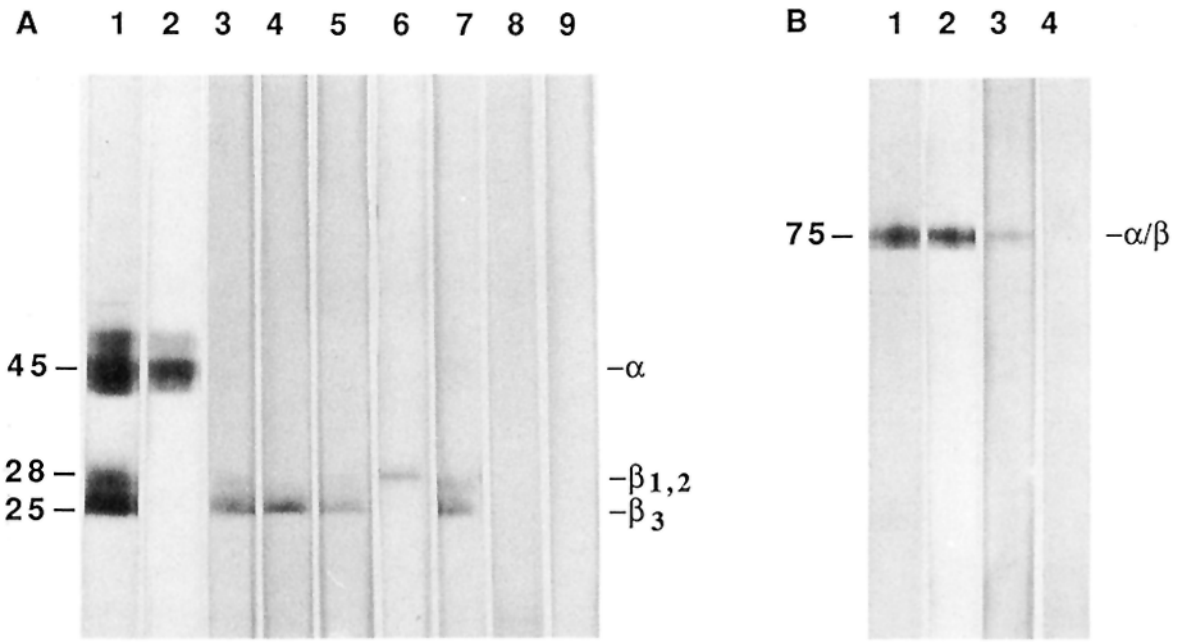

Figure 1. PH-30 is an SDS-resistant complex of two immunologically distinct proteins. Immunoblot analysis of nonreduced epididymal sperm extracts that were either boiled in 2% SDS (*A*) or treated at RT with 0.5% SDS (*B*) before electrophoresis. The primary antibodies used in *A* were: rabbit PH-30 polyclonal antiserum (lane *1*), affinity-purified α-specific antibodies (lane *2*), affinity-purified β-specific antibodies (lane *3*), mouse monoclonal antibodies mAB PH-30 (lane *4*), and mAB PH-1 (lane *5*). Lanes *6* and *7* were probed with affinity-purified β-specific antibodies and represent two fractions of an immunodepletion experiment: PH-30 protein that does not bind to mAB PH-30 (lane *6*) and PH-30 protein that does bind to mAB PH-30 (lane *7*). As controls, an epididymal sperm extract was probed with nonspecific mouse IgG (lane *8*) or with preimmune rabbit IgG (lane *9*). The primary antibodies in *B*, lanes *1–3*, were the same as in *A*, lanes *1–3*; preimmune rabbit antiserum was used as control in *B*, lane *4*.

blots of sperm cell extracts. Since the polyclonal antibody does not react well with reduced PH-30 protein, all immunoblots presented here are of nonreduced cell extracts.

When used to probe an extract of epididymal sperm, the unfractionated polyclonal antiserum recognized both the α chain and the β chain of PH-30 (Fig. 1 *A*, lane *1*). The apparent molecular masses of these subunits were ~45 and ~27 kD, respectively. These values differ slightly from those reported previously (14), since they were calculated with reference to prestained molecular mass markers. Affinity-purified antibodies against the α chain reacted only with the α chain (Fig. 1 *A*, lane *2*); affinity-purified antibodies against the β chain reacted only with the β chain (Fig. 1 *A*, lane *3*). The two chains are therefore immunologically distinct. Both the fusion inhibitory monoclonal antibody, mAb PH-30, as well as the control monoclonal antibody, mAB PH-1, coimmunoprecipitate α and β from nondenaturing detergent cell lysates (14). Immunoblot analysis with mAb PH-30 and mAb PH-1 revealed that both of these monoclonal antibodies react with the β subunit of PH-30 (Fig. 1 *A*, lanes *4* and *5*, respectively). It is therefore plausible that mAb PH-30 inhibits fusion by binding to a functionally important epitope of the β chain of PH-30.

