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Inhibition of the Mouse Sperm Surface α-D-Mannosidase Inhibits Sperm-Egg Binding in Vitro[1]

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ABSTRACT

In previous reports from this laboratory, we identified the presence of a novel α-D-mannosidase on the surface of rat, mouse, hamster, and human spermatozoa [J Cell Biol 1989; 109:1257–1267 and Biol Reprod 1990; 42:843–858]. Since it has been suggested that mannosyl residues on the egg zona pellucida may be important for sperm-egg binding, studies were undertaken to examine the potential role of the sperm α-D-mannosidase during fertilization. Incubation of mouse spermatozoa in the presence of increasing concentrations of the inhibitory sugars, α-methyl mannoside, α-methyl glucoside, D-mannose, or D-mannitol, resulted in a dose-dependent decrease in the number of spermatozoa bound per egg without a deleterious effect on sperm motility or on the sperm acrosome, and a dose-dependent inhibition of the sperm mannosidase activity. Galactose, however had no effect on sperm-egg binding or on sperm mannosidase activity. Two nucleotide sugars (UDP-GlcNAc and UDP-gal) were also tested and shown to reduce sperm-egg binding but with only a minimal effect on sperm mannosidase activity. In additional studies, spermatozoa incubated in the presence of a mannose-containing oligosaccharide exhibited a dramatic reduction in sperm-egg binding that correlated with a similar inhibition of sperm mannosidase activity. The oligosaccharide substrate did not affect sperm motility or the sperm acrosome. These studies suggest that the sperm α-D-mannosidase may play an important role during fertilization.

INTRODUCTION

The early events of fertilization involve the recognition and interaction between complementary molecules present on spermatozoa and the egg zona pellucida. While the precise nature of these complementary molecules has not been well-defined, several lines of evidence suggest that glycoconjugates present on the gamete surface are involved [1–3]. Specifically, it has been proposed that carbohydrate moieties present on the zona pellucida act as ligands for receptor-like components present on the surface of capacitated spermatozoa.

In the mouse, several sperm surface enzymes have been implicated as possible binding sites or receptors for the zona pellucida ligand including galactosyltransferase [4–6] and fucosyltransferase [7]. In addition, other sperm proteins including trypsin-like protease [8] and a 95-kDa protein [9] have also been suggested to serve as receptors during sperm-zona binding. It is critical to point out that throughout the literature the complementary molecules on the spermatozoa and zona pellucida have both been interchangeably referred to as ligands or receptors. The conventions of cell biology dictate that the small binding molecule or functional group should be designated the ligand, and the plasma membrane protein binding to that molecule or functional group should be designated the binding site or receptor. Therefore, throughout this report we refer to the sperm surface enzymes that bind to the zona pellucida as binding sites or receptors, and the complementary molecule on the zona pellucida is referred to as the ligand.

Recently, our laboratory identified a novel α-D-mannosidase on the surface of rat, mouse, hamster, and human spermatozoa. Initial characterization studies revealed this enzyme to be quite different from previously characterized mannosidases. In contrast to the sperm acrosomal acidic mannosidase, which has a pH optimum of 4.4 and is active primarily towards the synthetic substrate *p*-nitrophenyl α-D-mannopyranoside, the newly discovered mannosidase has a pH optimum of 6.5 and is active primarily toward mannose-containing oligosaccharides [10, 11]. Since several reports have suggested that mannose residues on the rat and mouse zona pellucida may be involved in sperm-zona binding [12, 13], studies were undertaken to examine the potential role of the novel sperm mannosidase during fertilization. In this report we demonstrate that incubation of mouse spermatozoa in the presence of the inhibitory sugars α-methyl mannoside, α-methyl glucoside, D-mannose, or D-mannitol resulted in a dose-dependent inhibition of the sperm surface mannosidase and a dose-dependent decrease in the number of spermatozoa bound per egg as measured by an in vitro sperm-egg binding assay. Moreover, incubation of mouse spermatozoa with a mannose-containing oligosaccharide resulted in an inhibition of sperm surface mannosidase activity and sperm-egg binding. These sugars, however, had no effect on sperm motility or acrosomal integrity. These studies suggest that the sperm surface α-D-mannosidase may have an important role during fertilization.

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MATERIALS AND METHODS

Materials

Methyl-α-D-mannoside, methyl-α-D-glucoside, D-mannose, UDP-*N*-acetylglucosamine (UDP-GLcNAc), UDP-galactose (UDP-gal), chlortetracycline, eCG, and hCG were purchased from Sigma Chemical Co., St. Louis, MO; D-mannitol was obtained from Aldrich Chemical Co., Milwaukee, WI; and galactose was obtained from Mann Research Laboratories, New York, NY. All other reagents used were of the highest grade available.

In Vitro-Sperm-Zona Binding Assay

Mature male C57B16 mice (30–35 g body weight) purchased from Sasco (St. Louis, MO) and ICR female mice (22–25 g body weight) purchased from Harlan (Indianapolis, IN) were housed under a constant 16L:8D cycle and allowed free access to food and water.

Male mice were killed by an overdose of ether and each cauda epididymidis was excised and minced in Toyoda's medium [14] containing 20 mg/ml BSA, pH 7.4, to allow the spermatozoa to disperse. The minced tissue was incubated for 30 min at 37°C, 5% CO_2 after which the tissue pieces were removed and an aliquot of the sperm suspension was withdrawn to determine the sperm concentration. Incubation of the spermatozoa continued for an additional 30 min before being used in the zona binding studies.

