

Crypticity and Functional Distribution of the Membrane Associated α -L-Fucosidase of Human Sperm

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ABSTRACT Two distinctive isoforms of the enzyme α -L-fucosidase are found within human semen in substantial amounts, suggesting specialized functions during reproduction. The membrane-associated isozyme of human sperm cells was previously characterized biochemically, and here we report on its subcellular localization. Intact, detergent permeabilized, capacitated, and acrosome-reacted sperm were investigated using antifucosidase immunofluorescence, binding of the fluorescent fucosylated glycoconjugate RITC-BSA-fucose (RBF), and enzyme activity in the presence and absence of selected inhibitors. Both immunolocalization and RBF binding show that fucosidase is broadly distributed over the membrane systems of human sperm, but is relatively enriched within the equatorial segment. Upon detergent treatment or induction of acrosome reaction (AR), a portion of enzyme activity is recoverable in the supernatant, presumably associated with released remnants of the outer acrosomal membrane. Surprisingly, cell-bound enzyme activity increases sharply following permeabilization of intact sperm, representing cryptic fucosidase that is relatively stable and corresponds with strong fluorescence in the equatorial segment and other sperm membranes. These observations support the notion that the fucosidase has a role in the intimate species signature interactions between sperm and oocyte. *Mol. Reprod. Dev.*

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Key Words: fertilization; gamete; equatorial segment; acrosome; fucose; DFJ

INTRODUCTION

In recent years, there has been a growing focus on the essential roles of polysaccharide moieties in the functions of mammalian gametes including sperm-oocyte recognition and fertilization. Carbohydrate containing cytoarchitectural features distinguish species from each other, and enable species specific recognition and interaction between gametes at the molecular level (Benoff, 1997; Shalgi and Raz, 1997; Hoodbhoy and Dean, 2004). For humans and several other species, numerous lines of evidence suggest that fucose-containing polysaccharides or fucosidases are important in sperm functions. In the ascidian

Halocynthia roretzi, α -L-fucosidase mediates sperm-egg binding via an interaction of α -L-fucosidase on the sperm and the complimentary L-fucose residues on glycoproteins in the vitelline coat of the eggs (Matsumoto et al., 2002). In *Drosophila*, it was recently reported that spermatozoa of several species contain a plasma membrane associated α -L-fucosidase, sharing commonalities with sperm α -L-fucosidases of other animals (Intra et al., 2006). Cytochemical studies showed homogenous distribution of fucose residues throughout the zona pellucida and cortical granules of metaphase II human oocytes using fucose specific lectin from *Aleuria aurantia*, and incubation with 0.2 M fucose abolished staining of the zona pellucida and cortical granules (Jimenez-Movilla et al., 2004). Flechon et al. (2003) showed active incorporation of fucose during in vitro maturation of porcine oocytes, resulting in its accumulation both at the zona surface and in the perivitelline space, paralleling their observations on in vivo specimens. Tesarik et al. (1993) reported that fucosylated neoglycoprotein and solubilized zona pellucida protein compete for binding sites on the surface of human sperm. Lucas et al. (1994) reported that fucose-directed lectins and antibodies compete for binding of human sperm to the zona pellucida using the hemizona assay.

The important general roles of fucosylated glycans in normal human physiology and disease conditions, as well as the biochemistry of fucose-containing molecules, have been reviewed in depth (Staudacher et al., 1999; Becker and Lowe, 2003). In humans, there are at least 13 different genes for fucosyltransferases (Becker and Lowe, 2003), underlying the robust and complex composition of fucose-containing polysaccharides that occur. Fucosidases [EC 3.2.1.51] are typically characterized as lysosomal acid hydrolases, glycoproteins that are specialized for removal of terminal fucose residues from

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saccharide-containing substrates (Johnson and Alhadeff, 1991). In addition, they may also have transglycosylation activities (White et al., 1987; Berteau et al., 2004), which might be important for cell to cell interactions. The human enzyme is essential for normal physiology, and the enzyme synthesized in most tissues is encoded by the *FUCA1* gene on chromosome 1 (Kretz et al., 1992). Homozygous malfunction of this gene results in the rare lethal genetic disease fucosidosis in humans (e.g., Michalski and Klein, 1999; Kanitakis et al., 2005). Homozygous mutation in the homologous gene of English Springer Spaniels results in a viable condition with male infertility and defective spermatogenesis (Veeramachaneni et al., 1998). Recent efforts in this laboratory have demonstrated that human semen contains two distinct isoforms of the glycoprotein enzyme α -L-fucosidase (Alhadeff et al., 1999), both of which have since been purified and characterized biochemically (Khunsook et al., 2002, 2003). By contrast with the typical lysosomal acid hydrolase that occurs in human liver and other tissues (Johnson and Alhadeff, 1991), both seminal isoforms display enzyme activity over a broad range of pH values. The known isozyme subunits are of similar M_r , and differ from each other primarily in their glycosylation, presumably all transcribed from the *FUCA1* gene. The seminal plasma isoform is a soluble glycoprotein, present in substantial amount, which can be separated from the sperm-associated isoform. The spermatozoal enzyme is present in lesser amounts, and during purification it becomes less stable than the seminal plasma isoform. The membrane-associated α -L-fucosidase of human sperm has been purified 8,600-fold to homogeneity. SDS-PAGE and Western analyses showed this α -L-fucosidase to be comprised of a single subunit 51 kDa in size, with the characteristics of a single-pass membrane associated glycoprotein that is detectable on the sperm plasma membrane (Khunsook et al., 2003). Liang et al. (2004) have presented direct evidence of selective expression of the *FUCA1* gene in human primary spermatocytes.