Multiple β Chains Exist on Mature Sperm

Under nonreducing conditions the α chain migrates as a broad band, and the β chain migrates as a doublet. However, if purified PH-30 is analyzed by SDS-PAGE under reducing conditions, the α chain runs as a single sharp band. The β chain, however, retains its appearance as a doublet even under reducing conditions (not shown). On nonreducing SDS-PAGE the β doublet appears as a strong band of apparent molecular mass 25 kD and a weaker band of apparent molecular mass 28 kD (Fig. 1 *A*, lanes *3–5*). The upper β band (28 kD) is apparently composed of two species. To show this, a sperm extract was immunodepleted of mAb PH-30 reactive material as described in Materials and Methods. The mAb PH-30 immunodepleted extract was then incubated with Sepharose beads coupled with IgG from the rabbit polyclonal antiserum. The bound material was subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with affinity-purified β-specific polyclonal antibodies. This procedure revealed a single band, which we will call β_1 (M_r 28 kD, Fig. 1 *A*, lane *6*). The β_1 band comigrated with (or ran slightly slower than) the upper of the two bands of material that react with mAb PH-30, which we will term β_2 (M_r 27–28 kD, Fig. 1 *A*, lane *7*). The upper band of what appears as a doublet when whole sperm extracts are blotted with the polyclonal antiserum will therefore be referred to as $\beta_{1,2}$ (Fig. 1 *A*, lanes *1* and *3*). We term the lower band reactive with mAb PH-30, β_3 (M_r 25 kD, Fig. 1 *A*, lane *7*). Thus, on mature epididymal sperm there appear to be at least three β subunits, β_1, β_2, and β_3. All three β subunits are recognized by polyclonal anti–PH-30 antibodies on immunoblots (Fig. 1 *A*, lanes *6* and *7*). Preimmune rabbit serum (Fig. 1 *A*, lane *8*) and nonspecific mouse IgG (Fig. 1 *A*, lane *9*) did not bind to any material comigrating with the PH-30 bands. At present the functional significance of the various β species found on mature epididymal sperm is not clear.

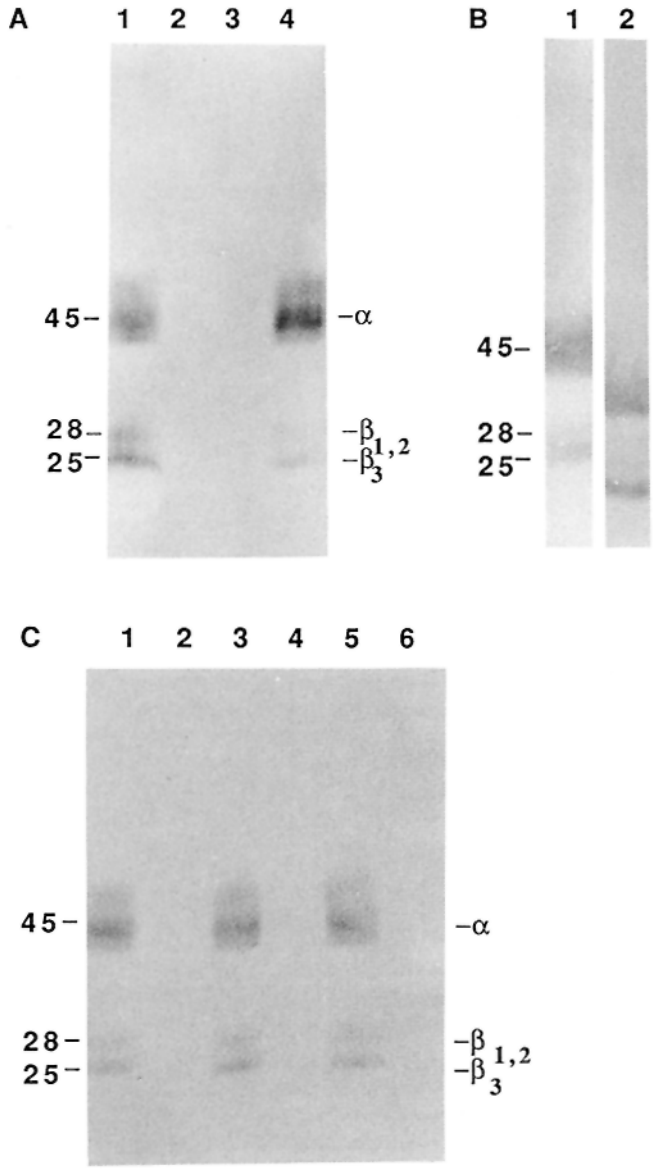

Figure 2. PH-30 is an integral membrane glycoprotein. All samples were run on nonreducing SDS-PAGE and immunoblotted with rabbit polyclonal anti-PH-30 antiserum. (*A*) Binding of PH-30 from mature epididymal sperm extracts to Con A-Sepharose CL-4B. Material bound (lane *1*) or in the supernatant (lane *2*) after incubation with Con A-Sepharose CL-4B beads. Material bound (lane *3*) or in the supernatant (lane *4*) after incubation with Sepharose CL-4B beads. (*B*) Treatment with endoglycosidase F. Epididymal sperm extracts were mock digested (lane *1*) or digested (lane *2*) with endoglycosidase F as described in Materials and Methods. (*C*) Membrane extractability of PH-30 from mature epididymal sperm. Mature epididymal sperm were treated with high salt (0.5 M KCl, lanes *1* and *2*), moderate salt (0.1 M KCl, lanes *3* and *4*), or with alkali (0.1 M $NaHCO_3$, pH 11.5, lanes *5* and *6*) for 30 min on ice and then pelleted at 200,000 *g* for 30 min in a model TLA 100.3 rotor (Beckman Instruments, Inc., Palo Alto, CA). Equal volumes of pellets (lanes *1*, *3*, and *5*) and supernatants (lanes *2*, *4*, and *6*) were analyzed for PH-30 protein as described above.