Female mice were induced to superovulate by injection of 10 IU eCG followed by injection of 10 IU hCG 48 h later. Fifteen to sixteen hours after hCG injection, the mice were killed by ether overdose and the oviducts were removed and placed into Biggers, Whitten and Whittingham media (BWW), pH 7.4. Under a dissecting microscope, each oviduct was pricked with a needle and the cumulus mass was removed. The cumulus masses from each of five animals were pooled and treated with 0.1% hyaluronidase to disperse the cumulus cells. The denuded eggs were then washed through three changes of BWW medium before being allotted to 100-μl droplets of BWW under silicon oil.

Approximately 1×10^5 spermatozoa (1×10^6 spermatozoa/ml) were added to each droplet of eggs and incubation of spermatozoa and eggs was carried out for 60 min under oil at 37°C, 5% CO_2. At this time the eggs were removed from each droplet and washed three times in BWW; a narrow-bore pipette was used to transfer the eggs. The eggs were then mounted onto glass slides and the number of spermatozoa bound per egg was determined using a Zeiss phase-contrast microscope.

To determine if various sugars or a mannose-containing oligosaccharide ($Man_5GlcNAc$), prepared in our laboratory from the kidneys of rats administered swainsonine [15, 16], could affect the binding of spermatozoa to the egg zona pellucida, stock solutions of specific sugars or the oligosaccharide were prepared in BWW, and a small volume (5–50 μl) was added to the droplet of BWW to yield 5–50 mM final concentrations of the sugars or 0.7–3.8 mM concentrations of the $Man_5GlcNAc$. The eggs and spermatozoa were then added to the droplet, resulting in a final droplet volume of 100 μl. Incubation of the spermatozoa and eggs was carried out for 60 min at 37°C, 5% of CO_2 under oil, after which the eggs were processed as described above. The number of spermatozoa bound per egg was determined for each treatment group and was compared to the number of spermatozoa bound per egg for the control group. Control incubations were carried out in the absence of sugars or oligosaccharide.

Sperm Motility Assay

To determine if the sugars or the $Man_5GlcNAc$ substrate adversely affected sperm motility, $1–2 \times 10^5$ cauda epididymal spermatozoa were incubated in Toyoda's buffer, 37°C, 5% CO_2 for 60 min to allow capacitation. At this time, the various sugars or $Man_5GlcNac$ were added to the spermatozoa to a final concentration of 50 mM or 3.8 mM, respectively. These amounts of sugars or substrate represented the highest concentrations tested in the sperm-egg binding assay. Immediately after the addition of the sugar or substrate (Time 0) and again at 15-min intervals over 1 h, the percentage of progressively motile spermatozoa was determined subjectively by determining out of 100 total spermatozoa the number of spermatozoa that were progressively motile. In addition, for each of the sugars or substrate the quality of the sperm motility was estimated at each of these time points and was rated from 1 (motile with no forward progression) to 4 (vigorous forward motility).

Chlortetracycline Fluorescence Assay

To examine if the sugars or the $Man_5GlcNAc$ substrate affected the integrity of the sperm acrosome, the percentage of cauda spermatozoa with intact acrosomes was determined after incubation in the presence of 50 mM concentrations of sugars or 3.8 mM $Man_5GlcNAc$. Briefly, $1–2 \times 10^5$ spermatozoa were allowed to capacitate in Toyoda's buffer at 37°C, 5% CO_2 for 60 min. At this time, a sugar or $Man_5GlcNAc$ was added to the spermatozoa to a final concentration of 50 mM or 3.8 mM, respectively; immediately thereafter and again 60 min later, an aliquot of spermatozoa was removed and placed onto a glass slide. Ten microliters of a 500 μM solution of chlortetracycline prepared in 20 mM Tris, 130 mM NaCl, and 5 mM cysteine, pH 7.8, was added to the spermatozoa and gently mixed [17]. After 10 sec 12.5% glutaraldehyde in 1 M Tris buffer, pH 7.8, was added to a final concentration of 0.1%. The spermatozoa were then examined using a Zeiss fluorescent microscope with the excitation wavelength set at 390 nm and the emission wavelength at 520 nm. The spermatozoa were scored for fluorescence on the basis of criteria established by Ward and Storey [17]. Spermatozoa with intact acrosomes exhibited a bright green fluorescence over the entire sperm head

or on the anterior portion of the head. Spermatozoa that had undergone the acrosome reaction and had lost their acrosomes showed a barely detectable fluorescence over the entire sperm head.

Sperm α-D-Mannosidase Assay

A uniformly labeled high mannose oligosaccharide ($Man_9GlcNAc$) was obtained from a [^{3}H]mannose-labeled glycopeptide prepared from rat epididymal epithelial cells cultured in the presence of [2-^{3}H]mannose as described previously [16]. In preliminary studies, it was found that the rate of hydrolysis of [^{3}H]$Man_9GlcNAc$ was directly proportional to the sperm concentration ($0.25–1.0 \times 10^6$ spermatozoa). The reaction mixture containing 0.5×10^6 spermatozoa was linear for at least 4 h. Since the K_m for the substrate is not yet known, the assay was always done using a longer incubation time (2–4 h) than the 1 h used in the in vitro sperm-zona binding assay described above. Due to the longer incubation time, the sperm surface mannosidase was able to hydrolyze 10–15% of the added oligosaccharide as free [^{3}H]mannose, a number much more reliable than the 2–6% obtained with shorter incubation times.