During recent years, several laboratories have contributed to a growing understanding of the sperm equatorial segment, which appears to have critical roles in the recognition and fusion events between mammalian sperm membranes and the conspecific oolemma. The basics of the organization and compartmentalization of the mammalian sperm head have been recently updated (Ramalho-Santos et al., 2002; Wolkowicz et al., 2003; Yoshinaga and Toshimori, 2003; James et al., 2004). A number of specific proteins and glycolipids have been localized to the equatorial segment after acrosomal exocytosis, and remain in this relatively stable environment where they may be involved in sperm-ocyte interactions (e.g., Allen and Green, 1995; Gadella et al., 1995; Auer et al., 2004; Da Ros et al., 2004; Saxena and Toshimori, 2004; Busso et al., 2005; Herrero et al., 2005; Jagadish et al., 2005). In addition, other proteins may move away from the equatorial segment as sperm complete acrosomal exocytosis (Spinaci et al., 2005).

These developments have made it more compelling to test our hypothesis that the membrane α -L-fucosidase is richly represented within the equatorial segment of human sperm cells, and that a portion of this glycoprotein remains in that subcellular location following the acrosome reaction (AR).

We now report further analysis of the subcellular distribution within human sperm for the membrane associated α -L-fucosidase by comparing cells that were differentially prepared to expose different membranes and compartments of the sperm cell. Direct assays of enzyme activity, analysis of the subcellular distribution of binding sites for the fucosylated glycoconjugate RITC-BSA-fucose (RBF), and anti-fucosidase-directed immunolocalization studies were completed. All results support the conclusion that a substantial portion of the membrane-associated human sperm α -L-fucosidase is cryptically localized and enriched within the base of the acrosomal compartment. Substantial amounts of the α -L-fucosidase remain within the equatorial segment following exocytosis of the acrosome, which would make this enzyme available for species signature functions during sperm-egg interaction.

MATERIALS AND METHODS

Semen Specimens and Preparations

Human semen specimens were obtained from healthy, male volunteers over 18 years of age with informed, written consent, in accord with protocols approved by the Lehigh University Institutional Review Board. All experiments reported here have been repeated on multiple independent occasions using specimens from at least three different donors. Except as otherwise specified, assessments of semen and sperm characteristics were consistent with those World Health Organization (WHO, 1999). For experiments involving capacitation and AR, the general methods of Suarez et al. (1986) were used with modifications of centrifugation specifications.

Percoll[®] gradient fractionation. Briefly, sperm cells were washed and enriched by centrifugation through a Percoll[®] (Sigma, St. Louis, MO P-1644) gradient (isotonic 47.5 over 95% Percoll[®]) for 25 min at 500g. Top and interface layers were removed and HSM was added to remaining volume to yield final volume of 10 ml, and the sample was centrifuged 10 min, 500g. The resulting pellet was resuspended in HSM to a final cell concentration of 20–40 million per ml. For some experiments, subsequent washes in HSM (Suarez et al., 1986), or pH 7.4 phosphate buffered saline were used. For preparation of cell free seminal plasma, to be used as a source of the soluble seminal plasma isoform of α -L-fucosidase, whole semen was subjected to centrifugational washes with HSM at 10,000 and 60,000g.

Capacitation

Percoll[®] washed sperm were incubated in a 35-mm Petri dish in the presence of 3.5 or 1.75% bovine serum albumin (BSA, Sigma A-7906), for 3 or 4 hr, 37°C, 5%

CO₂. After incubation, cells were washed in 4 ml HSM by centrifugation for 5 min, 500g, and the resulting pellet was resuspended to 20–40 million sperm per ml.

