Biochemical Characterization of a Glycosylphosphatidylinositol-Linked Hyaluronidase on Mouse Sperm†

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ABSTRACT: On the basis of DNA homology to bee venom hyaluronidase, it was recently suggested that the GPI-linked mammalian sperm antigen, PH-20, may function as a cell surface hyaluronidase [Gmachl, M., & Kreil, G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 3569−3573]. We have quantified the activity of the soluble acrosomal hyaluronidase of mouse sperm and further demonstrate the existence of a membrane-bound hyaluronidase, detected on both acrosome-intact and acrosome-reacted mouse sperm, distinct from the soluble form of the enzyme. The membrane-bound hyaluronidase was specifically released by PI-PLC, indicating that it is GPI linked. Acrosome-intact and acrosome-reacted sperm released several polypeptides (68, 44, 39, 34, 17, and 15 kDa) when treated with PI-PLC. In addition, GPI-linked polypeptides unique to acrosome-intact or to acrosome-reacted sperm were identified. Fractionation of the PI-PLC-released components from acrosome-reacted sperm using size exclusion chromatography revealed a single peak of hyaluronidase activity which comigrates with a 68 kDa GPI-linked protein present in these fractions. Taken together, these data demonstrate the existence of at least two isoforms of hyaluronidase: a soluble form within the acrosomal vesicle which is released during acrosomal exocytosis and a GPI-linked form which is present on the surface of both acrosome-intact and acrosome-reacted sperm. Both forms may be necessary for successful penetration of the extracellular vestments that surround the egg prior to fertilization.

Before successful gamete fusion can occur, mammalian sperm must first pass through two structures surrounding the egg: the cumulus complex and the zona pellucida. The major extracellular component of the cumulus complex which also permeates the zona pellucida and the perivitelline space is hyaluronic acid (Ball *et al.*, 1982; Talbot, 1984; Dandekar & Talbot, 1992), a large polymer of alternating *N*-acetylglucosamine and glucuronic acid residues. It has been proposed that to successfully negotiate the viscous intercellular milieu presented by hyaluronic acid in the cumulus matrix a sperm-associated hyaluronidase would hydrolyze the hyaluronic acid en route to the zona pellucida and the egg plasma membrane (Yanagamachi, 1981). Indeed, a great deal of research has been devoted to the characterization of a soluble hyaluronidase which is a component of the acrosomal vesicle (Hechter & Hadidian, 1947; Swyer, 1947; Borders & Raferty, 1968; Rogers & Morton, 1973; Brown, 1975; Gadella *et al.*, 1991) and which would be released to the extracellular environment after exocytosis of acrosomal contents (the acrosome reaction) prior to sperm−egg plasma membrane fusion.

Paradoxically, many mammalian sperm, including those from mouse, undergo the acrosome reaction at the zona pellucida subsequent to penetration of the cumulus complex (Saling *et al.*, 1979), suggesting that acrosomal hyaluronidase may serve some role other than penetration of the cumulus matrix. In addition to the soluble enzyme, previous reports have indicated the presence of a sperm surface associated hyaluronidase (Brown, 1975) that may be released into the surroundings prior to fertilization (Rogers & Morton, 1973) or may be due to leakage from the acrosomal vesicle (Talbot & Franklin, 1974a). However, more recent data have demonstrated the presence of a glycosylphosphatidylinositol-linked (GPI[1]-linked) sperm membrane protein which shares significant DNA sequence homology with bee venom hyaluronidase (Gmachl & Kreil, 1993). This GPI-linked protein was initially identified on the posterior head of acrosome-intact guinea pig sperm (Phelps *et al.*, 1988; Lathrop *et al.*, 1990) and was suggested to be involved in adhesion to the zona pellucida (Primakoff *et al.*, 1985; Myles *et al.*, 1987). Subsequently, a cloned PH-20 homologue isolated from a human testis cDNA library exhibited hyaluronidase activity (Gmachl *et al.*, 1993). Additional reports indicate that PH-20 possesses hyaluronidase activity and suggest that the hyaluronidase and zona binding activities of this protein may represent two distinct functional roles in fertilization (Hunnicutt *et al.*, 1993; Lin *et al.*, 1994). However, in the mouse no substantial biochemical characterizations have been performed on any GPI-linked mouse sperm surface proteins. In particular, we wondered whether mouse sperm contain a GPI-linked hyaluronidase and whether it is present on acrosome-intact or acrosome-reacted cells. Knowledge about the enzymatic activity and localization of this enzyme would reveal important insights into its role during fertilization.

In this paper we demonstrate that, in addition to the soluble acrosomal form of the enzyme, mouse sperm possess a GPI-linked hyaluronidase on both acrosome-intact and acrosome-

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[1] Abbreviations: BSA, bovine serum albumin; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

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reacted sperm. Treatment of acrosome-reacted sperm with PI-PLC specifically released hyaluronidase activity into the supernatant. Multiple polypeptides are released by PI-PLC treatment of both acrosome-intact and acrosome-reacted sperm, but hyaluronidase activity was restricted to one component as demonstrated by a single peak of activity from size exclusion chromatography. The presence of the mouse sperm surface GPI-linked hyaluronidase on both acrosome-intact and -reacted cells suggests that it may play multiple roles in fertilization.