Cauda epididymal spermatozoa ($0.5–1 \times 10^6$ spermatozoa) in Toyoda's buffer were incubated in a 50-μl incubation mixture containing 4000 cpm [^{3}H]mannose-labeled oligosaccharide substrate ($Man_9GlcNAc$) and 100 mM cacodylate buffer, pH 7.4. Spermatozoa were incubated for 2–4 h at 37°C to allow hydrolysis of the substrate, after which the reaction was stopped by boiling the assay mixture for 5 min (Fig. 1). The released [^{3}H]mannose was separated from the labeled oligosaccharide by gel filtration on a column of Bio-Gel P-2 and quantified as described previously [18]. One unit is the amount of enzyme that catalyzes the release of 1000 cpm of [^{3}H]mannose/h at 37°C.

To determine the effect of different sugars or the $Man_5GlcNAc$ on sperm mannosidase activity, spermatozoa were incubated with varying concentrations of the sugars for 10 min on ice prior to the addition of the radiolabeled substrate.

Statistical Analysis

To determine if the number of spermatozoa bound per egg in the presence of the various sugars or mannose-containing oligosaccharide was significantly different from that observed in control spermatozoa (incubated in the absence of sugars), a Wilcoxon signed rank test was utilized.

RESULTS

To test the involvement of the sperm mannosidase in fertilization, initial studies were undertaken to determine if simple mannose sugars could inhibit the sperm mannosidase activity as well as the binding of spermatozoa to the zona pellucida. Presumably, these sugars would compete with the substrate in the enzyme assay or with the glycoconjugate on the zona pellucida for the sperm mannosidase, thereby causing inhibition of enzyme activity or sperm-egg binding. Other simple non-mannose-containing sugars were used in the sperm mannosidase assay and sperm-egg binding assay as controls.

Cauda epididymal spermatozoa were incubated in the presence of increasing concentrations of α-methyl mannoside, α-methyl glucoside, D-mannose, D-mannitol, or galactose and the sperm-egg binding or the sperm mannosidase activity was measured. As shown in Figure 2, α-methyl mannoside, α-methyl glucoside, D-mannose, and D-mannitol caused a dose-dependent decrease in the number of spermatozoa bound per egg (Fig. 2a), which correlated with a dose-dependent decrease in the sperm mannosidase activity (Fig. 2b). As reflected by both the sperm-egg binding assay and the sperm mannosidase assay, the most potent inhibitors of the sperm mannosidase were α-methyl mannoside and D-mannose. Alpha-methyl mannoside at 50 mM resulted in a 60–80% reduction ($p < 0.05$ vs. control) in mannosidase activity and sperm-egg binding, and 50 mM D-mannose caused a 60% reduction ($p < 0.05$ vs. control) in activity and binding. D-mannitol also caused a dramatic inhibition of sperm-egg binding (60% at 50 mM; $p < 0.05$), although a less striking effect on the sperm mannosidase activity. Since glucose is the structural epimer of mannose,