Induction of Acrosome Reaction (AR)

Following capacitation, capacitated cells in HSM were treated with a final concentration of 0.01 or 0.05 mM A23187 (Sigma C-7522) ionophore at 37°C, 5% CO₂ for 30 min. After incubation, ionophore-treated cells were washed with HSM. In some experiments, and exclusively in preparations for fluorometry, Bromo-A23187 (BrA23187) (Molecular Probes, Eugene, OR, B-1494) was used at 0.01 mM for induction of AR and gave results comparable to those for A23187. Stock solutions of these ionophores were made in dimethylsulfoxide (DMSO, Fisher D-128), which were freshly diluted at the time of treatment to give a final concentration of DMSO of 1% or less. Control studies confirmed that DMSO alone did not influence results reported here.

Permeabilized Washed Sperm

For some studies on membrane permeabilization, BrA23187 was used on noncapacitated cells at 0.01 mM to deliver gentle perturbation of membranes without disruption of sperm motility, while minimizing interference with fluorescence of 4-MU-based enzyme assays. Such treatments did not significantly alter AR status. In other permeabilization studies, washed cells were treated with Triton[®] X-100 (Sigma T8787) at final concentrations of 0.1 or 0.01% as specified.

Microscopy

Glycoconjugate localization. RBF (Sigma A5918) was stored as a frozen stock solution in glass distilled water at 1 mg/ml, and was prediluted in appropriate medium and added to live unfixed cells to give a final concentration of 10 µg/ml for each of the cell preparations studied. During incubation, suspensions were examined microscopically as wet mounts. For detergent treatments, washed cells were treated with Triton[®] X-100 at final concentrations of 0.1, 0.06, 0.03, 0.01, and 0.001% and incubated 30 sec to 5 min, 37°C, depending upon detergent concentration. Reduced Triton[®] X-100 (Sigma X-100-RS) was also used at concentrations of 0.1, 0.01, and 0.001%, with comparable results. Cells washed in buffer, Percoll[®] washed, capacitated, or acrosome-induced preparations (as described above) were examined for various incubation times, with or without further treatments as specified. For observation, 7 µl of sample was placed on an ESCO Fluoro slide (VWR), covered with a No. 1 coverslip and evaluated using epifluorescence or confocal microscopy. For capacitated and acrosome-induced preparations, fluorescence was monitored at 0, 10, 20, 30, 40 min. The specificity of binding for the fucosyl moiety of RBF was challenged in control experiments in which cells were preincubated for 5 min at 37°C with 0.1% BSA or with FITC-BSA (Sigma A-9771) at 10 µg/ml, neither of which showed significant competition with RBF binding. Slides were evaluated using an Olympus IMT2-RFL microscope, or a

Nikon Eclipse E800, or a Zeiss LSM 510 Meta confocal microscope. Images presented here were captured using the Zeiss confocal system with 63× oil or 100× oil immersion objectives, and a Helium-Neon-1 (HeNe1) laser. Figure images were configured using Adobe Photoshop 7.0 without alteration of original image characteristics.

Evaluation of acrosome status. For each population sample, 8 µl was dried on an Esco Fluoro slide. Slides were fixed for 10 min in 100% methanol, washed three times with PBS and once with distilled water. Each slide was incubated with 20 µl of FITC-PSA (40 µg/ml) (Sigma L-0770) for 30 min at room temperature in a dark moisture box. Slides were washed three times with PBS, once with distilled water, and mounted with 8 µl Mowiol (Calbiochem, San Diego, CA 475904) and a No. 1 coverslip. At least 200 cells were scored using an Olympus IMT2 fluorescence microscope using the criteria of Mortimer et al. (1987).

Immunolocalization of α-L-fucosidase. Slides were prepared with 8 µl specimens, dried, and fixed as above. Each slide was incubated with 35 µl of primary, polyclonal goat-anti-human liver α-L-fucosidase antibody (1:100) (Andrews-Smith and Alhadeff, 1982) for 30 min at 37°C, 5% CO₂. After incubation, slides were washed with PBS, and 35 µl of secondary RITC-conjugated rabbit-anti-goat IgG antibody (1:5,000) (KPL 03-13-06) was added to each slide and incubated 30 min, 37°C, 5% CO₂. Slides were again rinsed with PBS. When double labeling for acrosome status was desired, 25 µl of 10 µg/ml FITC-PSA (Sigma) was added to each slide for 5 min at room temperature. Slides were washed three times with PBS, followed by one rinse with distilled water. Slides were mounted with 8 µl of Mowiol and a No.1 coverslip. Images were captured with a Zeiss LSM 510 Meta confocal microscope with 63× oil immersion objective. HeNe1 and Argon lasers were used for scanning under multi-track conditions, recording temporally separated FITC and RITC signals. Control preparations that omitted primary antibody, or substituted nonimmune antibody preparations, or single fluor preparations, did not show comparable immunofluorescent signals. Maximum projections of confocal z stacks were generated using Zeiss LSM 510 Version 3.2 SP2 software. Adobe Photoshop 7.0 was used for image configuration without alteration of image characteristics.