PH-30 Is a Tight Complex of the α and β Chains

Since both monoclonal antibodies, mAB PH-30 and mAB PH-1, coimmunoprecipitate the α and β chains of PH-30 (14), but bind only to the β chain on immunoblots (Fig. 1 *A*, lanes *3* and *4*), it appears that α and β form a complex. Further evidence that this is, indeed, the case was obtained by analyzing extracts of epididymal sperm that were treated with SDS at RT (rather than at 100°C) before electrophoresis on nonreducing SDS-PAGE. When a sample treated with 0.5% SDS at RT was subjected to SDS-PAGE and then blotted with the polyclonal PH-30 antiserum, the only band detected was of apparent molecular mass 75 kD (Fig. 1 *B*, lane *1*). Affinity-purified antibodies against both the α chain and the β chain recognized the 75-kD band (Fig. 1 *B*, lane *2* and *3*, respectively). The 75-kD band was not recognized by the rabbit preimmune antiserum (Fig. 1 *B*, lane *4*). The 75-kD band thus represents a complex of the α and β chains. This complex was completely stable in 0.5% SDS at RT and largely stable (≥75%) in SDS concentrations as high as 4% at RT. PH-30 of epididymal sperm thus appears to be a tight but not covalently linked complex of two distinct subunits, α and β.

PH-30 Is an Integral Membrane Glycoprotein

To determine whether the α and β chains of PH-30 are glycoproteins, mature epididymal sperm extracts were incubated with Con A-Sepharose. The bound glycoproteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the polyclonal antiserum against PH-30. Both the α and the β chain bound quantitatively to Con A-Sepharose CL-4B (Fig. 2 *A*, lane *1*). No PH-30 remained in the Con A-Sepharose CL-4B supernatant (Fig. 2 *A*, lane *2*). Control Sepharose CL-4B beads did not bind PH-30 (Fig. 2 *A*, lane *3*), and therefore both chains remained in the Sepharose CL-4B supernatant (Fig. 2 *A*, lane *4*). These data suggest that at least one subunit of PH-30 is a glycoprotein.

To determine which chain(s) of PH-30 contain N-linked carbohydrate moieties, a mature epididymal sperm extract was treated with endoglycosidase F, subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the polyclonal PH-30 antiserum. After endoglycosidase F treatment, the mature α and β chains of PH-30, which normally migrate at 45 and 25–28 kD, respectively (Fig. 2 *B*, lane *1*), migrated with molecular masses of 38 and 21–24 kD, respectively (Fig. 2 *B*, lane *2*). Therefore, both the α and the β chain of PH-30 contain N-linked carbohydrates.

To assess whether PH-30 is an integral membrane protein, its extractability from mature epididymal sperm by high salt (0.5 M KCl) or alkali (0.1 M $NaHCO_3$, pH 11.5) was monitored (6). Sperm were incubated for 1 h on ice with either high salt or alkali and then pelleted at 200,000 *g* for 30 min. Pellets of the extractions with 0.5 M KCl, 0.1 M KCl (as control), and 0.1 M $NaHCO_3$ were run on SDS-PAGE (Fig. 2 *C*, lanes *1*, *3*, and *5*, respectively) next to their corresponding supernatants (Fig. 2 *C*, lanes *2*, *4*, and *6*, respectively), transferred to nitrocellulose, and immunoblotted with the polyclonal PH-30 antiserum. In each case, PH-30 was quantitatively recovered in the membrane pellet fraction; no PH-30 was detected in the respective supernatants. PH-30 is therefore resistant to extraction by either high salt or alkaline conditions. These results, together with the binding of PH-30 to Con A-Sepharose and the removal of N-linked carbohydrate chains after endoglycosidase F digestion, show that PH-30 is an integral membrane glycoprotein.