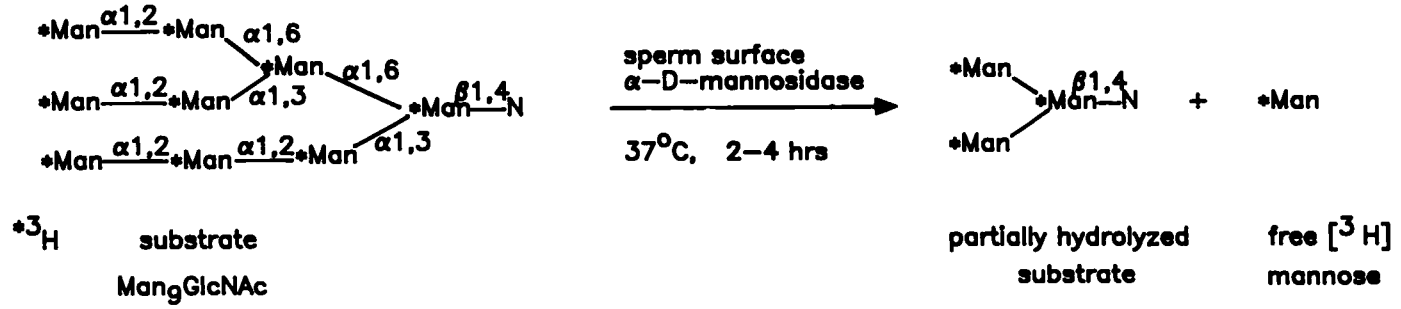
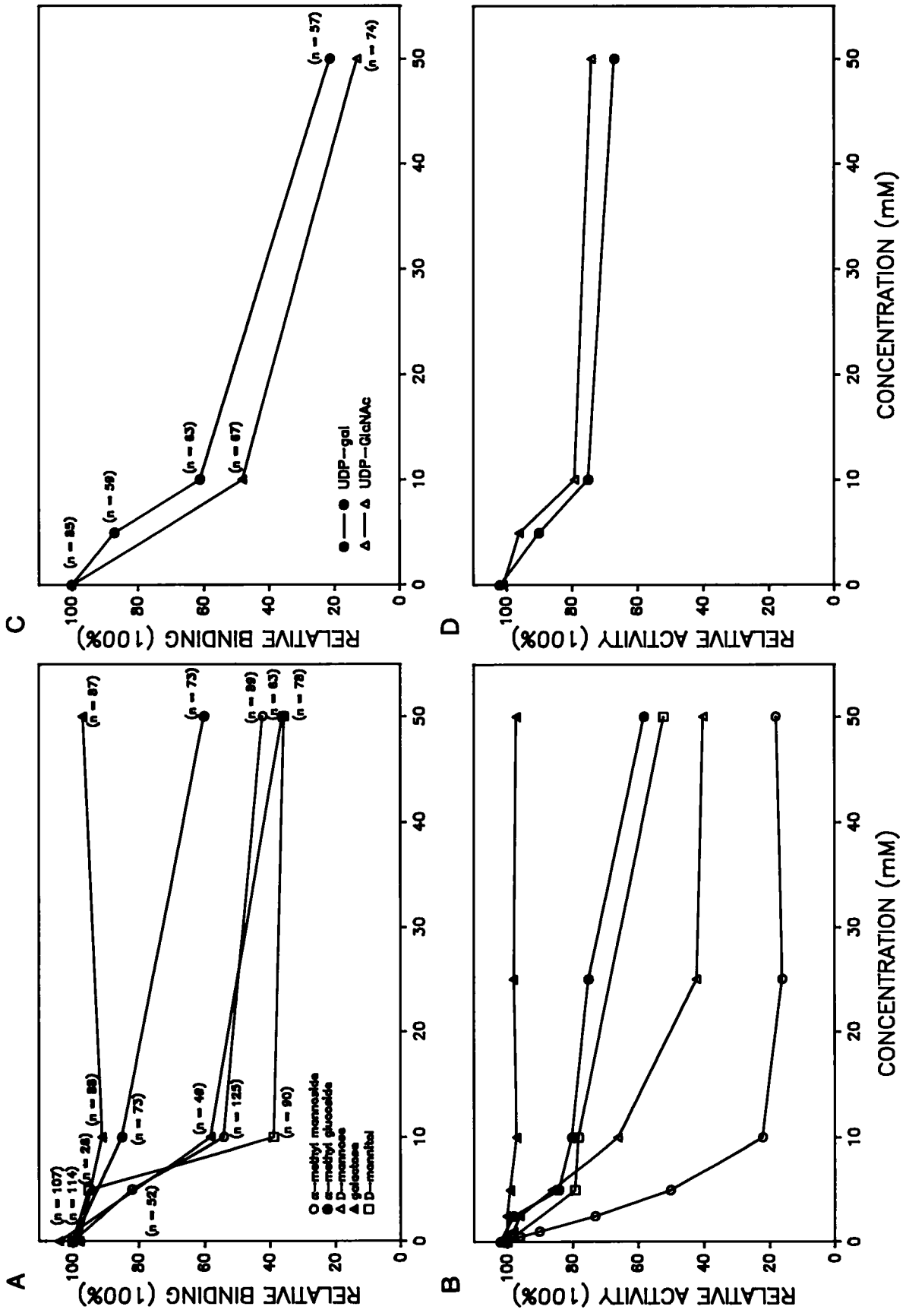


FIG. 1. Sperm α-D-mannosidase assay. Cauda epididymal spermatozoa ($0.5–1 \times 10^6$ spermatozoa) in Toyoda's buffer were incubated in a 50 μl incubation mixture containing 4 000 cpm [^{3}H]mannose-labeled oligosaccharide substrate, $Man_9GlcNAc$, and 100 mM cacodylate buffer, pH 7.4. After 2–4 h at 37°C to allow hydrolysis of the substrate the reaction was stopped by boiling the assay mixture for 5 min. The released [^{3}H]mannose was separated from the labeled oligosaccharide and quantified. One unit is the amount of enzyme that catalyzed the release of 1000 cpm of [^{3}H]mannose/h at 37°C.



it was not surprising that α-methyl glucoside inhibited the sperm mannosidase, although not as effectively as the mannose-containing sugars. Galactose, however, showed no effect on the sperm mannosidase activity or on sperm-egg binding ($p > 0.05$ vs. control). In addition, two nucleotide sugars, UDP-GlcNAc and UDP-gal, were tested for their ability to affect sperm mannosidase activity and sperm-egg binding. One of these nucleotide sugars, UDP-gal, has previously been shown to reduce sperm-egg binding in the mouse by inhibition of the sperm surface galactosyltransferase [4, 5]; therefore, this sugar served as a positive control for our assays. In contrast to other investigators [4, 5, 26], we used higher concentrations of the UDP sugars in our assays for two reasons. First, we wanted to compare the effects of these nucleotide sugars at a concentration similar to that used for D-mannose and other simple sugars. Second, since AMP was not included in the assay to prevent hydrolysis of the nucleotide sugars by sperm phosphatases, an excess of the nucleotide sugars ensured that an adequate level of these sugars was present at the completion of the sperm-zona binding and sperm mannosidase assay. Figure 2c demonstrates that incubation of spermatozoa in the presence of increasing concentrations of UDP-GlcNAc or UDP-gal resulted in a dose-dependent reduction ($p < 0.05$ vs. control at 10 and 50 mM) in the number of spermatozoa bound to the zona pellucida, with only a slight inhibitory effect on the sperm mannosidase activity (Fig. 2d).

