Sperm from β1,4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly

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SUMMARY

A variety of sperm surface components have been suggested to mediate gamete recognition by binding to glycoside ligands on the egg coat glycoprotein ZP3. The function of each of these candidate receptors is based upon varying degrees of circumstantial and direct evidence; however, the effects on fertilization of targeted mutations in any of these candidate receptors have not yet been reported. In this paper, we describe the effects of targeted mutations in β1,4-galactosyltransferase, the best studied of the candidate receptors for ZP3. Surprisingly, galactosyltransferase-null (gt−/−) males are fertile; however, sperm from gt−/− males bind less radiolabeled ZP3 than wild-type sperm, and are unable to undergo the acrosome reaction in response to either ZP3 or anti-galactosyltransferase antibodies, as do wild-type sperm. In contrast, gt−/− sperm undergo the acrosome reaction normally in response to calcium ionophore, which bypasses the requirement for ZP3 binding. The inability of gt−/− sperm to undergo a ZP3-induced acrosome reaction renders them physiologically inferior to wild-type sperm, as assayed by their relative inability to penetrate the egg coat and fertilize the oocyte in vitro. Thus, although ZP3 binding and subsequent induction of the acrosome reaction are dispensable for fertilization, they impart a physiological advantage to the fertilizing sperm. A second strain of mice was created that is characterized by a loss of the long galactosyltransferase isoform responsible for ZP3-dependent signal transduction, but which maintains normal levels of Golgi galactosylation. Sperm from these mice show that the defective sperm-egg interactions in gt−/− mice are due directly to a loss of the long galactosyltransferase isoform from the sperm surface and are independent of the state of intracellular galactosylation during spermato genesis.

Key words: galactosyltransferase, fertilization, sperm, zona pellucida, mouse, ZP3

INTRODUCTION

Species-specific gamete recognition is initiated when sperm bind and penetrate the zona pellucida, a thick extracellular matrix surrounding the egg. In mouse, the zona pellucida contains three predominant glycoproteins, ZP1, ZP2 and ZP3, of which ZP3 is thought to possess the sperm-binding and acrosome-reaction activities of the zona pellucida (Bleil and Wassarman, 1980; 1983). The sperm-binding activity of ZP3 resides on a class of serine/threonine-linked oligosaccharide chains (Florman and Wassarman, 1985; Miller et al., 1992), which aggregate the sperm surface receptor thus triggering the acrosome reaction. Release of hydrolytic enzymes from the acrosome is believed to enable the sperm to penetrate through the zona pellucida (Miller et al., 1993a,b).

Although great efforts have been made to elucidate the mechanisms of sperm-egg interactions, the mouse sperm receptor(s) for ZP3 remains controversial (Snell and White, 1996). Several sperm components have been suggested to possess ZP3-binding activity. Those that have received the most attention are sp56 (Bleil and Wassarman, 1990; Cheng et al., 1994; Bookbinder et al., 1995), p95 (Leyton and Salin, 1989a) and β1,4-galactosyltransferase (GalTase) (Shur and Hall, 1982a,b; Lopez et al., 1985; Miller et al., 1992). Recent studies suggest that neither sp56 nor p95 are, in fact, sperm surface receptors for ZP3 (Foster et al., 1997; Kalab et al., 1994; Bork, 1996; Tsai and Silver, 1996). In contrast, the ability of GalTase to function as a sperm surface receptor for ZP3 oligosaccharides is supported by considerable evidence (Miller and Shur, 1994).

In somatic cells, GalTase exists in two distinct subcellular pools, where it serves two distinct biological functions (Shur, 1993). Most GalTase is localized to the trans Golgi complex, where it participates in glycoconjugate biosynthesis. A considerably lesser amount of GalTase is expressed on the plasma membrane, where it functions as a receptor for extracellular glycoside ligands. The Galtase (gt) gene encodes two isoforms that differ only in the length of their cytoplasmic domains (Shaper et al., 1988; Russo et al., 1990). There is evidence suggesting that the longer cytoplasmic domain is responsible, at least in part, for the ability of GalTase to function as a cell surface receptor for extracellular glycosides (Lopez et al., 1992; Evans et al., 1995).