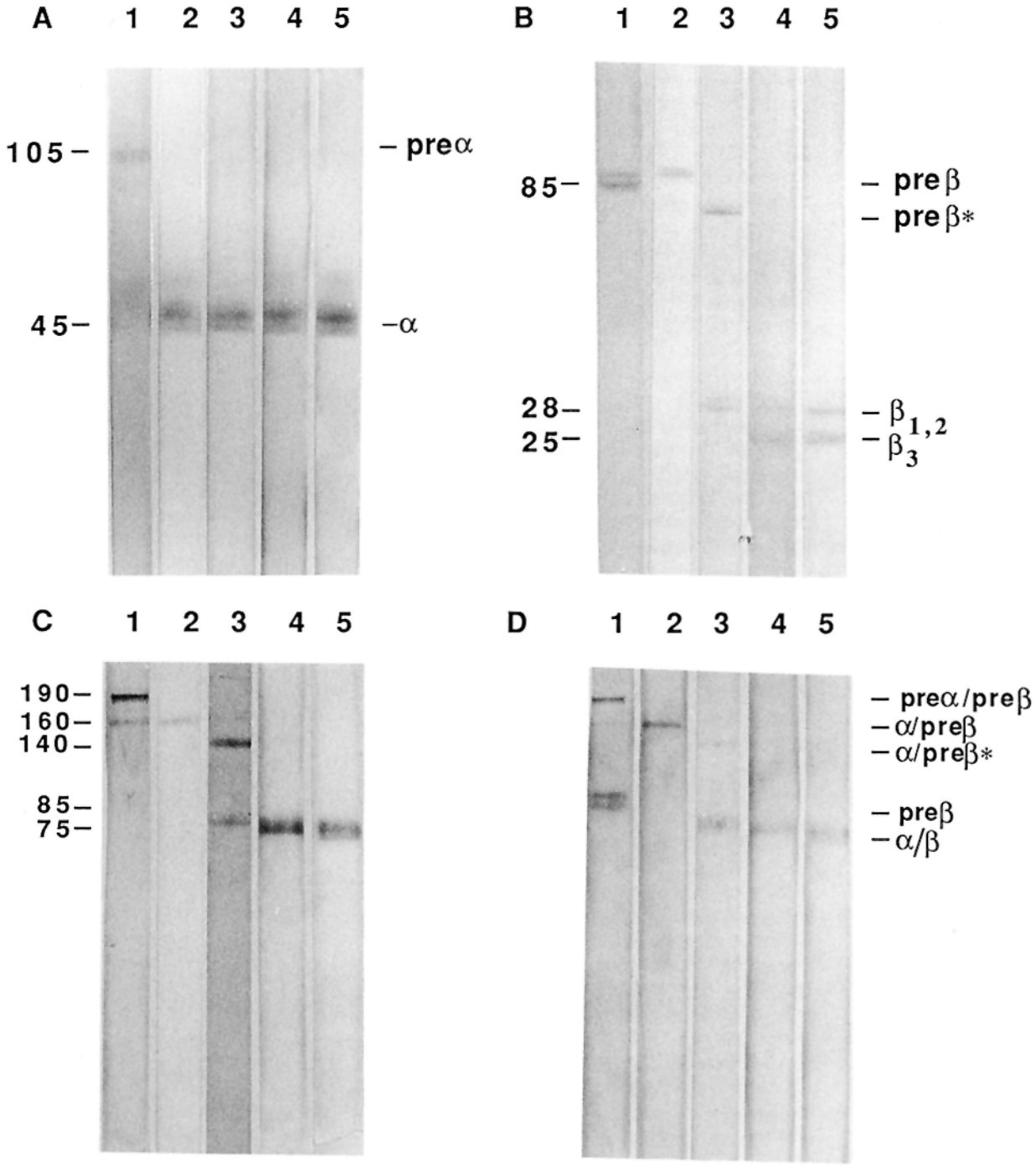

Figure 3. Both subunits of PH-30 are made as larger precursors. Cell extracts from sperm at different developmental stages were run on nonreducing SDS-PAGE and immunoblotted with affinity-purified α-specific antibodies (*A* and *C*) and affinity-purified β-specific antibodies (*B* and *D*). Extracts in *A* and *B* were boiled in 2% SDS; extracts in *C* and *D* were treated at RT with 0.5% SDS. Lane *1*, testicular cells; lane *2*, testicular sperm; lane *3*, distal corpus epididymal sperm; lane *4*, proximal cauda epididymal sperm; lane *5*, distal cauda epididymal sperm.

The PH-30 α and β Chains Are Made as Larger Precursors

We next determined the molecular forms of the α and β subunits on sperm at different developmental stages. Two populations of testicular cells were analyzed. The first was a pool of all testicular spermatogenic cells that we refer to here as testicular cells. This pool has been largely depleted of testicular sperm. The second consisted of isolated testicular sperm, the most fully developed cells in the testis. In addition we analyzed sperm from three stages of epididymal maturation: the distal corpus, the proximal cauda, and the distal cauda epididymis. In the three rodent species analyzed to date (rat, mouse, and hamster), fertilization competence is first observed in the distal corpus epididymis and then increases dramatically in the proximal cauda epididymis (3,

11). Cell extracts were separated by SDS-PAGE and subjected to immunoblot analysis with affinity-purified antibodies against the individual α (Fig. 3, *A* and *C*) or β subunits (Fig. 3, *B* and *D*). One set of extracts was boiled in 2% SDS to detect the individual subunits (Fig. 3, *A* and *B*), whereas another set was treated with 0.5% SDS at RT to resolve complexes of α and β (Fig. 3, *C* and *D*; see also Fig. 1 *B*).

As seen in Fig. 3 *A*, α was present on testicular cells predominantly as a precursor, pre α, of molecular mass 105 kD although some mature α was also visible (Fig. 3 *A*, lane *1*). However, on testicular sperm (Fig. 3 *A*, lane *2*), only the mature α chain (45 kD) was present. This implies that the pre α chain (105 kD) is processed to mature α (45 kD) in the testis before the testicular sperm stage. The size of the α chain does not change upon continued passage through the distal corpus epididymis (Fig. 3 *A*, lane *3*) or the proximal (Fig. 3 *A*, lane *4*) or distal cauda epididymis (Fig. 3 *A*, lane *5*).

The maturation pattern of β was quite different from that of α. On testicular cells antibodies specific for β detected a precursor, pre β, that appeared as a doublet with an average molecular mass of ~85 kD (Fig. 3 *B*, lane *1*). On testicular sperm, β antibodies detected a single band migrating at 88 kD, slightly higher than the doublet on testicular cells (Fig. 3 *B*, lane *2*). Unlike α, however, β did not appear to be proteolytically processed in the testis. Rather, it was observed to be processed separately and later during passage of sperm through the epididymis. In the distal corpus epididymis (Fig. 3 *B*, lane *3*), β antibodies detected a 75-kD band that we will refer to as pre β*, and a band at 28 kD corresponding to β_1 and/or β_2. Mature β_3 (25 kD), in addition to β_1/β_2 (28 kD), was found only in the proximal and distal cauda epididymis (Fig. 3 *B*, lanes *4* and *5*, respectively).