To examine more specifically the role of the sperm mannosidase in sperm-egg binding, cauda epididymal spermatozoa were incubated in the presence of increasing concentrations of $Man_5GlcNAc$, and the effect on sperm-egg binding and sperm mannosidase activity was determined. Unlike the simple monosaccharides examined in the initial studies, $Man_5GlcNAc$ is a mannose-containing oligosaccharide, the structure of which renders it a specific substrate for the sperm mannosidase (Fig. 3a insert). Indeed, it is structurally very similar to the labeled substrate, $Man_9GlcNAc$, used to assay the sperm mannosidase activity (Fig. 1). Moreover, from our previous studies we know that the $Man_5GlcNAc$ is a substrate for both rat [10] and human [11] spermatozoa. Therefore, the $Man_5GlcNAc$ should act as a very effective competitor for the labeled substrate used in the sperm mannosidase assay as well as be able to compete effectively for the recognition of mannose-containing oli-

FIG. 2. The effect of the sugars α-methyl mannoside, α-methyl glucoside, D-mannose, D-mannitol, galactose, UDP-GLcNAc, and UDP-galactose on sperm-egg binding and sperm mannosidase activity. (A and C) Cauda spermatozoa (1×10^5) were incubated for 60 min at 37°C, 5% CO_2 under oil in a 100-μl droplet of BWW buffer containing zona-intact eggs and 5–50 mM concentrations of the sugars. The eggs were then washed three times in BWW buffer and the number of spermatozoa bound per egg was determined for each sugar and at each concentration: (A) Mean ± SE: control, 26.7 ± 1.8; 10 mM galactose, 24.3 ± 2.4; 50 mM galactose, 26.0 ± 2.2; 5 mM α-methyl mannoside (α-MM), 22.8 ± 1.8; 10 mM α-MM, 13.7 ± 1.4; 50 mM α-MM, 11.1 ± 1.7; 10 mM α-methyl glucoside (α-MG), 22.4 ± 2.4; 50 mM α-MG, 15.5 ± 1.6; 10 mM D-mannose, 10.5 ± 1.2; 50 mM D-mannose, 8.7 ± 0.9; 5 mM D-mannitol, 25.4 ± 1.9; 10 mM D-mannitol, 10.3 ± 1.2; 50 mM D-mannitol, 9.5 ± 1.4 (C) Control, 16.1 ± 2.4; 5 mM UDP-gal, 14.0 ± 2.8; 10 mM UDP-gal, 9.7 ± 1.3; 50 mM UDP-gal, 3.5 ± 0.7; 10 mM UDP-GlcNAc, 7.7 ± 0.8; 50 mM UDP-GlcNAc, 2.1 ± 0.1. One hundred percent binding represents the number of spermatozoa bound per egg for control samples, i.e. spermatozoa and eggs incubated in the absence of sugars. (n) = number of eggs. (B and D) Cauda spermatozoa were incubated in the presence of 5–50 mM concentration of sugars for 10 min at 4°C, after which the radiolabeled oligosaccharide substrate ($Man_9GlcNAc$) was added. After 2 h, the reaction was stopped and the released [^{3}H]mannose was quantified. One hundred percent activity represents the release of 500 cpm as free [^{3}H]mannose from the spermatozoa incubated in the absence of sugars (control). Values represent the average of two experiments with a range of 10% for each experiment.