Alpha-L-fucosidase enzyme assays. Assays for α-L-fucosidase used the fluorometric method previously described (Alhadeff et al., 1999; Khunsook et al., 2002, 2003), except that reaction volumes were proportionately reduced to 60 µl, and outcomes were quantified using a Turner Biosystems TBS-380 fluorometer equipped for miniature cuvettes. Assays were conducted at pH 7.0 at room temperature, and monitored at discrete time points (usually 0, 5, and 10 min) without termination of reaction by addition of stop reagents. Standard curves for 4-methylumbelliferone (4MU) product concentrations were determined using reagent 4MU (Sigma M-1381), and showed linearity over all

relevant ranges. Conversion of substrate 4-methylumbelliferyl- α -L-fucopyranoside (4MU-FUC, Sigma M8527) to the fluorescent product 4MU showed linear proportionality with time of incubation or dilution of enzyme source, as expected for enzyme-limited assay conditions. For this report, enzyme activity was measured as nanomoles per minute of product generated at room temperature, except as otherwise specified. Deoxyfuconjirimycin (DFJ), a specific competitive inhibitor of α -L-fucosidase with $K_i = 10^{-8}$ M for the human liver enzyme (Winchester et al., 1990), was obtained from Calbiochem EMD. All enzyme activity data reported here could be inhibited by 94–100% by inclusion of DFJ at a final concentration of 1 mM, confirming that generation of 4MU was fucosidase-dependent. Stock solutions of 10 mM DFJ were prepared in degassed HSM and stored for up to 1 year in liquid nitrogen.

Partitioning of alpha-L-fucosidase. Experiments were conducted to determine where fucosidase activity resided after high-speed centrifugation. For partitioning studies, Percoll[®] washed cells were treated with either 0.01% Triton[®]X-100 or 0.01mM BrA23187 for 10 or 30 min, respectively. After incubation, 500 μ l HSM was added to cell mixture and centrifuged 5 min, 10,000g. The top 20 μ l of supernatant was removed and reserved for enzyme assay and the remaining volume of supernatant was measured and removed. The resultant pellet was resuspended to 500 μ l with HSM and assayed for enzyme activity. Enzyme partitioning was evaluated as the relative total enzyme activity in the supernatant versus cell associated enzyme in the pellet.

RESULTS

Immunolocalization studies using polyclonal antibodies against human liver α -L-fucosidase showed different amounts and patterns of distribution of this enzyme that varied depending upon the life history and acrosome status of the population. Figure 1 shows typical results of confocal microscopy. Images are represented as maximum projections of z stacks created using Zeiss LSM 510 software. Cells were doubly labeled for simultaneous visualization of antifucosidase immunofluorescence using a RITC-conjugated secondary antibody, and FITC-PSA to show acrosome status. Three distinctive immunolocalization patterns became evident in these experiments. Alterations of the sperm membrane system generated these different α -L-fucosidase staining patterns. Cells washed through a Percoll[®] gradient showed diffuse, faint α -L-fucosidase labeling in the head and midpiece regions (Fig. 1A). Capacitated cells revealed brighter labeling localized to the anterior head and faint staining of the midpiece and some tails (Fig. 1B). By contrast, as also shown in Figure 1A,B, there was no change in FITC-PSA-binding patterns on Percoll[®] washed versus capacitated cells. Note that one of the cells captured in the microscopic field of Figure 1B has undergone spontaneous acrosomal exocytosis, and anticipates the staining pattern revealed in Figure 1C,D. Acrosome-reacted cells, pro-