MATERIALS AND METHODS

Materials. The calcium ionophore A23187 was purchased from Molecular Probes (Eugene, OR). ^{125}I ($Na^{125}I$, pH 8−10) was purchased from New England Nuclear (Boston, MA). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* was purchased from Boehringer-Mannheim (Indianapolis, IN). Coomassie Blue-G250 was purchased from Kodak (Rochester, NY); BSA, hyaluronic acid, hyaluronidase, sodium lactate, sodium pyruvate, soybean trypsin inhibitor, apigenin, and gel filtration standards were purchased from Sigma (St. Louis, MO). Acrylamide and SDS−PAGE protein standards were purchased from Bio-Rad (Hercules, CA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Sperm Preparation. Epididymal sperm were collected from 12−15 week old Swiss−Webster CD-1 mice (Harlan Sprague-Dawley, San Diego, CA) by removing the epididymides, mincing the tissue in Whittingham's buffer (100 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM glucose, 2.6 mM KCl, 1.56 mM Na_2HPO_4, 0.5 mM sodium pyruvate, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM sodium lactate; Whittingham, 1971), gently agitating for 10 min to release sperm, and separating sperm from epididymal tissue by centrifugation (500*g*, 1 min). Sperm were concentrated by centrifugation for 10−15 min at 500*g* and resuspended either in Whittingham's without BSA (NC medium) and used immediately for experiments using noncapacitated sperm (Figures 4−7) or, alternatively, in Whittingham's containing 30 mg/mL BSA (C medium) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 2 h (Figures 1−3). Capacitated sperm were pelleted twice in NC medium by centrifugation (750*g*, 2 min) to remove BSA prior to experimental manipulations. Acrosome-reacted sperm were obtained by addition of the calcium ionophore A23187 (final concentration 10 μM for capacitated sperm, or 20 μM for noncapacitated sperm) for 30 min following the normal 2-h capacitation (Figures 1b, 2b, 3) or for 45−60 min after collection for noncapacitated sperm (Figures 4−7). Acrosome-reacted sperm were gently pelleted at least three times (750*g*, 2 min) until there was no detectable hyaluronidase activity in the medium. Sperm cell concentration was determined using a standard hemacytometer. Samples were collected before and after experimental treatments to determine acrosomal status as described below. Typically, sperm were 85%−95% acrosome reacted following treatment with ionophore.

Hyaluronidase Assay. Hyaluronidase activity of sperm and media were assayed according to Dorfman (1955). Briefly, dilutions of hyaluronidase standards (bovine testis hyaluronidase, Sigma No. H-3631) or experimental samples were incubated with a solution of hyaluronic acid (0.15 mg/mL in 150 mM Na_2PO_4, pH 5.35) at 37 °C for 45 min (Figures 2 and 3), 2 h (Figure 4), or 12 h (Figure 7). A solution of acidified albumin (1 mg/mL, pH 3.75 at 37 °C) was added to develop turbidity. Turbidity resulting from the albumin−hyaluronic acid complex was allowed to develop for 5 min, and the percent transmittance at 600 nm was measured with a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA) using distilled water as a blank (100% *T*) and buffer samples to define the greatest turbidity (~40% *T*). Sperm-containing samples were centrifuged briefly (10 000*g* for 1 min) to pellet sperm, which interfered with the formation of the hyaluronic acid−albumin complex, and the assay sample (supernatant) was removed and incubated with acid albumin. Decreased turbidity was correlated with the hydrolysis of hyaluronic acid. Percent transmittance was normalized and presented as turbidity reducing units/sperm (TRU/sperm) using the equation $TRU = (\% T - \% T_{min})/(\% T_{max} - \% T_{min})$ and adjusted to a per sperm basis so that the data could be compared among assays. Hyaluronidase activity present in incubation media was also calculated as TRU/sperm, based on the number of sperm exposed to the media. Control assays demonstrated that media alone did not have any contaminating hyaluronidase activity (data not shown). Hyaluronidase activity of gel filtration samples was expressed as a percentage of the maximum activity measured (100% *T*).

Acrosomal Status. Acrosomal status was determined by Coomassie Blue staining, modified from Moller *et al.* (1990). Sperm samples were fixed in 20 mM Na_2HPO_4 and 150 mM NaCl containing 7.5% formaldehyde (pH 7.5) for 10 min and centrifuged briefly (8000*g*, 1 min) to pellet the sperm. The sperm pellet was washed by resuspension in ammonium acetate (100 mM, pH 9.0) and centrifugation. Sperm samples, placed on poly(L-lysine)-coated glass slides, were air-dried and stained with 0.04% Coomassie Blue-G250 (Kodak, Rochester, NY) in 3.5% perchloric acid for 10 min. Slides were washed with distilled water, air-dried, and mounted in Permount (Fisher Scientific, Pittsburgh, PA). At least 100 sperm per sample were scored at random as acrosome intact (dark blue staining of the acrosomal vesicle), acrosome reacted (no staining in the acrosomal region), or equivocal (<2%).

Phospholipase C Treatments. Acrosome-reacted sperm were incubated with a phosphatidylinositol-specific phospholipase C (PI-PLC, 0.05 unit/10^6 sperm) for 1−3 h, or without the addition of enzyme for the comparable time, at 37 °C. At the end of the incubation times samples were centrifuged to pellet sperm, and the supernatants were collected. For hyaluronidase assays of PI-PLC-released sperm proteins, acrosome-reacted sperm were treated with PI-PLC for 60 min at 37 °C and centrifuged, and the supernatant and pellet fractions were collected and diluted to appropriate volumes for hyaluronidase assay. The hyaluronidase assay was performed as described above for sperm or sperm supernatant samples. Protease inhibitors were used throughout the preparation to avoid proteolysis of any of the components. Sperm proteins released by the PI-PLC treatment were also identified by gel autoradiography as described below.

Gel Autoradiography and Densitometry. Sperm surface proteins on acrosome-intact or acrosome-reacted sperm were radioiodinated using Chloramine-T and $Na^{125}I$. Free ^{125}I was removed by centrifugation of the sperm through 10% sucrose.