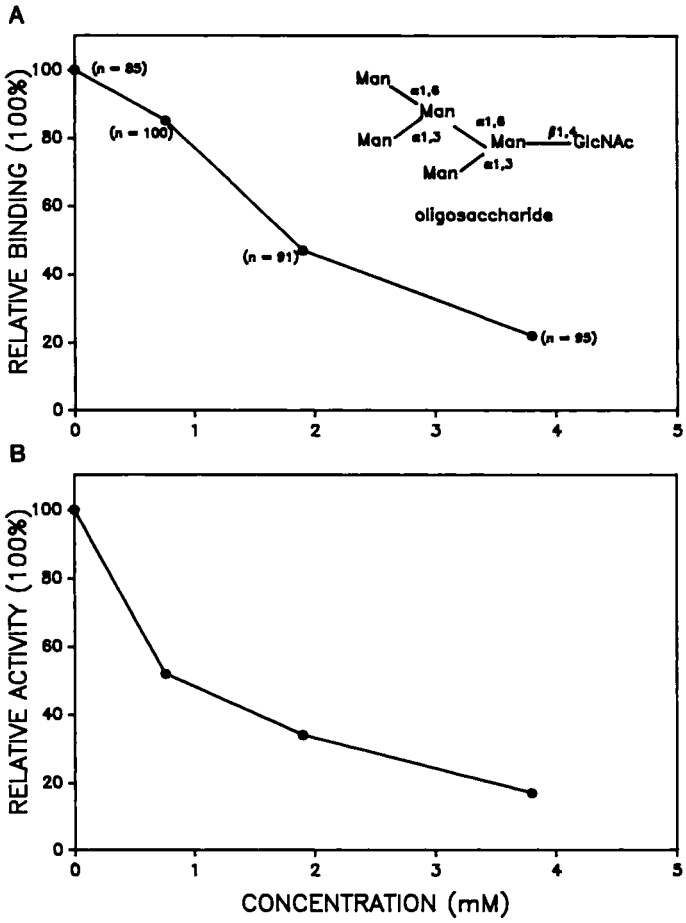


FIG. 3. The effect of the mannose-containing oligosaccharide substrate ($Man_5GlcNAc$) on sperm-egg binding and sperm mannosidase activity. (A) Cauda spermatozoa 1×10^5 were incubated for 60 min at 37°C, 5% CO_2 under oil in 100 μl BWW buffer in the presence of 0.76, 1.9, or 3.8 mM concentration of $Man_5GlcNAc$ and zona-intact eggs. Eggs were then washed three times in BWW and the number of spermatozoa bound per egg was determined: Mean ± SE: control, 16.1 ± 2.4; 0.76 mM $Man_5GlcNAc$, 13.7 ± 1.7; 1.9 mM $Man_5GlcNAc$, 7.5 ± 1.0; 3.8 mM $Man_5GlcNAc$, 3.6 ± 0.7. One hundred percent binding represents the number of spermatozoa bound per egg for control samples incubated in the absence of $Man_5GlcNAc$. (Insert) Structure of the oligosaccharide substrate $Man_5GlcNAc$. (B) Cauda spermatozoa were incubated in the presence of $Man_5GlcNAc$ for 10 min at 4°C, after which the radiolabeled substrate was added. After 2 h, the reaction was stopped and the released [^{3}H]mannose was quantified. One hundred percent activity represents the release of 500 cpm as free [^{3}H]mannose from spermatozoa incubated in the absence of $Man_5GlcNAc$ (control). Values represent the average of two experiments with a range of 10% for each experiment.

gosacchrides on the zona pellucida during the sperm-zona binding assay. These oligosaccharide residues on the zona pellucida are presumably the recognition site(s) for the sperm surface mannosidase.

As shown in Figure 3, increasing concentrations of $Man_5GlcNAc$ caused a dose-dependent reduction in the number of spermatozoa bound per egg (Fig. 3a), which correlated with a dose-dependent reduction in the activity of the sperm mannosidase (Fig. 3b). At 3.8 mM concentrations of the substrate, an 80% reduction in sperm-egg binding ($p < 0.05$) vs. control) and in sperm mannosidase activity was observed. These studies are consistent with our initial observations on the inhibitory effects of simple monosaccharides and support our belief that sperm surface mannosidase may be important for sperm-egg binding.

To confirm that the observed inhibition in sperm-egg binding and sperm mannosidase activity is not due to the effect of these compounds on sperm motility, the effect of the sugars on sperm motility was examined. Cauda epididymal spermatozoa were incubated in the presence of the highest concentrations of sugars tested in the sperm-egg binding assay, 50 mM concentrations of the monosaccharides or 3.8 mM $Man_5GlcNAc$, and the percentage of progressively motile spermatozoa was determined over 60 min. As shown in Figure 4, the percentage of spermatozoa that were progressively motile after incubation in the presence of the monosaccharides or the $Man_5GlcNAc$ was not different from that of control spermatozoa incubated in the absence of sugars. UDP-GlcNAc, however, was the only nucleotide sugar in which the percentage of progressively motile spermatozoa was lower than that in control spermatozoa. Moreover, when the quality of the sperm motility was examined for all the sugars as well as the $Man_5GlcNAc$,

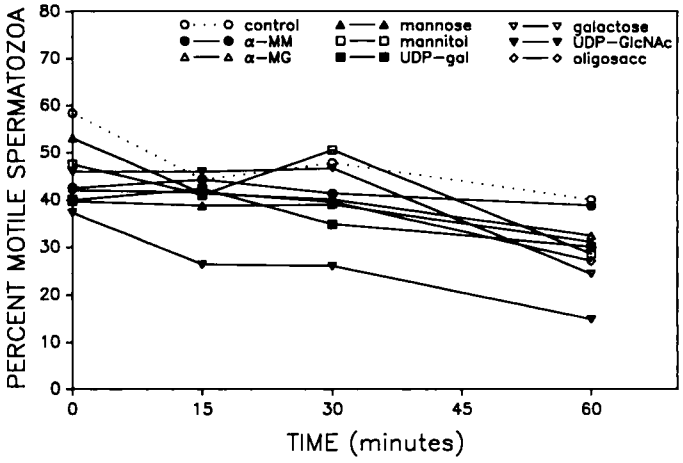


FIG. 4. The percentage of spermatozoa progressively motile after incubation in the presence of monosaccharide sugars, nucleotide sugars, or $Man_5GlcNAc$. Fifty-millimolar concentrations of the sugars or 3.8 mM concentration of the $Man_5GlcNAc$ were added to 1-2 × 10^6 cauda spermatozoa and motility was examined over 60 min. Values represent the mean of four experiments with the average fractional standard error of the mean ranging from 0.01–0.4. α-MM, α-methylmannoside; α-MG, α-methylglucoside; oligosacc, $Man_5GlcNAc$.

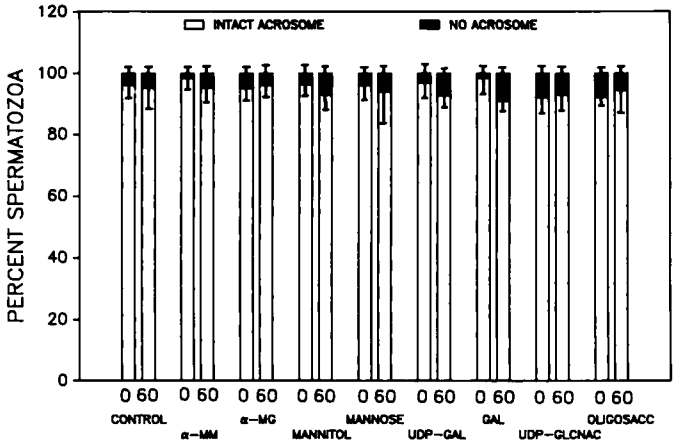


FIG. 5. The percentage of cauda spermatozoa with intact acrosomes after incubation in the presence of monosaccharide sugars, nucleotide sugars, or $Man_5GlcNAc$. Fifty-millimolar concentrations of the sugars or 3.8 mM concentration of the $Man_5GlcNAc$ were added to 1–2 × 10^6 spermatozoa; the integrity of the sperm acrosome was determined by chlortetracycline fluorescence immediately thereafter and again 60 min later. Spermatozoa with intact acrosomes exhibited a bright green fluorescence over the entire sperm head or on the anterior portion of the head. Spermatozoa that had lost their acrosome show a barely detectable fluorescence over the entire sperm head. Values represent the mean ± SEM of 3–4 experiments. α-MM, α-methylmannoside; α-MG, α-methylglucoside; oligosacc, $Man_5GlcNAc$.