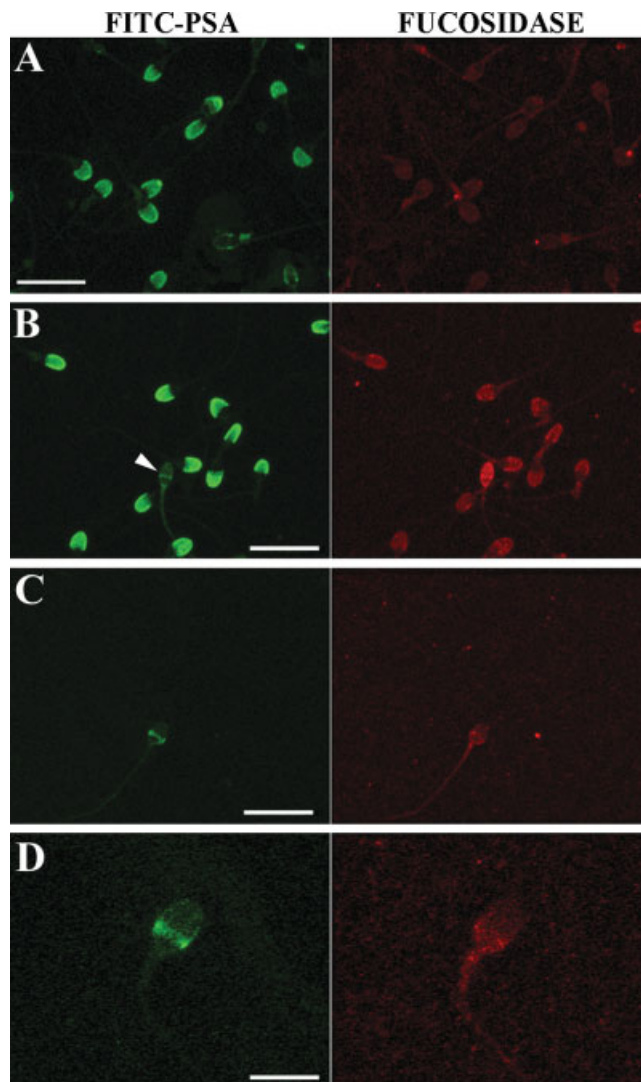


Fig. 1. Immunolocalization of α -L-fucosidase in human sperm cells. Confocal maximum projections of z stacks of washed (A), capacitated (B), and acrosome induced (C, D) human sperm cells. Cells were doubly labeled with FITC-PSA (green) and RITC conjugated secondary antibodies against polyclonal goat-anti-human liver α -L-fucosidase (red). No labeling was observed in the controls. Scale bar represents 10 microns (A–C) or 5 microns (D).

duced by treatment of a capacitated population with BrA23187 and Ca^{2+} , showed α -L-fucosidase labeling in the posterior head region (Fig. 1C,D), and the midpiece. Release of the acrosomal compartment, apparent by FITC-PSA staining, revealed this third distinctive immunolocalization pattern. Exocytosis of the acrosomal membrane exposed cryptic stores of α -L-fucosidase. These results were consistently observed for specimens from multiple donors and for multiple specimens from the same donor. Omission of the FITC-PSA double label yielded the same fluorescent localization pattern of fucosidase and no signal was detected on control slides.

Parallel results were obtained using RBF. Representative images obtained with laser scanning microscopy

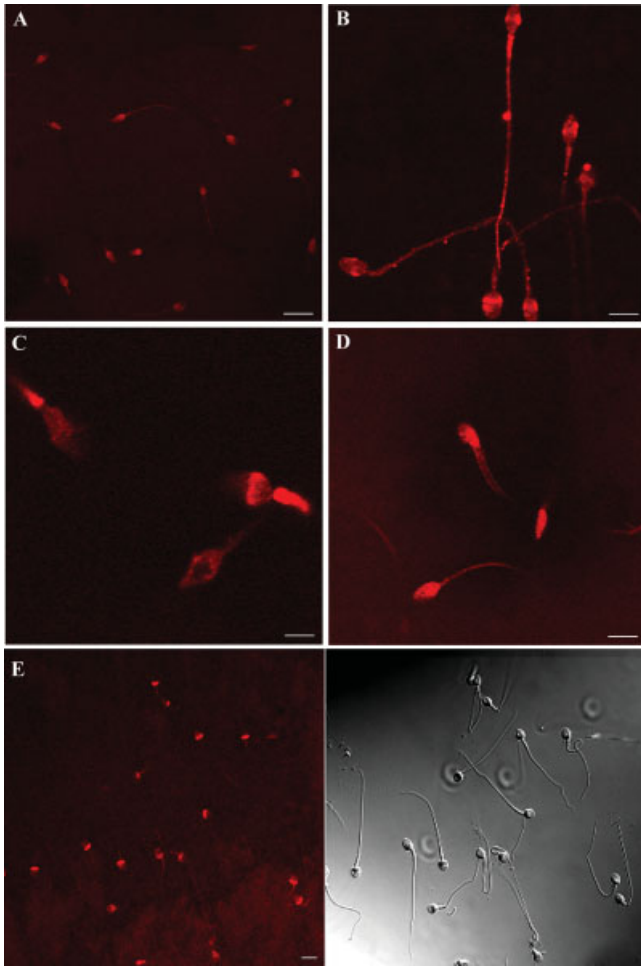


Fig. 2. Localization of fucose binding sites on human sperm. Percoll[®] washed (A), 0.1% (B) and 0.01% (C) Triton[®] X-100 treated, capacitated (D), and acrosome-induced (E) human sperm using RITC-BSA-fucose (RBF). All images acquired using Zeiss LSM 510 Meta confocal microscope at 63 \times oil (A–D) or 100 \times oil (E). Scale bar represents 10 microns (A, B, D, E) or 5 microns (C).