Sperm were treated with PI-PLC, and supernatants from iodinated samples were separated by SDS−PAGE. Gels were fixed overnight in 50% methanol/0.37% formaldehyde, silver stained, exposed to X-ray film (Kodak XRP-1) for 1−8 days, and developed in a Kodak X-Omat automatic film developer. The autoradiographic profile of each sample was scanned with a laser densitometer (LKB, Piscataway, NJ) to quantify the proteins present in the PI-PLC-released supernatants. These data were normalized against the background levels of protein release in the absence of PI-PLC and presented as fractional change. Molecular weights of reduced samples were calculated by interpolation from a linear regression of the mobility of protein standards (Bio-Rad, Hercules, CA) electrophoresed on the same gels. Values stated in the text are the average values with a maximum observed deviation of 5%.

Fractionation of PI-PLC-Released Proteins. Acrosome-reacted sperm (95% acrosome reacted, $n = 3$) were washed six times by centrifugation (750*g*, 2 min) to remove soluble hyaluronidase. Sperm were divided into two aliquots and incubated in the presence or absence of PI-PLC (0.02 unit/10^6 sperm) for 3 h at 37 °C in 5% CO_2. Soybean trypsin inhibitor (SBTI, 20 μg/mL) was added to the ionophore and PI-PLC incubation steps to prevent proteolysis during incubations. The supernatants from both the treated and control sperm were collected and fractionated by HPLC on a Superose-12 gel filtration column (Pharmacia, Piscataway, NJ) operated at a flow rate of 0.4 mL/min and 10 atm, and 0.4-mL fractions were collected. All fractions were assayed for hyaluronidase activity as described above. The Superose-12 column was calibrated using gel filtration molecular mass standards (2000−12.4 kDa range) from Sigma (St. Louis, MO), fitted to a linear regression ($r^2 = 0.99$), and used to estimate the apparent molecular weight of the hyaluronidase peak (elution time = 30.5 min). A second size exclusion chromatography matrix, Superdex-75, was used to obtain greater resolution of the molecular size of the GPI-linked hyaluronidase. The column was run at 0.4 mL/min, and 0.2-mL fractions were collected. The Superdex-75 column was also calibrated with molecular weight standards (Sigma, St. Louis, MO). All fractions were assayed for hyaluronidase activity, and fractions containing the hyaluronidase were assayed for protein content as described below.

Colloidal Gold Protein Staining. Fractions from the hyaluronidase peak of the Superdex-75 column were dehydrated using vacuum centrifugation, resuspended in sample buffer, and separated by SDS−PAGE. Proteins were blotted onto nitrocellulose and stained with colloidal gold according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Apparent molecular weights were determined by comparison to molecular weight standards as above. Proteins were quantified by laser densitometry (LKB, Piscataway, NJ).

RESULTS

The central question addressed here is whether mature mouse sperm possess a GPI-linked hyaluronidase. Other investigators have shown that the guinea pig sperm antigen PH-20 is a GPI-linked protein, present on both acrosome-intact and acrosome-reacted sperm (Primakoff *et al.*, 1985; Myles *et al.*, 1987), with sequence homology to bee venom hyaluronidase (Gmachl & Kreil, 1993). To determine if a GPI-linked hyaluronidase is present on mouse sperm, we have determined the presence of membrane-associated hyaluronidases in both intact and acrosome-reacted sperm and demonstrated the PI-PLC release of this hyaluronidase activity. The mouse sperm GPI-linked proteins have been visualized by gel autoradiography and quantified by densitometry, and finally, the composition of the GPI-linked hyaluronidase was determined by size exclusion chromatography.

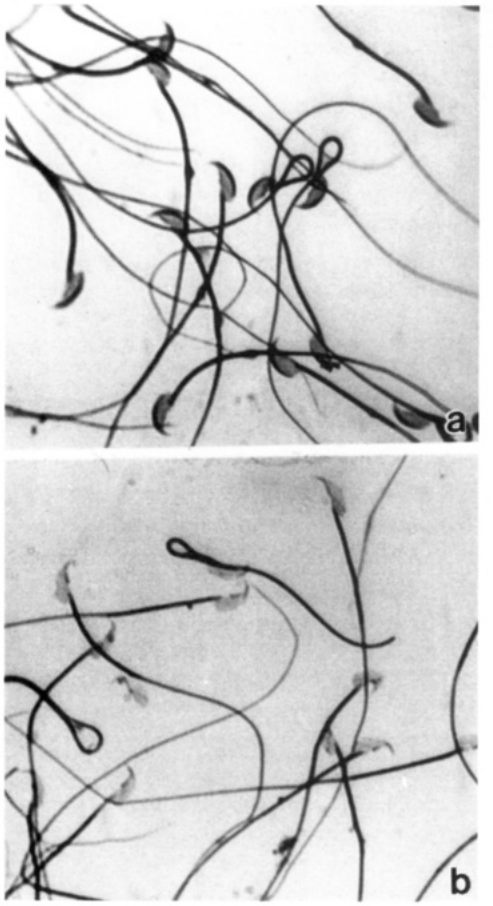

FIGURE 1: Determination of acrosomal status using Coomassie Brilliant Blue. Acrosome intact sperm were characterized by intense staining over the acrosomal crescent (a), while acrosome-reacted sperm did not display this staining pattern (b). Sperm were 8% (a) and 85% (b) acrosome reacted in these samples.

Hyaluronidase Activity in Mouse Sperm. Previous studies have shown that a soluble hyaluronidase is localized within the acrosome of mammalian sperm including bull (Borders & Raferty, 1968), boar (Gadella *et al.*, 1991), ram (Brown, 1975), hamster (Rogers & Morton, 1973; Talbot & Franklin, 1974a), and guinea pig (Talbot & Franklin, 1974b). Surprisingly, little is known about mouse sperm hyaluronidase, although it is the most studied system in terms of mammalian fertilization mechanisms. Therefore, our initial studies characterized mouse sperm hyaluronidases.