During spermatogenesis, only the long GalTase isoform is expressed and, on mature epididymal sperm, all of the protein is confined to the dorsal, anterior aspect of the plasma
membrane overlying the intact acrosome (Shur and Neely, 1988). In this location, GalTase is thought to function as a gamete receptor by binding to terminal N-acetylgalcosamine (GlcNAc) residues on ZP3 (Miller et al., 1992). Aggregation of GalTase by ZP3 oligosaccharides, or by anti-GalTase antibodies, activates a pertussis toxin (PTx)-sensitive heterotrimeric G-protein complex that associates with the cytoplasmic domain of the long GalTase isoform, triggering the acrosome reaction (Leyton and Saling, 1989b; Ward et al., 1992; Miller et al., 1992; Macek et al., 1991; Gong et al., 1995). Overexpressing GalTase on the sperm surface leads to increased ZP3 binding, accelerated G-protein activation and precocious acrosome reactions (Youakim et al., 1994). Upon egg activation by the fertilizing sperm, the egg cortical granules release N-acetylgalcosaminidase into the perivitelline space, which destroys the GalTase recognition motif on ZP3 and produces the block to polyspermic binding (Miller et al., 1993b).

Inevitably, the ultimate test of any putative ZP3 receptor is to examine the effects of null mutations on sperm-egg interactions. We therefore created two strains of mice by homologous recombination; those that are devoid of both GalTase isoforms and those devoid of the long isoform, but which express the short isoform ectopically in sperm in lieu of the long isoform. The cumulative results presented here show that GalTase is required for optimal ZP3 binding, ZP3-mediated induction of the acrosome reaction and the subsequent penetration of the zona pellucida. However, despite their inability to bind ZP3 or undergo ZP3-dependent acrosomal exocytosis, GalTase-null sperm are fertile. Thus, ZP3 binding and induction of the acrosome reaction are dispensable for fertilization, although their presence confers a physiologically advantageous to the fertilizing sperm, at least as assayed in vitro. Controls eliminate the possibility that the defect in ZP3-dependent acrosomal induction is the secondary result of altered galactosylation during spermatogenesis. Furthermore, the ability of GalTase to function as a signal transducing receptor on the sperm surface is dependent upon the expression of the long GalTase isoform.

**MATERIALS AND METHODS**

**Creation of total and long-form GalTase-deficient mice**

Mice devoid of both GalTase isoforms were created by homologous recombination as previously described (Lu et al., 1997). In order to eliminate the long GalTase isoform without affecting the expression of the short isoform, an insertion type targeting vector was constructed by creating a point mutation into the first ATG translation initiation codon that initiates translation of long GalTase isoform.

The point mutation was introduced by a PCR-mediated site-specific mutagenesis procedure (Ho et al., 1989; Silver et al., 1995). Two pairs of primers were used to amplify two small GalTase fragments, one of which contains a point mutation at the first ATG. (primer 1: GGATGCCCCTCAACCCCAA; primer 2: GCTCACGAAACCTAGCTTCCCGGC [bolded sequences represent the mutated ATG codon including the new HindIII cut site]; primer 3: AGACTCCGCCTCCCCAGTT; primer 4: AGAAGCTGGACAGGCTTGGAA). In the first step, the PCR reaction mixture was denatured at 94°C for 5 minutes, and amplified for 35 cycles of 94°C, 1 minute; 52°C, 1 minute; 72°C, 1 minute. 10 μl of the first PCR products were fractionated on a 1.5% low melting point agarose gel. 1 μl of each product in the gel block was subjected to a secondary PCR amplification process using two flanking primers under the same PCR conditions. The final product was a 858 bp fusion DNA fragment containing a novel HindIII site at the mutated first ATG codon. The Nael/Narl fragment in the 858 bp PCR product was subcloned into Nael/Narl-digested genomic DNA isolated as described (Lu et al., 1997). The presence of the mutation and the integrity of the surrounding sequences were confirmed by DNA sequencing.