are shown in Figure 2. Washed cells displayed faint, diffuse head labeling, which developed gradually and was best observed after 20 min of incubation with 10 μ g/ml RBF glycoconjugate (Fig. 2A). Washed cells pretreated for 5 min with high concentrations (0.1% final concentration) of Triton[®] X-100 had bright equatorial band fluorescence immediately upon addition of RBF (Fig. 2B). This detergent concentration also resulted in loss of cell motility. Populations treated with lower detergent concentrations (0.01% Triton[®] X-100) and RBF displayed diffuse head labeling that was brighter than seen for washed cells, along with a low incidence of equatorial band labeling (Fig. 2C). This signal developed more rapidly (<20 min) than for washed cells. Very low concentrations (0.001% final) of Triton[®] X-100 gave results similar to those seen in the washed cell control. This concentration did not affect cell motility (not illustrated).

Capacitated cells stained with RBF (Fig. 2D) showed diffuse head labeling brighter than the signal seen in washed cells. Staining also appeared faster (<20 min). Following treatment for induction of AR, populations showed a variety of RBF staining patterns (Fig. 2E). The patterns included (1) bright equatorial band, (2) bright diffuse head, (3) posterior head, or (4) no signal. RBF staining patterns were scored along with the frequency of acrosome-reacted cells as visualized using FITC-PSA, and the specific results for scoring of over 200 cells per specimen, for five different populations, are reported in Table 1. On average, the staining patterns were 44.8% equatorial band, 49.2% diffuse head, 2.4% posterior head, and 3.6% no RBF staining. In such experiments, the proportion of cells that showed bright RBF staining of the equatorial segment was not significantly different from the proportion of acrosome-reacted cells.

Additional variables were introduced to evaluate the specificity for binding of RBF. Washed cells were pretreated with 0.1% Triton[®] X-100, followed by either 0.1% BSA or 10 μ g/ml FITC-BSA. Subsequently, RBF was added. Neither of these compounds (which lack fucose residues) showed significant alteration of the normal patterns of RBF binding that were described above.

For both approaches to subcellular localization of α -L-fucosidase, either by anti-fucosidase immunolocalization or by analysis of RBF binding sites, it was noted that significant staining of nonsperm cells (leucocytes, spermatids, tissue cells) is present. Immature sperm cells, sperm with cytoplasmic remnants (especially if broken or permeabilized), and abnormal forms of sperm do stain with the procedures used here. To minimize the influence of such complications, enrichment for normal and mature sperm was achieved through the Percoll[®] washing process.

Further analyses bearing on the subcellular location and amounts of the membrane-associated α -L-fucosidase were conducted using direct fluorometric assays of enzyme activity. These assays can be grouped into four categories: (a) comparison of the relative enzyme activity within populations that have been subjected to different preparative treatments including Percoll[®] washed, capacitated, and acrosome-induced populations, (b) evaluation of the overall crypticity for enzyme activity by contrasting measurable amounts of α -L-fucosidase in washed intact cells versus a duplicate specimen that had been permeabilized by treatment with either 0.01% Triton[®] X-100 or with BrA23187 (Table 2), (c) determination of the partitioning of total enzyme activity between solubilized versus cell-associated material following permeabilization and centrifugation at 10,000g (Fig. 3), and (d) monitoring of the stability of sperm-associated α -L-fucosidase over extended periods.

Alpha-L-fucosidase activity of cell populations sequentially Percoll[®] washed, capacitated, and acrosome-reacted were measured. Enzyme activity, beginning with Percoll[®] washing, persisted through capacitation and AR for any given cell population. Most specimens

TABLE 1. Patterns of Staining by RBF in Populations of Sperm Following Treatment for Induction of Acrosome Reaction

Donor	% Acrosome-reacted	RITC-BSA fucose staining			
		Equatorial band	Diffuse head	Posterior head	No staining
Donor 1	65.9%	68.8%	24.0%	2.7%	4.5%
Donor 2	52.1%	49.3%	46.1%	1.5%	3.1%
Donor 3	40.0%	38.1%	53.3%	5.3%	3.3%
Donor 4	40.0%	25.7%	69.0%	1.1%	4.1%
Donor 5	54.0%	42.1%	53.6%	1.4%	2.9%
Average	50.4%	44.8%	49.2%	2.4%	3.6%

Quantification of RBF staining in acrosome-induced sperm populations. Following Percoll[®] fractionation and capacitation, sperm populations were treated for induction of acrosome reaction. Five such populations were scored in detail for acrosome status and distribution of RBF signal.