In all experiments, unequivocal determination of acrosomal status was necessary to determine the relative contributions from acrosome-intact and acrosome-reacted sperm. We used the Coomassie Brilliant Blue staining technique to determine acrosomal status. Mouse sperm that were acrosome intact showed intense staining over the acrosomal crescent on the sperm head (Figure 1a) whereas acrosome-reacted sperm lacked this staining pattern (Figure 1b). Typically, capacitated sperm populations were 70%−80% acrosome intact after the 2-h incubation period while sperm that had been

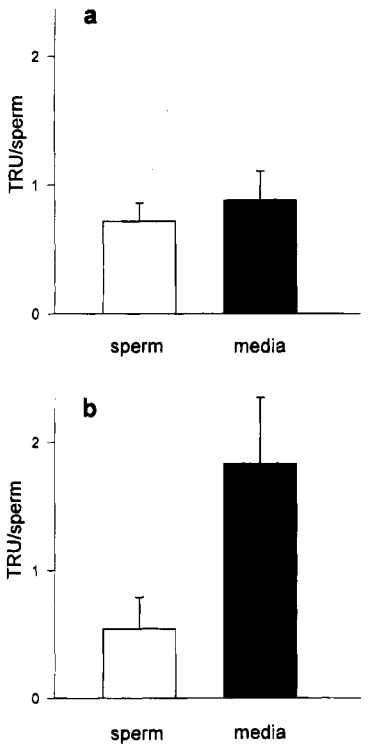

FIGURE 2: Hyaluronidase activity of intact and acrosome-reacted mouse sperm. (a) Intact, capacitated sperm (□) have an associated hyaluronidase activity. Additionally, hyaluronidase activity was detected in the incubation medium (■) of intact sperm, suggesting the presence of a soluble hyaluronidase activity. (b) When sperm were induced to undergo the acrosome reaction in the presence of the calcium ionophore A23187, a large detectable increase of hyaluronidase activity was released into the medium (■). Sperm-associated hyaluronidase activity was retained even in acrosome-reacted sperm (□). Data represent the mean ± SD for five different trials.

incubated with the calcium ionophore were >90% acrosome reacted.

Hyaluronidase activity (0.7−0.9 TRU/sperm) was detected in association with both capacitated mouse sperm and the media in which sperm had been incubated (Figure 2a). These data suggest that acrosome-intact sperm possess a hyaluronidase activity in addition to the previously documented hyaluronidase activity that dissociates from sperm during *in vitro* incubation (Rogers & Morton, 1973; Talbot & Franklin, 1974a). Release of hyaluronidase into the medium could be due to release of a weakly associated hyaluronidase from the sperm surface (Gadella *et al.*, 1991), leakage of the acrosomal vesicle prior to normal acrosome reactions (Rogers & Morton, 1973; Talbot & Franklin, 1974a), or a result of spontaneous acrosome reactions during incubation *in vitro* (Talbot & Franklin, 1974a; Zao *et al.*, 1985), consistent with our findings that approximately 30% of the population was acrosome reacted.

Ionophore treatments in the presence of Ca^{2+} induced the acrosome reaction in large percentages of capacitated sperm. Following treatment with the ionophore there was a 1.9-fold increase in hyaluronidase activity in the media compared to levels in media incubated with intact sperm, suggesting that hyaluronidase was released during the acrosome reaction. Furthermore, washed acrosome-reacted sperm retained levels of hyaluronidase activity similar to intact sperm, indicating the presence of a second, nonsoluble hyaluronidase associated with mouse sperm (Figure 2b).

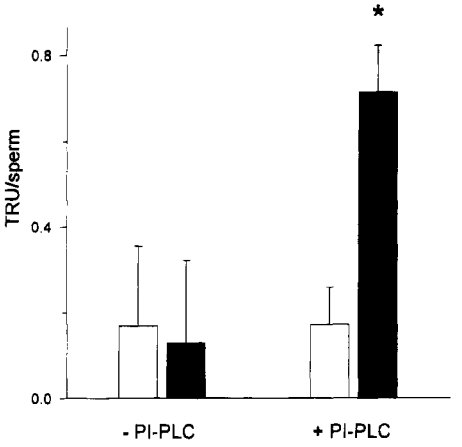

FIGURE 3: Hyaluronidase activity released by PI-PLC treatment. Acrosome-reacted sperm were incubated for 1 h in the presence (+) or absence (−) of PI-PLC and the resulting supernatants (■) and sperm (□) assayed for hyaluronidase activity. Although neither sperm sample had substantial levels of hyaluronidase activity, the +PI-PLC treatment released significantly greater (*, *t*-test, $P < 0.05$) hyaluronidase activity into the supernatant than samples incubated in the absence of PI-PLC. This activity was 5.5-fold greater than hyaluronidase activity released by acrosome-reacted sperm incubated in buffer alone.

Hyaluronidase Activity of GPI-Linked Components. The presence of a nonsoluble hyaluronidase in both intact and acrosome-reacted sperm suggested that a membrane-associated form of this enzyme was present. In order to determine if the membrane-associated hyaluronidase was attached by a GPI linkage, acrosome-reacted sperm were treated with PI-PLC to release GPI-linked sperm proteins. The PI-PLC released fractions were assayed for hyaluronidase activity. In our initial studies, soluble hyaluronidase activity was detected in the supernatant of sperm incubation media (Figure 2). This hyaluronidase could be due to spontaneous acrosome reactions or cell lysis during experimental manipulations, and these sources of hyaluronidase would interfere with specific detection of a GPI-linked, membrane-associated hyaluronidase released by PI-PLC treatment. However, soluble acrosomal hyaluronidase released into the medium by ionophore treatment of sperm could be completely removed from samples of acrosome-reacted sperm by three or more wash steps (data not shown). Therefore, these experiments were conducted using acrosome-reacted sperm that were washed extensively to remove all soluble hyaluronidase. This ensured that the proteins detected were components specifically released by PI-PLC treatment and were not a contaminating acrosomal hyaluronidase.