only spermatozoa incubated in the presence of UDP-GlcNAc or UDP-gal at concentrations higher than 10 mM showed reduced values; grade 2–2.5 for the nucleotide sugars versus grade 3.5–4 for control and other treated spermatozoa (data not shown). While it is important to assess the sperm motility parameters in greater detail, i.e. velocity, these studies suggest that inhibition of the sperm-egg binding in the presence of the monosaccharides or the $Man_5GlcNAc$ is not due to their nonspecific effect on sperm motility.

Additional experiments were carried out to determine if the presence of the monosaccharides, nucleotide sugars, or $Man_5GlcNAc$ removed the sperm acrosome. Since it has been shown that mouse spermatozoa require the presence of an intact acrosome for zona binding [19], removal of the acrosome by the mannose sugars or substrates would result in an artifactual reduction in sperm-egg binding. Cauda epididymal spermatozoa were incubated in the presence of 50 mM concentrations of the monosaccharide or nucleotide sugars or 3.8 mM $Man_5GlcNAc$, and the integrity of the sperm acrosome was determined by chlortetracycline staining immediately after the addition of the sugars or 60 min later. Figure 5 shows that even after 60 min incubation in the presence of the sugars or the $Man_5GlcNAc$, 90% or more of the spermatozoa possessed intact acrosomes. These observations confirm that the reduction in sperm-egg binding and the inhibition of the sperm mannosidase activity is not due to loss of the sperm acrosome.

DISCUSSION

Several lines of evidence suggest that the novel α-D-mannosidase may play a role in the fertilization process. First,

our initial characterization studies of the mannosidase have shown the enzyme to possess several criteria that are fundamental for a functional role in sperm-zona binding. These criteria include the observations that the sperm mannosidase is a plasma membrane component and that the catalytic domain of the enzyme is oriented towards the outer surface of the spermatozoa [10]. Moreover, the sperm mannosidase activity has been detected on the spermatozoa of several species including rat, mouse, hamster [10], and human [20], and has been shown to increase on rat and hamster epididymal spermatozoa as they mature in the epididymis [20].

In addition to our initial characterization studies of the sperm mannosidase, the studies presented in this report further supported a putative role for the sperm mannosidase in sperm-egg binding. When mouse cauda epididymal spermatozoa were incubated in the presence of increasing concentrations of the mannose sugars α-methyl mannoside, D-mannose, or D-mannitol or in the presence of a structural epimer of mannose, α-methyl glucoside, the number of spermatozoa that bound per egg as well as the sperm mannosidase activity decreased in a dose-dependent manner. Since the sperm mannosidase showed substrate specificity with mannose-containing oligosaccharides, these mannose sugars most likely bind to the catalytic site(s) of the sperm surface mannosidase, thereby causing inhibition of the enzyme. Galactose, however, did not bind to the sperm surface mannosidase and, as reflected in the sperm-zona binding assay and enzyme assay, did not cause inhibition. Also, control experiments revealed that these sugars did not alter sperm motility or sperm acrosome integrity. Therefore, the inhibition of sperm-egg binding observed in the presence of the sugars was likely a consequence of the inhibition of sperm surface mannosidase. It is worth mentioning that although the mannose sugars showed a dose-dependent inhibition of both the sperm surface mannosidase and sperm-zona binding, the magnitude of inhibition was somewhat different with some of the sugars. In particular, D-mannitol at 10 mM showed a 60% inhibition of sperm-egg binding but only a 20% inhibition of sperm surface mannosidase (Fig. 2). We have no explanation for the observed differences other than perhaps that the binding of D-mannitol to the catalytic site(s) prevented the enzyme from recognizing high-molecular-weight zona glycoprotein(s). Additional studies are needed and may resolve this issue.

Two nucleotide sugars, UDP-GlcNAc and UDP-galactose, were also tested in the sperm-egg binding assay and the sperm mannosidase assay. Upon initial observation, it appears that at 50 mM concentrations, both UDP-GlcNAc and UDP-galactose were more potent than the mannose sugars in inhibiting sperm-egg binding, with a less dramatic effect on the sperm mannosidase activity. However, unlike all other sugars tested previously, at 50 mM concentrations of UDP-GlcNAc or UDP-gal the motility of the spermatozoa was impaired and therefore the inhibition of sperm-zona binding observed at this concentration could have been, in part, due to impaired motility. Fifty-millimolar concentrations of the UDP sugars, however, did not alter the integrity of the sperm acrosome. At 10 mM UDP-GlcNAc or UDP-gal, the sperm motility was not affected, and a 40–50% reduction in sperm-zona binding and a 20–25% inhibition of sperm mannosidase activity was observed. While the inhibition of sperm-egg binding most likely is due to the inhibition of galactosyltransferase by the UDP sugars [4, 5], the mechanism by which these nucleotide sugars affect the sperm mannosidase is unclear. One possible explanation is that if galactosyltransferase and mannosidase are positioned close to each other on the sperm plasma membrane, then the binding of the UDP sugars to the galactosyltransferase could sterically mask the mannosidase and prevent it from binding to the radioactive mannose substrate used in the sperm mannosidase assay. A second, less plausible, explanation is that some component in the UDP sugars is recognized by the sperm mannosidase.