Percent acrosome-reacted was determined by selective FITC-PSA staining. All percentages were based on scoring of at least 200 cells.

showed an increase in α -L-fucosidase activity, lowest to highest, progressing from Percoll[®] washed, capacitated, and acrosome-reacted cells. Some populations showed no increase or small decreases in enzyme activity, however the enzyme remained actively stable throughout all three preparations. Enzyme activity in all preparation types was inhibitable by 1 mM DFJ.

Crypticity for α -L-fucosidase activity was examined by assaying enzyme activity in suspensions of cells that

had been Percoll[®] washed followed by either one or two washes in HSM, with and without subsequent permeabilization to reveal intracellular enzyme activity. Both 0.01% Triton[®] X-100 and 0.01 mM BrA23187 were separately used as agents to permeabilize membranes with minimal short-term effects on motility and without detriment for fluorometric measurements. For each specimen tested, a crypticity ratio (CR), the ratio of measurable enzyme activity following permeabilization relative to that of the untreated sample, is reported in Table 2. For most specimens tested, there was a significant increase in measurable activity of α -L-fucosidase following permeabilization with either treatment, as reported for 10-min time points in Table 2. Treatment with BrA23187 showed an average CR of 3.0, while 0.01% Triton[®] X-100 gave an average CR of 3.7.

As human sperm are manipulated, and particularly when treated with surfactants, some of the fucosidase enzyme becomes solubilized (Alhadeff et al., 1999; Khunsook et al., 2003). Some of this activity may be associated with membrane fragments or vesicles, while some remains tightly associated with the sperm cells. To distinguish the partitioning of enzyme between cellular and solubilized material, we exposed various Percoll[®] fractionated specimens to 0.01 mM BrA23187, followed by centrifugation at 10,000g, and quantified enzyme activity in the supernatant versus sedimented material. Figure 3 summarizes the partitioning of α -L-fucosidase activity between cells and supernatant for 14 specimens. Under these conditions, an average of 60% of enzyme activity remained associated with the cellular material.

Preliminary experiments were conducted to evaluate the stability of plasma membrane-associated α -L-fucosidase enzyme activity. For example, one experiment performed in duplicate revealed after 24 hr incubation of Percoll[®] washed sperm at 37°C, 5% CO₂, 70% of the original fucosidase activity remained, and after 72 hr 48% of the fucosidase activity was detectable. These observations reflect a surprising persistence of enzyme activity which may be important within the context of human fertilization.

TABLE 2. Crypticity Ratio (CR) in Percoll[®] Washed Cells Treated with 0.01% Triton[®] X-100 (A) or 10 μ M BrA23187 Ionophore (B)

Specimen	CR
A	
31DT	7.7
41DT	4.6
26ZZ	3.2
36ZZ	4.4
5PM	2.4
51CM	1.1
46ZZ	1.8
66ZZ	6.3
71DT	2.8
3HJ	1.1
4HJ	3.1
Average	3.7
B	
51HJ	3.9
4GS	4.1
61BJ	2.1
71SP	2.9
5GS	6.6
8SD	1.2
81SP	4.4
1MX	1.4
2MX	1.9
48ZZ	1
Average	3.0

Enzyme activity was measured in human sperm cells before and after permeabilization with either 0.01% Triton[®] X-100 for 10 min or 10 μ M BrA23187 ionophore for 30 min. Average CRs were 3.7 and 3.0 for 0.01% Triton[®] X-100 and BrA23187 ionophore, respectively.

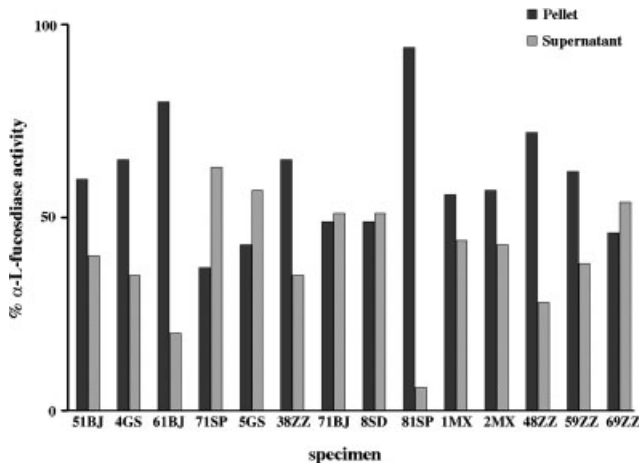


Fig. 3. Partitioning of alpha-L-fucosidase in human sperm cells. Following permeabilization with 10 μ M BrA23187 ionophore, percent relative fucosidase activity was calculated for the pellet and supernatant for each donor. On average, 59.6% of the fucosidase activity was associated with the pellet and 40.4% was associated with the supernatant.