As shown in Fig. 1 *B*, complexes of α and β can be resolved when extracts of distal cauda epididymal sperm are treated with 0.5% SDS at RT and run on nonreducing SDS-PAGE. Analysis of nonboiled and nonreduced samples of testicular cells, testicular sperm, and sperm from the epididymis (distal corpus, proximal cauda, distal cauda) revealed complexes of the respective PH-30 precursors. These precursors were recognized by both α and β-specific antibodies (Fig. 3, *C* and *D*, respectively).

On unboiled testicular cells, α and β antibodies detected bands of molecular mass 190 and 160 kD (Fig. 3, *C* and *D*, lane *1*). The 190- and 160-kD bands, therefore, most likely represent complexes of the PH-30 α and β precursors. The presence of uncomplexed pre β on testicular cells (Fig. 3 *D*, lane *1*) suggests that pre β might be present in excess of pre α.

On unboiled testicular sperm, the α and β antibodies detected only one band at 160 kD (Fig. 3, *C* and *D*, lane *2*). We established above that testicular sperm contain only pre β and mature α (Fig. 3, *A* and *B*, lane *2*). Thus, the 160-kD band represents a complex of α and pre β. We therefore interpret the 190-kD band seen on nonboiled testicular cells to be a complex of pre α and pre β.

On unboiled extracts of sperm from the distal corpus epididymis two bands, at 140 and 75 kD, were recognized by affinity-purified α and β antibodies (Fig. 3, *C* and *D*, lane *3*). Sperm from the proximal and the distal cauda epididymis contained only one band at 75 kD (Fig. 3, *C* and *D*, lanes *4* and *5*, respectively; see also Fig. 1 *B*). Given that the 75-kD band represents a complex of mature α and β, the band at 140 kD on distal corpus sperm must be a complex of pre β* with mature α. Collectively these results illustrate that both the α and the β chain of PH-30 are made as larger precursors. Complexes of pre α and pre β exist at early developmental stages. Proteolytic processing of the α and β chains occurs independently at different stages of sperm maturation.

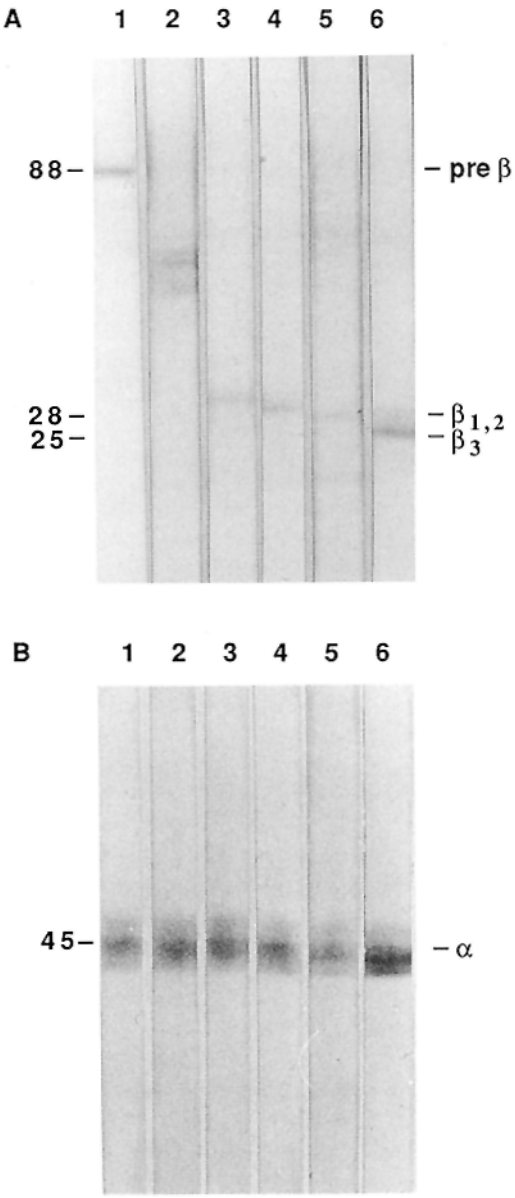

Figure 4. Proteolytic processing of PH-30 on testicular sperm in vitro. Extracts of testicular sperm or distal cauda epididymal sperm were boiled in 2% SDS, run on nonreducing SDS-PAGE, and probed with β-specific (*A*) or α-specific antibodies (*B*). Testicular sperm were mock digested (lane *1*) or treated with 20 μg/ml proteinase K (lane *2*), chymotrypsin (lane *3*), TPCK-treated trypsin (lane *4*), or 100 μg/ml dispase (lane *5*) for 2 h at 37°C. An extract of untreated distal cauda epididymal sperm is shown in lane *6*.