Washed, acrosome-reacted sperm were incubated in the presence or absence of PI-PLC and the resulting supernatants tested for hyaluronidase activity. A hyaluronidase activity was released into the supernatant by PI-PLC treatment (Figure 3). This activity was 4.5-fold greater than the hyaluronidase activity remaining associated with the acrosome-reacted mouse sperm following PI-PLC treatment and was significantly greater ($P < 0.05$) than activity detected in the supernatant of untreated sperm (Figure 3). These data demonstrate that a hyaluronidase is present among the proteins released from acrosome-reacted sperm by treatment with PI-PLC and indicate that the membrane-associated mouse sperm hyaluronidase is covalently attached to the membrane by a GPI linkage.

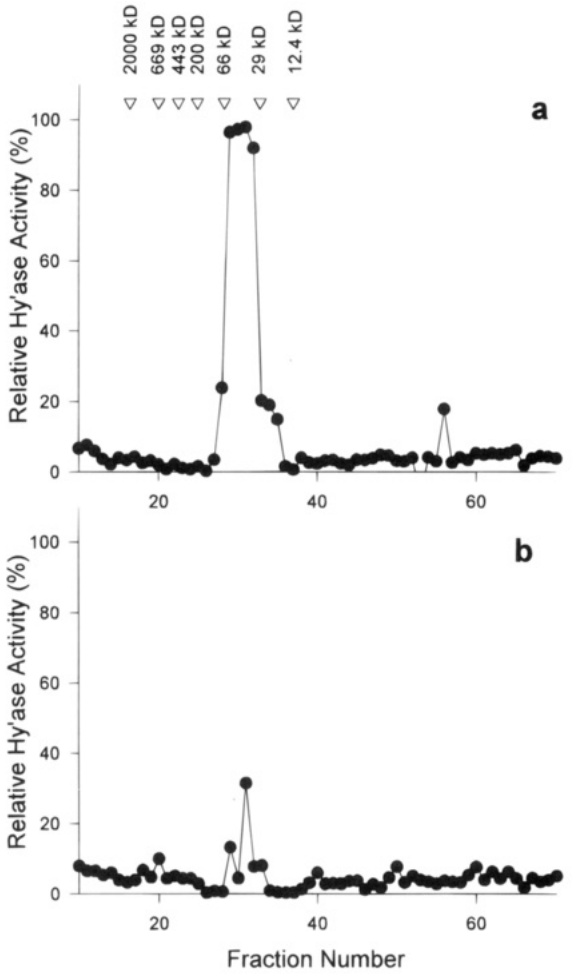

FIGURE 4: GPI-linked sperm membrane proteins contain a single hyaluronidase activity. (a) Supernatant from PI-PLC-treated acrosome-reacted sperm was fractionated by size exclusion chromatography. Each fraction was assayed for hyaluronidase activity (●). A single peak of hyaluronidase activity was detected in fractions 29−32. The peak value corresponds to a molecular mass of 53 ± 30 kDa. The elution positions of the molecular weight standards used to calibrate the column are indicated at the top of the plot (▽). (b) Control supernatants from sperm incubated without PI-PLC did not show significant hyaluronidase activity (●), although a minor peak probably due to contamination with acrosomal hyaluronidase appears in fraction 31.

Fractionation of the Hyaluronidase. In order to determine the molecular size of the native enzyme, we used size exclusion chromatography to fractionate the components of the PI-PLC supernatant. Each fraction was assayed for hyaluronidase activity. Hyaluronidase activity was restricted to a single peak in fractions 29−32 (Figure 4A). Control supernatants treated identically, but incubated in the absence of PI-PLC, had only trace hyaluronidase activity (Figure 4B), confirming that the hyaluronidase activity detected in the whole supernatant from PI-PLC-treated mouse sperm is indeed a GPI-linked protein specifically released by PI-PLC treatment.

The peak hyaluronidase activity has a predicted molecular mass of 53 kDa, determined from nonlinear regression of elution time vs molecular mass for protein standards separated on the same column. This molecular mass is within the range of previously published hyaluronidases (Aronson & Davidson, 1967; Borders & Raferty, 1968; Kemeny *et al*., 1984; Gmachl & Kreil, 1993); however, the size exclusion column used in these experiments has a broad

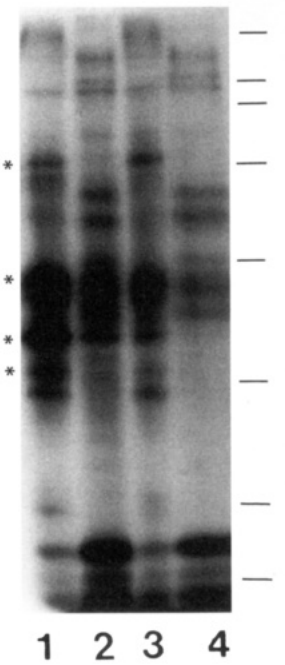

FIGURE 5: Gel autoradiography of PI-PLC-released components. Both acrosome-intact (lane 1) and acrosome-reacted (lane 2) sperm contain multiple surface components that are released by a 60-min incubation with PI-PLC. Acrosome-intact (lane 3) and -reacted (lane 4) sperm samples incubated in the absence of PI-PLC were used to quantify background levels, since sperm have many noncovalently associated surface components acquired during epididymal transit and lost during subsequent *in vitro* incubations. Four polypeptides in the predicted molecular mass range of the GPI-linked hyaluronidase are enriched in the PI-PLC supernatants from both acrosome-intact and acrosome-reacted sperm (*). Molecular mass standards are indicated at the right: 200, 116, 97, 66, 45, 31, 21.5, and 14.4 kDa.