DISCUSSION AND CONCLUSIONS

Previous investigations in this laboratory generated results of detailed biochemical characterization the distinctive isoforms of α -L-fucosidase that exist in human semen (Alhadeff et al., 1999; Khunsook et al., 2002, 2003). By contrast, the present report turns the focus toward the possible functions of the membrane-associated fucosidase of human sperm cells. Where possible, experiments reported here approach living cells and preparations of human sperm that retain relevance for reproductive function in nature or in assisted reproductive technologies. Three primary lines of evidence indicate that the membrane-associated α -L-fucosidase is present to some degree in most of the sperm membranes, with a portion of that glycoprotein detectable on the outer surface of intact sperm. By several criteria, however, larger quantities of this enzyme are present within cells, and particularly within the acrosomal compartment, and notably in the equatorial segment of the human sperm cell. Visualization of the enzyme, as well as direct measurement of enzyme action, vary as a function of the life history of the specimen, and depend particularly on the status or integrity of the membrane system of the cells. Results from studies on antifucosidase immunolocalization and analysis of the distribution of fucose-binding sites on sperm are in agreement, both showing compelling association with the equatorial segment. Two very different methods were used to alter access to the acrosomal compartment, namely (1) permeabilization of uncapacitated human sperm with nonionic detergent or (2) treatment of capacitated cells for induction of acrosomal exocytosis. Both treatments result in striking enhancement of visualization of α -L-fucosidase, and in measurable enzyme activity.

For quantitative comparisons, direct chemical assays for fucosidase activity are most appropriate. By introducing a simple and well-defined variable, namely treatment with Triton[®] X-100 or with BrA23187, permeabilization is achieved, making available enzyme molecules (or at least active sites) that were previously masked. Demonstration of crypticity for fucosidase activity in human sperm is reported here, with permeabilization resulting in a substantial multiplication in the amount of enzyme activity that is measurable. By contrast, the unpurified soluble seminal plasma isoform of this enzyme shows no comparable enhancement. Nevertheless, caution is appropriate since treatments that induce permeability may also cause disaggregation of protein complexes and repartitioning of ions or other substances within cells. To avoid serious damage to cells, we have selected treatment conditions that do not extensively compromise cellular motility. Taken together, our results strongly support the notion that the fucosidase is already well represented within the equatorial band of mature sperm, and that it presumably arrives there during earlier stages of sperm development. In addition, there may be some redistribution of enzyme within the sperm membrane system during capacitation and AR.

In addition to the enzyme that is detectable in the equatorial segment, it is also represented in the general membrane system of the sperm. Relatively little is exposed on the outer surface of the outer acrosomal membrane, but when that membrane is permeabilized or released by detergent treatment or by acrosomal exocytosis, enzyme activity is evident. These observations suggest that some of the enzyme is probably present on the outer acrosomal membrane, facing into the acrosomal compartment.

The very occurrence of distinct isoforms of α -L-fucosidase in the seminal plasma and in the sperm membrane system, and the impressive amounts of enzyme activity present, are provocative. When considered in the context of evidence for the involvement of fucose moieties in the intimate intracellular interactions of reproduction in humans and other animals, the roles of fucosidase become compelling topics for further analysis. It remains possible that both isoforms of seminal fucosidase have functions that advance the likelihood for reproduction. The seminal plasma enzyme may function to ease the passage of sperm through the cervical mucus and reproductive tract, while the sperm membrane fucosidase may have roles in recognition and penetration at the level of the zona pellucida or oolemma. With recognition of the enrichment of this enzyme within the equatorial segment, a role that facilitates membrane to membrane interaction becomes more likely than previously thought. However, since α -L-fucosidase is common in many human tissues, it is unlikely to be a useful target for immunocontraception.

The results presented here enhance the concept of the sperm equatorial segment. Fucosidase must be added to the list of distinctive structural elements that make up this key region sequestered within the sperm cell. This

membranous surface normally only becomes exposed following exocytosis of the acrosomal membranes and soluble components. Its architecture seems relatively stable, and has key features of both a compartment and a membranous surface poised for signature functions during fertilization. The equatorial segment is a compartment by virtue of its limited diffusion and communication with other regions of the sperm cell. The equatorial segment is prefabricated for surface function, but sequestered and protected from exposure until key moments in fertilization. During extended incubation in situ, as reported here, the α -L-fucosidase retains enzyme activity. This observation is especially provocative since the enzyme deteriorates quickly following extraction from sperm cells, as previously reported (Khunsook et al., 2003). The stability of this enzyme for extended periods corresponds with the expected time requirements for effective sperm transport and opportunity to encounter the oocyte. We imagine that fucosidase could be one of the first essential functions in membrane to membrane interaction, with removal or exchange of fucose residues on the oocyte surface being critical for the initiation of further steps in specific molecular interactions between these cells.

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