In Vitro Proteolysis of Testicular Sperm Leads to Processing of PH-30

We next wished to determine whether the precursors of PH-30 could be converted to their mature forms by proteolysis

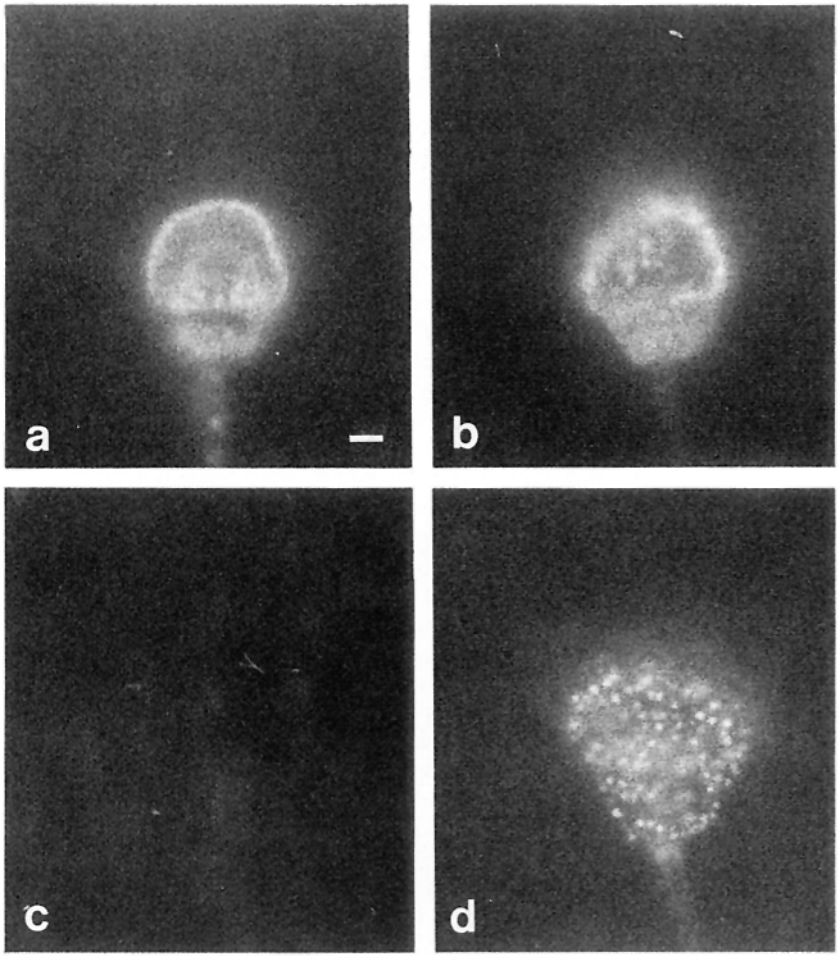

Figure 5. In vitro trypsinization of testicular sperm unmasks the mAB PH-30 epitope. Immunofluorescence micrographs of mock digested testicular sperm (*A* and *C*), and testicular sperm treated with 100 μg/ml TPCK-treated trypsin for 1 h on ice (*B* and *D*). Samples in *A* and *B* were stained with the rabbit PH-30 polyclonal antiserum; samples in *C* and *D* were stained with mAB PH-30. Cells shown are representative of the vast majority of the cells viewed. Primary antibodies were added to live cells on ice. Bar, 2 μm.

in vitro. Untreated testicular sperm (Fig. 4, *A* and *B*, lane *1*) were run on SDS-PAGE next to testicular sperm that had been treated at 37°C with either proteinase K, chymotrypsin, trypsin, or the metalloendoprotease, dispase (Fig. 4, *A* and *B*, lanes *2–5*, respectively). The protease-treated testicular sperm samples were then subjected to immunoblot analysis with β- and α-specific antibodies (Fig. 4, *A* and *B*, respectively).

All four proteases tested affected the size of pre β found in testicular sperm extracts. Treatment with proteinase K yielded bands at ~47 kD (Fig. 4 *A*, lane *2*). Treatment with chymotrypsin almost quantitatively converted the pre β chain to a 30-kD band (Fig. 4 *A*, lane *3*) that migrated slightly above $\beta_{1,2}$ on mature distal cauda epididymal sperm (Fig. 4 *A*, lane *6*). Treatment with trypsin (Fig. 4 *A*, lane *4*) generated a 28-kD band comigrating with $\beta_{1,2}$ on epididymal sperm. Finally, treatment with the metalloendoprotease dispase (Fig. 4 *A*, lane *5*) converted pre β to a 27-kD band migrating between $\beta_{1,2}$ (28 kD) and β_3 (25 kD) on mature sperm. Thus, pre β of testicular sperm can be converted into lower molecular mass species by proteolysis. Treatment with chymotrypsin, trypsin, or dispase resulted in products that were similar in size to the β chains found on mature epididymal sperm. A 20-fold increase in concentration of these three proteases did not lead to further processing or degradation of β (not shown). In contrast to their effects on pre β, treatment of testicular sperm with proteinase K, chymotrypsin, trypsin, or dispase had no effect on the α subunit of PH-30 (Fig. 4 *B*, lanes *2–5*).

Treatment of testicular cells with proteinase K led to the disappearance of the pre α band and reduced (by ~10 kD) the molecular mass of >50% of the pre β molecules detected on immunoblots. Treatment of testicular cells with chymotrypsin or trypsin, however, had no effect on pre α or pre β (not shown). These data indicate that, on testicular cells, pre α and at least the majority of pre β are accessible to external protease. However, pre β found on testicular cells (as opposed to that on testicular sperm) could not be converted by in vitro proteolysis to species that migrate in the vicinity of the β bands found on mature epididymal sperm. Correct processing of pre α to α may therefore be a prerequisite for correct processing of pre β.