fractionation range, and thus the actual size of the GPI hyaluronidase could be expected to vary from the mean value by as much as 30 kDa. This molecular mass range actually brackets the range of reported hyaluronidases: 85 kDa (liver; Aronson & Davidson, 1967) to 40 kDa (bee venom; Gmachl & Kreil, 1993). Therefore, the subsequent experiments were performed to identify the molecular masses of PI-PLC-released proteins and to more precisely delineate the molecular mass of the GPI-linked hyaluronidase.

GPI-Linked Components of the Sperm Surface Membranes. The polypeptide composition of the PI-PLC-released sperm membrane components was determined by incubating ^{125}I-labeled acrosome-intact or acrosome-reacted sperm in the presence or absence of PI-PLC, collecting the supernatant fractions, and separating the proteins by reducing SDS−PAGE. Autoradiography (Figure 5) revealed a complex profile of proteins in both intact (lane 1) and reacted (lane 2) sperm. Electrophoresis of supernatants from sperm incubated in the absence of PI-PLC shows the background levels of release (lanes 3 and 4), and these were used to normalize the densitometric quantitation and to identify polypeptides specifically released in the presence of PI-PLC. All of the polypeptides, designated by their apparent molecular masses, were quantified by laser densitometry and normalized as fractional change from the corresponding background (Figure 6). Several polypeptides are enriched in the PI-PLC supernatants from both intact and acrosome-reacted sperm: 68, 44, 39, 34, 17, and 15 kDa. Other proteins were present but not enriched in the PI-PLC supernatants

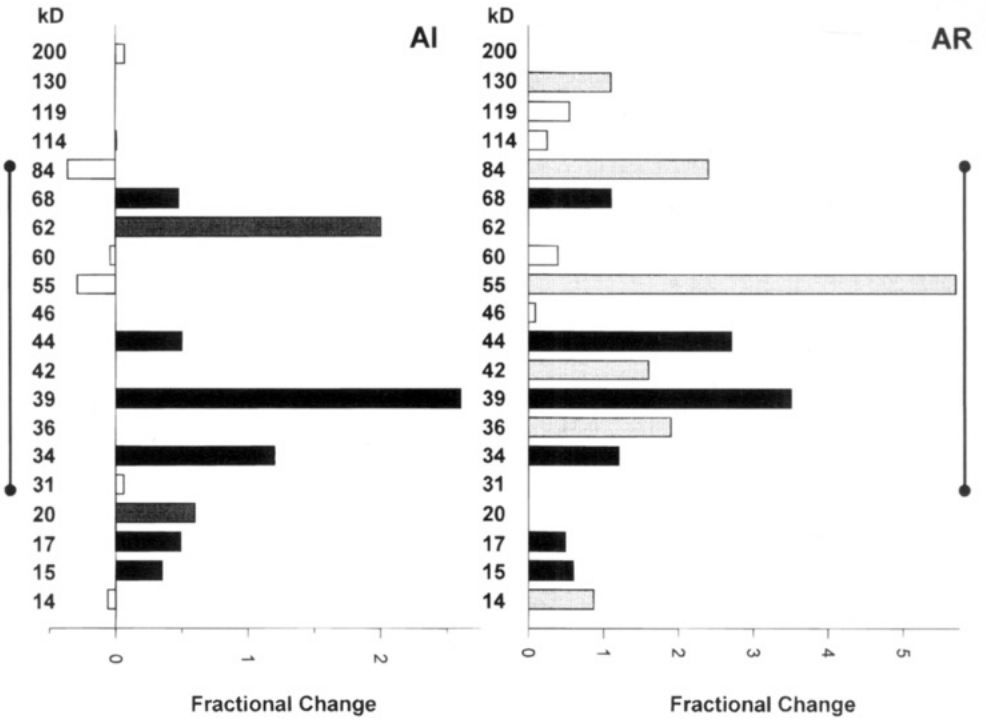

FIGURE 6: Densitometric quantitation of PI-PLC-enriched polypeptides. The fractional increase of all polypeptide bands in the PI-PLC supernatants of acrosome-intact (AI) or acrosome-reacted sperm (AR) was quantified by laser densitometry. Several polypeptides are enriched in both AR and AI sperm (■). In addition, polypeptides uniquely enriched in either AI (dark hatch) or in AR (light hatch) sperm were detected. A few polypeptides appeared in either AI, AR, or both the PI-PLC-treated supernatants but did not appear to be enriched (□). These may represent loosely associated sperm surface components that are nonspecifically released over time.

obtained from acrosome-intact and/or -reacted sperm: 200, 119, 114, 60, 46, and 31 kDa. In addition, some bands were enriched in only one of the preparations: 62 and 20 kDa (acrosome intact) and 130, 84, 55, 42, 36, and 14 kDa (acrosome reacted).

Although the profiles of PI-PLC-released proteins are complex, some important trends are apparent. There are several components that appear only in the samples from acrosome-reacted sperm, suggesting that unique GPI-linked proteins are revealed on the inner acrosomal membrane following acrosomal exocytosis. Further, two components, 62 and 20 kDa, are present in the acrosome-intact samples but lost in the acrosome-reacted samples, suggesting that these components are in the membrane areas lost during acrosomal exocytosis (i.e., the plasma membrane overlying the acrosome). Additional studies of nonreduced gel autoradiography revealed high molecular weight components not present in the reduced samples, suggesting that some of the polypeptides enriched in the PI-PLC-treated supernatants are disulfide linked (data not shown). The relationship of these polypeptides will be addressed in future studies.