For positive and negative selection, the neo and TK genes were added in the vector outside the homologous region. The targeting vector was linearized inside the homologous sequences with BamHI, prior to electroporation. The point mutation in the first GalTase translation initiation codon was introduced into ES cells by a two-step recombination procedure (Hasty et al., 1991). In the first step, targeting vector containing both positive and negative selection markers integrated homologously into the genome generating a duplicate genomic sequence. Cells carrying such a vector are resistant to G418-positive selection because of the presence of the neo cassette, but sensitive to FIAU, a substrate for the negative selection marker, TK gene. The surviving cells were screened for homologous integration by Southern blot using a 5′-external probe as described (Lu et al., 1997). Positive clones were subjected to a secondary FIAU-negative selection procedure, since any clones that have undergone spontaneous intrachromosomal recombination between two duplicate homologous sequences survive FIAU selection. The intrachromosomal recombination event leaves only the point mutation in the genome, excising the remainder of the targeting vector. The existence of the point mutation in ES clones was identified by HindIII digestion and Southern analysis of genomic DNA using a probe corresponding to the first exon. Positive clones were used to produce chimeric mice as described (Lu et al., 1997; Bradley, 1987).

**Sperm GalTase activity and immunofluorescence**

Cauda epididymal sperm were collected and capacitated at 35°C, 5% CO2 for 1 hour in dmKRBT (120 mM NaCl, 2 mM KCl, 2 mM CaCl2, 10 mM NaHCO3, 0.36 mM Na2HPO4·H2O, 1.2 mM MgSO4·7H2O, 5.6 mM glucose, 1.1 mM sodium pyruvate, 25 mM TAPSO [3-{N-tris(hydroxymethyl)-methylamino}-2-hydroxy propane sulfonic acid], 18.5 mM sucrose, 1x penicillin/streptomycin, 6 mg/ml BSA [fatty acid free], pH 7.4).

For GalTase enzyme assay, sperm were subsequently washed three times by centrifugation at 1,000 g for 4 minutes in Medium B (0.14 M NaCl, 4 mM KCl and 20 mM Hepes, pH 7.3) containing protease inhibitors (PIC) (Youakim et al., 1994). The washed sperm pellet was assayed for GalTase activity towards exogenous GlcNAc substrates by high voltage borate electrophoresis as previously described (Lu et al., 1997).

For immunofluorescence, capacitated sperm were washed three times in PBS by centrifugation at 1,000 g for 4 minutes before being dried onto polysine pre-coated slides. Sperm were fixed for 10 minutes at ~20°C in 95% ethanol/5% glacial acetic acid. The fixed sperm were rinsed three times in PBS and then incubated in PBS with 1:1000 dilution of fluorescein-labelled avidin (Vector Laboratories, Inc.) for 45 minutes at 23°C. The slides were washed three times in PBS, once in PBS/5% NGS before they were incubated in biotinylated goat anti-rabbit IgG for 45 minutes at 23°C. The slides were washed three times in PBS and then incubated in fluorescein-labeled avidin (Vector Laboratories, Inc.) for 45 minutes at 23°C. After brief rinsing with PBS, the slides were mounted in glycerol/ PBS (9:1) containing 4% n-propyl gallate.

**Sperm-egg binding assay**

After capacitation, sperm were filtered through a nitex membrane and adjusted to 2x10⁶ sperm/ml. While sperm were undergoing capacitation, eggs from the oviducts of supersaturated females were collected into fresh dmKRBT and incubated in 0.2% hyaluronidase at 37°C for 10 minutes to remove cumulus cells. Cumulus cells were removed by transferring eggs through several drops of fresh dmKRBT. 15-20 eggs
were added to 50 μl drops of dmKRBT under paraffin oil. Three to five 2-cell-stage embryos flushed from the oviducts of 30 hours p.c. superovulated females were included in each drop as control. 50 μl of sperm suspension were added to each drop containing eggs. Binding was performed at 37°C for 30 minutes. The unbound or loosely bound sperm were washed off from eggs by transferring the eggs through 200 μl drops of dmKRBT with a wide-bore pipette. Washing was stopped when <4 sperm remained bound to 2-cell embryos. The eggs with tightly bound sperm were fixed in freshly prepared 4% paraformaldehyde for 5 minutes. The number of bound sperm per egg was recorded.

**Induction of the sperm acrosome reaction**

Sperm were prepared as described above. 25 μl sperm were incubated with 25 μl anti-GaTase antisemur or preimmune serum at a 1:100 dilution for 0, 30 or 60 minutes at 37°C. Positive controls included sperm incubated in the presence of 10 μM A23187(Sigma Chemical Co.) or ~20 ng zona pellucida glycoproteins, prepared as described below. After incubation, sperm were fixed in 100 μl of fresh 4% paraformaldehyde for 10 minutes at 23°C. Fixed sperm were collected at 1000 g for 4 minutes and resuspended in 500 μl of 0.1 M ammonium acetate, pH 9.0. Sperm were washed once in this buffer and resuspended in 100 μl of 0.1 M ammonium acetate. 10 μl of this suspension was dried onto glass slides and stained for 2 minutes at 23°C in 0.22% Coomassie blue G250 in 50% methanol/10% glacial acetic acid. After staining, the slides were rinsed with tap water, air dried and mounted. The acrosome-reacted sperm were counted under ×400 magnification by the loss of intense staining on the anterior aspect of the sperm head.