Protease Treatment of Testicular Sperm Exposes Epitopes on the PH-30 Protein

Immunofluorescence experiments have revealed that neither the fusion inhibitory monoclonal antibody (mAb PH-30) nor the noninhibitory control monoclonal antibody (mAb PH-1) reacts with native PH-30 on testicular sperm whereas both monoclonal antibodies react with native PH-30 on mature epididymal sperm (14). The polyclonal antibody raised against PH-30 protein does bind to live untreated testicular sperm (13, and see below). We therefore tested whether treatment of testicular sperm with proteases that cleave pre β in vitro

Table I. Binding of the Monoclonal Antibodies mAb PH-30 and mAb PH-1 to Testicular Sperm After Treatment with Different Proteases

Protease	Immunofluorescence	
	mAb PH-30	mAb PH-1
None (mock digestion)	–	–
Proteinase K	++	++
Chymotrypsin	++	++
Trypsin	++	++
Dispase	+	++

Testicular sperm were either mock digested or treated with 100 μg/ml of proteinase K, chymotrypsin, or trypsin, or with 500 μg/ml dispase for 1 h on ice. Samples were stained with the indicated antibodies as described in Materials and Methods. The results were the same in three separate experiments. A minus indicates no staining. A double plus indicates strong, and a single plus indicates weaker staining. In all positive samples (+, ++), punctate staining was seen over the whole head of virtually all sperm cells.

(proteinase K, chymotrypsin, trypsin, and dispase, see Fig. 4) unmask the epitopes seen by mAb PH-30 and mAb PH-1.

Fig. 5 shows the results of a typical experiment in which testicular sperm were treated with trypsin on ice. Immunofluorescence photographs were taken of testicular sperm that were mock digested (Fig. 5, *A* and *C*) or trypsinized for 1 h on ice (*B* and *D*, 100 μg/ml trypsin). Fig. 5, *A* and *B* were stained with the polyclonal PH-30 antiserum, whereas *C* and *D* were stained with the monoclonal antibody mAB PH-30. Staining with the polyclonal antibody clearly revealed the presence of PH-30 on the whole head of untreated (Fig. 5 *A*) and on trypsin-treated (*B*) testicular sperm. The staining pattern with mAb PH-30 was different. As shown previously (13, 14) mAB PH-30 does not recognize untreated testicular sperm (Fig. 5 *C*). However, after trypsinization mAb PH-30 bound to testicular sperm (Fig. 5 *D*). The staining pattern shown is representative of virtually all of the trypsin-treated testicular sperm analyzed. The staining pattern in Fig. 5 *D* resembled that seen with the polyclonal antibody (*B*) in that staining was seen over the whole head. At present we cannot explain why the monoclonal antibody gave a punctate staining (Fig. 5 *D*) while the polyclonal antibodies gave a more diffuse staining pattern (*B*).

Results obtained with the other proteases and with both mAB PH-30 and mAB PH-1 are shown in Table I. Whereas the polyclonal antibody bound to PH-30 on testicular sperm, neither mAB PH-30 nor mAB PH-1 bound to testicular sperm. However, treatment of testicular sperm with either proteinase K, chymotrypsin, trypsin, or dispase (on ice) allowed binding of both mAb PH-30 and mAb PH-1. As described above (Fig. 5), in all cases following protease treatment staining was seen over the whole sperm head. Staining with both monoclonal antibodies was punctate in appearance and visible on virtually all protease treated testicular sperm. The staining intensity was roughly the same in all samples, with the exception of dispase-treated testicular sperm, which were stained relatively less intensely with mAB PH-30. Collectively these findings suggest that in vitro treatment of testicular sperm with several proteases leads to processing of pre β. Both in vivo and in vitro, processing unmasks at least two new epitopes on PH-30. One of the newly exposed epitopes appears to be important for sperm–egg fusion (14).

Discussion

The fusion reactions that occur between enveloped viruses and their host cells are exoplasmic events. Although viral membrane fusion proteins are well characterized (15, 18), very little is known about proteins mediating exoplasmic fusion events that occur between cellular membranes (19). In this paper we have further characterized PH-30, a protein that has been shown previously to play a role in the exoplasmic fusion event that occurs between the plasma membranes of guinea pig sperm and egg (14).

On mature epididymal sperm, PH-30 exists as a tight complex of two immunologically distinct subunits, α and β. Affinity-purified polyclonal antibodies against the α subunit react only with α. Affinity-purified polyclonal antibodies and two monoclonal antibodies, that we show here to be directed against the β subunit, react only with β. These findings confirm earlier results showing different polypeptide patterns after digestion of α and β with V8 protease (14). Analysis with several antibodies directed against the β subunit indicates that there are at least three different forms of β (β_1, β_2, and β_3) on mature epididymal sperm. The functional significance of the multiple β bands is not yet apparent. The PH-30 α and β chains form a complex that is resistant to treatment with SDS. This finding explains the ability of monoclonal antibodies directed against the β subunit to coimmunoprecipitate both α and β (14). The PH-30 α/β complex found on mature epididymal sperm behaves as an integral membrane glycoprotein. It is not extracted from the sperm membrane with high salt or alkali, it binds quantitatively to Con A–Sepharose, and the apparent molecular masses of both of its subunits are reduced upon treatment with endoglycosidase F.