The observations that a membrane-associated hyaluronidase is present in both acrosome-intact and acrosome-reacted sperm and that four of the polypeptides enriched by both AI and AR sperm (68, 44, 39, and 34 kDa) are within the molecular mass range delineated by initial size exclusion chromatography (~85−30 kDa, bracketed on Figure 6) suggest that one or more of these polypeptides are responsible for the GPI-linked hyaluronidase activity. Using a narrow-range size exclusion matrix to resolve these GPI-linked proteins, one peak of hyaluronidase activity was recovered (Figure 7a). Of the PI-PLC-released proteins, only the 68 kDa polypeptide was found to comigrate with the hyaluronidase activity, as shown by densitometric quantitation of the peak fraction proteins separated by reducing SDS−PAGE and stained with colloidal gold (Figure 7b). These data demonstrate that the 68 kDa GPI-linked protein which is present on both acrosome-intact and acrosome-reacted mouse sperm is the mouse sperm surface hyaluronidase.

DISCUSSION

There has been a growing body of evidence that there may be more than one form of a mammalian sperm associated hyaluronidase. In addition to the previously characterized soluble hyaluronidase found within the sperm's acrosomal vesicle (Hechter & Hadidian, 1947; Swyer, 1947; Borders & Raferty, 1968; Rogers & Morton, 1973; Brown, 1975; Gadella *et al.*, 1991). Gmachl and Kreil (1993) predicted that mammalian sperm would contain a GPI-linked sperm surface hyaluronidase. This conclusion was based on significant DNA homology between bee venom hyaluronidase and PH-20, a GPI-linked protein found on the surface of guinea pig sperm. In support of this hypothesis, Lin *et al.* (1994) showed that a polyclonal antibody against recombinant PH-20 recognized a 53.7 kDa GPI-linked protein localized to the surface of mouse sperm overlying the anterior head. However, until now, no direct confirmation of a GPI-linked hyaluronidase associated with the mammalian sperm surface has been reported. We have demonstrated that several polypeptides are released from the sperm surface by treatment with PI-PLC on both acrosome-intact and acrosome-reacted sperm, indicating that they are GPI linked and that one of these, a 68 kDa protein, is responsible for a sperm surface hyaluronidase activity.

Both acrosome-intact and acrosome-reacted sperm retained a membrane-associated hyaluronidase activity even after extensive washing to remove soluble forms of hyaluronidase.

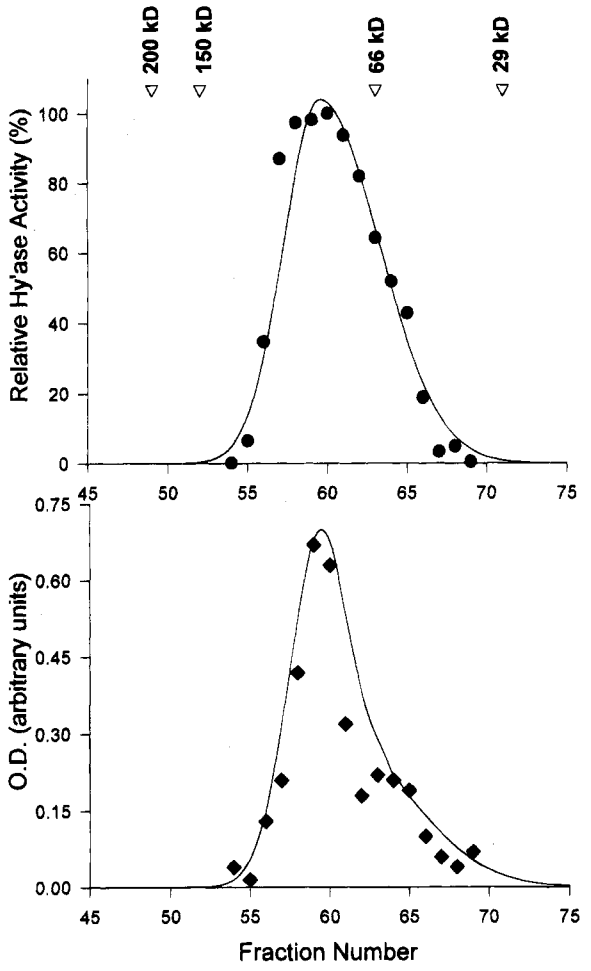

FIGURE 7: Fractionation and molecular mass determination of the GPI hyaluronidase. GPI-linked proteins were separated on Superdex 75, a narrow-range size exclusion matrix. One peak of hyaluronidase activity was detected (a, top). The 68 kDa GPI-linked protein showed an elution pattern coincident with the hyaluronidase activity, as revealed by colloidal gold protein staining of SDS–PAGE separated proteins from these fractions (b, bottom). The elution positions of the molecular mass standards used to calibrate the column are indicated at the top of the plot (▽).

The membrane-associated activity appears to be covalently attached to the mouse sperm surface by a GPI linkage since it was released only by treatment with PI-PLC. The fact that both acrosome-reacted and acrosome-intact sperm possess a GPI-linked hyaluronidase suggests that this enzyme plays an important role in sperm penetration of the egg's extracellular vestments prior to sperm–egg membrane fusion.