The potential role of the sperm mannosidase in sperm-egg binding was further supported by our inhibition studies utilizing a mannose-containing oligosaccharide substrate. Unlike the monosaccharides reported above, the $Man_5GlcNAc$ was expected to decrease enzyme activity by competing with the labeled substrate in the enzyme assay, and decrease the ability of the spermatozoa to bind to the zona pellucida by competing with putative ligands. These putative ligands are presumably glycoconjugates containing high mannose oligosaccharide chains on the zona pellucida. Indeed, incubation of spermatozoa with $Man_5GlcNAc$ resulted in a dramatic inhibition of sperm-egg binding (80%), which correlated with a similar reduction in enzyme activity. Moreover, the presence of the $Man_5GlcNAc$ did not impair the sperm motility or remove the sperm acrosome. These data further support our premise that sperm mannosidase is important for sperm-egg binding.

Our observations on the inhibition of sperm-egg binding in the presence of simple mannose sugars are in agreement with other published studies. For example, α-methyl mannoside inhibits sperm-egg binding in the rat [12] and in the mouse [13]. In addition, treatment of hamster eggs with concanavalin A, a lectin that binds to high mannose and/or hybrid oligosaccharides, prevents sperm-egg binding [21]. Other studies in which rat eggs were pretreated with jack-bean mannosidase, an enzyme that cleaves α-linked mannosyl residues from mannose-rich oligosaccharides, showed nearly complete inhibition of sperm-egg binding [12]. These studies, taken together with the results presented herein, strongly suggest that mannosyl residues on the egg zona pellucida and α-D-mannosidase on the spermatozoa may act in a ligand-receptor interaction to facilitate sperm-egg binding.

The ligand activity of the mouse zona pellucida has been shown to be associated with two of the three glycoproteins associated with the zona pellucida (ZP1, ZP2, and ZP3). Evi-

dence by Bleil et al. [22] suggests that ZP3 is the primary ligand that binds to capacitated spermatozoa prior to the acrosome reaction, whereas ZP2, the secondary ligand, binds to spermatozoa after the acrosome reaction. Even though ZP3 contains both *N*-linked and *O*-linked oligosaccharides, the ligand activity has been shown by Florman and Wassarman [23] to be associated only with the *O*-linked oligosaccharides. In contrast to these studies, other reports have shown that treatment of mouse eggs with almond glycopeptidase, an endo-enzyme that cleaves the *N*-acetylglucosaminyl asparagine linkage in *N*-linked glycoproteins, drastically reduces sperm-egg binding [24]. These studies suggest that *N*-linked glycoproteins, containing high mannose/hybrid/complex-type oligosaccharide(s) present on the mouse zona pellucida, may also be involved in sperm-egg binding.

In the mouse, only one family of zona pellucida proteins possessing ligand activity (ZP1, ZP2, ZP3) has been well studied, but several potential binding sites or receptors on the surface of spermatozoa have been identified. Bleil and Wassarman [25] have identified a 56-kDa protein that binds ZP3 and is localized to the heads of acrosome-intact spermatozoa. Shur and Hall [4, 5] have suggested that galactosyltransferase present on the sperm head may mediate sperm-egg interactions by binding to *N*-acetylglucosamine residues on the zona pellucida. The potential role of this enzyme in sperm-egg binding has been supported by observations that UDP-dialdehyde and alpha-lactalbumin, two known inhibitors of galactosyltransferase, inhibit sperm-egg binding in vitro [4, 5]. Moreover, the presence of purified galactosyltransferase or antibodies against the bovine milk enzyme result in inhibition of sperm-egg binding [6]. These studies indicate a role for the sperm galactosyltransferase during sperm-zona binding. Other studies have suggested that a trypsin-like protease on mouse spermatozoa may be involved in sperm-egg binding [8]. In these studies it was shown that in the presence of various protease inhibitors the binding of capacitated mouse spermatozoa to the zona pellucida is prevented in a dose-dependent manner. Additional studies by Benau and Storey [26] suggest that the trypsin site and galactosyltransferase are independent binding sites. Recently, Leyton and Saling [9] identified a 95-kDa protein present on the mouse sperm plasma membrane that binds to whole zonae pellucidae or purified ZP3 as well as serving as a tyrosine kinase substrate. In addition, immunofluorescence staining of spermatozoa with an anti-phosphotryosine antibody localized the site of tyrosine phosphorylation to the acrosomal region of the sperm head. These investigators suggest that the 95-kDa protein may be involved in sperm-egg interaction by binding to ZP3, which subsequently stimulates tyrosine kinase activity leading to the sperm acrosome reaction.

The identification of several sperm-associated enzymes that appear to be involved in sperm-egg binding suggests that perhaps several receptor-ligand interactions must occur between the spermatozoa and the zona pellucida before a committed sperm-egg binding occurs that subsequently results in fertilization. Moreover, the precise order of these interactions or the dominant receptor-ligand interaction may vary among species and may contribute to the species-specificity of fertilization. The data presented in this report suggest that a sperm surface α-D-mannosidase may serve as one of these receptors.

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