Both subunits of PH-30 are made as larger precursors, pre α and pre β. Pre α and pre β are processed at different developmental stages and complexes of α and β exist throughout sperm maturation. The susceptibility of pre α and the majority of pre β to proteinase K indicates that both precursors are present on the surface of testicular cells. By the time the cells have matured to become testicular sperm, pre α has been processed to its mature size and does not undergo further detectable proteolytic modifications. Pre β, on the other hand, is present on the surface of both immature testicular cells as well as testicular sperm. Proteolytic processing of pre β occurs during passage through the epididymis and appears to occur in at least two steps. At an early stage of epididymal maturation (in the distal corpus epididymis), two bands of decreased molecular mass are seen, pre-β* and a band that comigrates with $\beta_{1,2}$ of mature epididymal sperm. Final processing of pre β, which leads to the appearance of β_3, occurs in, or in transit to, the proximal cauda epididymis.

Processing of PH-30 thus appears to be under tight developmental regulation. In rodents, sperm become fully fertilization competent when they reach the proximal cauda epididymis. Acquisition of fertilization competence is thought to be due to biochemical alterations of sperm surface proteins occurring during epididymal passage (4). Our results strongly suggest that proteolysis plays an active role in the maturation of functionally important sperm surface proteins. The final processing step that we observe for the β subunit of PH-30 occurs in, or in transit to, the proximal cauda epididymis. Coincident with this cleavage, the epitope recog-

nized by the fusion-inhibitory monoclonal antibody is revealed. Concomitantly, PH-30 localizes (Phelps, B. M., D. E. Koppel, P. Primakoff, and D. G. Myles, manuscript in preparation) to the posterior head, the region where fusion with the egg is thought to be initiated and/or continued (14, 22), and sperm become fertilization competent. Therefore, it is tempting to speculate that the final processing event that generates β_3 on epididymal sperm may activate PH-30 and may therefore be responsible, at least in part, for the acquisition of sperm fertilization competence.

Interestingly, we found that in vitro proteolysis of live testicular sperm with chymotrypsin, trypsin, or dispase cleaves pre β into distinct polypeptides of apparent molecular masses similar to those of $\beta_{1,2}$ and β_3 found on mature epididymal sperm. An attractive interpretation of these results is that the β subunit of PH-30 on testicular sperm may have two domains, a precursor domain that is sensitive to proteolysis and a second, perhaps functional, domain that is resistant to further proteolysis. Given that sperm may be exposed to a proteolytic environment in the epididymis and again following the acrosome reaction, it is not surprising that mature PH-30, which functions in fusion with the egg plasma membrane appears to be at least relatively protease resistant.

The monoclonal antibodies mAb PH-30 (which inhibits fusion) and mAb PH-1 (which does not inhibit fusion) do not bind to testicular sperm (14). However, treatment of testicular sperm with either proteinase K, chymotrypsin, trypsin, or dispase exposes the sites reactive with both monoclonal antibodies. Exposure of these epitopes could be due to removal of the precursor domain of pre β, to more complex conformational changes in the structure of β, and/or to modification or removal of the glycocalyx that covers the sperm surface (5). In this respect it is interesting that trypsinization of testicular sperm exposes an epitope on at least one other surface protein, AH-40 (Phelps, B. M., D. E. Koppel, P. Primakoff, and D. G. Myles, manuscript in preparation).

In the in vitro proteolysis experiments presented in Fig. 5, testicular sperm were treated with high concentrations of protease (100 μg/ml trypsin, chymotrypsin, proteinase K; 500 μg/ml dispase) for 1 h on ice. Under these conditions, the newly exposed epitopes recognized by mAbs PH-30 and PH-1 were found over the entire sperm head. Conversely, when testicular sperm were treated with a fivefold lower concentration of trypsin (20 μg/ml) for 5 min at RT followed by a 1-h incubation at RT, the epitope recognized by mAb PH-30 was found only in the posterior head region (Phelps, B. M., D. E. Koppel, P. Primakoff, and D. G. Myles, manuscript in preparation), where it is localized on mature epididymal sperm (14). The explanation for this difference requires further experimentation. Localization may be impeded at low temperature due to interference with an energy dependent process or to reduced lateral diffusion of PH-30. Alternatively, the harsher proteolysis conditions used here may have destroyed an auxiliary protein(s) involved in the localization process. In any event, the data shown here are consistent with the observation that exposure of the epitopes recognized by mAb PH-30 and mAb PH-1 precedes localization of PH-30 to the posterior head (13). PH-30 is present at high surface density ($\geq 5 \times 10^3$ α/β dimers per μm^2) in the posterior head of mature sperm (Primakoff, P., unpublished data). Localization of PH-30 may therefore help establish a critical concentration required for its function.

Since PH-30 is involved in an exoplasmic fusion event, we asked at the outset whether PH-30 shares features with viral membrane fusion proteins. Our results show that PH-30 does display properties similar to those of viral membrane fusion proteins. It is an integral membrane glycoprotein, it is composed of two tightly associated subunits, and both subunits are made as precursors. Strictly speaking, the processing of PH-30 from two separate precursors is more complex than that of any simple viral fusion protein analyzed to date. Furthermore, we are fully aware that the similarities of PH-30 and viral fusion proteins may be completely coincidental as these features are shared by other cell surface proteins. However, the basic biochemical features of PH-30 established here are consistent with what is known about exoplasmic membrane fusion proteins. Moreover, the finding that the PH-30 subunits are made as precursors suggests an important developmental regulation of PH-30 function. The correlation between the processing of the β subunit of PH-30 and the acquisition of fertilization competence strengthens the idea that PH-30 plays an important role in the fusion event between sperm and egg.

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