Characterization of the GPI-linked mouse sperm components themselves is complex. The data presented here are the first characterization of GPI-linked proteins from mammalian sperm. Previous work revealed that the guinea pig sperm antigen PH-20 was GPI linked (Phelps *et al.*, 1988). However, this work was done exclusively by means of immunoprecipitation of the PH-20 antigen from a PI-PLC supernatant fraction, and the presence or identity of additional GPI-linked components was not investigated. Therefore, it is important to note that mouse sperm have several prominent GPI-linked proteins, two of which appear exclusively in acrosome-intact sperm; several additional GPI-linked components appear in acrosome-reacted sperm, concomitant with the exposure of the inner acrosomal membrane following acrosomal exocytosis. It will be of interest to further characterize these GPI-linked proteins and determine their possible roles in fertilization.

Four polypeptides were enriched on both acrosome-intact and acrosome-reacted sperm, 68, 44, 39, and 34 kDa, and all fall within the predicted molecular mass range of the GPI hyaluronidase determined by size exclusion chromatography. However, quantitation of proteins in the fractions containing hyaluronidase activity revealed that only the 68 kDa GPI-linked protein comigrated with the hyaluronidase. Finally, we have shown that the GPI-linked mouse sperm hyaluronidase is inhibited by the flavonoid apigenin (Thaler & Cardullo, 1994), which has been previously shown to be a competitive inhibitor of bovine testicular hyaluronidase (Yuppusamy *et al.*, 1990) as well as the recombinant form of PH-20 (Lin *et al.*, 1994). The biochemical characteristics of this protein, hyaluronidase activity, molecular mass, and GPI linkage, and its presence on both acrosome-intact and acrosome-reacted sperm all suggest that the GPI-linked hyaluronidase is, in fact, identical to the PH-20-like antigen identified by Lin *et al.* (1994).

Taken together, these data demonstrate the presence of two distinct hyaluronidase populations on mouse sperm. The first is a soluble form within the acrosomal vesicle which is released into the surroundings following acrosomal exocytosis. The second is the GPI-linked membrane hyaluronidase that is found on both acrosome-intact and acrosome-reacted sperm. Our studies demonstrate that this enzyme was released by PI-PLC, indicating that the hyaluronidase is anchored by a GPI linkage. Thus, hyaluronidase joins a growing list of GPI-linked cell surface enzymes including alkaline phosphatase, 5′-nucleotidase, acetylcholinesterase, alkaline phosphodiesterase I, trehalase, and p63 proteinase [for a review, see Low (1987)].

A physiological role for multiple forms of sperm-associated hyaluronidase is still unclear. However, a clue to its role in the fertilization process may come from looking at the localization of hyaluronic acid surrounding the egg. In the mouse, acrosome-intact sperm first must penetrate the cellular layer surrounding the oocyte known as the cumulus complex. The major extracellular component of the cumulus complex is hyaluronic acid, which extends as a continuous matrix from the cumulus mass into the outer portion of the zona pellucida (Ball *et al.*, 1982; Talbot, 1984). In addition, hyaluronic acid has also been reported in the perivitelline space between the zona pellucida and the egg plasma membrane (Dandekar & Talbot, 1992).

Given the distribution of egg-associated hyaluronic acid, it is possible to propose a role for the different isoforms of mouse sperm surface hyaluronidase. Since mouse sperm are acrosome intact when negotiating the cumulus matrix, they might require a sperm surface hyaluronidase in order to break down the substantial matrix of hyaluronic acid between the cumulus cells. Once through the cumulus mass the sperm encounters the zona pellucida and binds to an 83 kDa glycoprotein, ZP3 (Bleil & Wassarman, 1980). In addition to its role as the sperm adhesion ligand ZP3 is also a potent agonist of acrosomal exocytosis which ultimately results in the release of acrosomal enzymes including the soluble hyaluronidase. It is probable that this soluble hyaluronidase would digest any remaining hyaluronic acid within the matrix, allowing the acrosome-reacted sperm to penetrate the zona pellucida. Finally, the remaining population of GPI-linked hyaluronidase on acrosome-reacted mouse sperm may

be needed to digest the last remnants of hyaluronic acid within the zona pellucida and the perivitelline space. In this context, the different isoforms of sperm-associated hyaluronidase may exist simply to ensure the digestion of hyaluronic acid which may otherwise present a substantial barrier to successful fertilization. Alternatively, it is possible that the membrane-associated forms of hyaluronidase may function in a more complex fashion as a binding molecule, as has been suggested for the PH-20 antigen (Primakoff *et al.*, 1985; Myles *et al.*, 1987). However, no egg-associated ligand other than the hyaluronic acid has been implicated in PH-20-mediated adhesion events during fertilization.

As a putative adhesion molecule, sperm surface hyaluronidase would join a list of other cell surface enzymes that are implicated in binding to the zona pellucida. These molecules include a mouse sperm β-1,4-galactosyltransferase (Miller *et al.*, 1992), a sperm surface fucosyltransferase (Cardullo *et al.*, 1989), and an α-D-mannosidase (Cornwall *et al.*, 1991). Of these enzymes, only the β-1,4-galactosyltransferase has been shown to have a specific reactivity toward the zona pellucida (Miller *et al.*, 1992).

Independent of its role as a digestive enzyme or an adhesion molecule, the attachment of sperm surface hyaluronidase by a GPI linkage may have profound consequences. In particular, Baltz and Cardullo (1989) have shown that the rate of association between a soluble ligand and its complementary receptor (or enzyme) is maximized by a high diffusion coefficient of the receptor on the sperm surface. Interestingly, PH-20, like most GPI-linked proteins, exhibits a high diffusion coefficient similar to membrane lipids (Cowan *et al.*, 1987; Phelps *et al.*, 1988). This unique membrane attachment may therefore ensure that hyaluronidase activity proceeds at a maximal rate to guarantee successful penetration of the egg vestments and fusion of the sperm and egg plasma membranes. The strategy of increasing the mobility of a cell surface protein to maximize enzymatic activity may be characteristic of other GPI-linked enzymes as well.

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