**Purification of zona pellucida glycoproteins**

The oocytes from 200 mice were trimmed using small scissors under a dissecting microscope to remove fat and connective tissue. The ovaries were homogenized with a Polytron at a setting of 4:75 for 5 seconds in 12 ml of homogenization buffer (25 mM triethanolamine HCl, 150 mM NaCl, 1 mM MgCl₂, 6H₂O and 1 mM CaCl₂, 2H₂O, pH 8.5) supplied with 12 μg of soybean trypsin inhibitor, 4 μg of bovine testicular hyaluronidase and 4 μg of DNase I. The homogenate was placed on ice for 1 minute and then homogenized twice as above. 200 μl of 0.2 g/ml deoxycholic acid solution was added to the homogenate and homogenized three additional times. 200 μl of 20% NP40 was added and three bursts given with the Polytron. The homogenate was further homogenized by 10 strokes in a glass homogenizer. About 14 ml of ovary homogenate with 9 ml of 72% Percoll/28% homogenization buffer was placed into a Beckman heat-seal tube and ultracentrifuged in a Ti-50 rotor for 70 minutes at 25,000 revs/minute at 5°C. The position of the bands in the tube was recorded in relationship to colored markers (Sigma Chemical Co.). The upper band containing the zona pellucida (above the blue marker) was aspirated and placed in another heat-seal tube. The zona pellucida sample was ultracentrifuged once again. The zona pellucida band was collected into 10 mM PBS with 1 M NaCl, pH 7.0 and pelleted by centrifuging at maximum speed in a microcentrifuge for 5 minutes. The zona pellucida pellet was washed twice in high salt PBS buffer (10 mM PBS plus 1 M NaCl, pH 7.0) and once in 10 mM phosphate buffer (no salt). The zona pellucida sample was finally resuspended in 10 mM phosphate buffer and stored in 50% glycerol at −20°C or used for iodination. Alternatively, the zona were solubilized in mild acid, neutralized and used for inducing the acrosome reaction.

**Iodination of zona glycoproteins using Bolton-Hunter reagent**

Immediately prior to use, 125I-labeled Bolton-Hunter reagent (ICN Biomedicals) was evaporated to dryness by inserting a needle through the rubber septum and admitting a gentle stream of dry nitrogen, following the manufacturer’s instruction. Zona pellucidae were washed once with 0.1 M sodium borate, pH 8.5, and chilled to 0°C on ice. The zona pellucida sample was added to vials containing dried 125I-labeled Bolton-Hunter reagent and incubated 30 minutes at 0°C. The reaction was quenched by adding 100 μl of 1 M glycerine in 0.1 M sodium borate, pH 8.5. The 125I-labeled ZP glycoproteins were separated on 10% SDS-PAGE gel. The areas containing ZP3 (83 kDa) and ZP2 (120 kDa) were cut from the gel and electroeluted. The 125I-labeled zona glycoproteins were dialyzed against three changes of 2 liters of 10 mM phosphate buffer containing 8 M urea at 23°C and then against three changes of 2 liters of distilled water at 5°C. The protein concentration was determined by BCA protein assay kit (Pierce, Inc.).

**125I-ZP3-binding assay**

Sperm were prepared as described except that PTx was added to the sperm suspensions to a final concentration of 100 ng/ml to inhibit acrosome reactions. ×10⁶ cts/minute of 125I-labeled-ZP3 was mixed with the sperm suspension and incubated at 37°C for 10 minutes. After incubation, the sperm were fixed 10 minutes with 4% paraformaldehyde before they were washed three times with dmKRBT/BSA buffer. The bound 125I-ZP3 in sperm pellets was counted in a gamma counter. The level of nonspecific binding was determined by replacing 125I-ZP3 with deglycosylated 125I-ZP3 (25 mM NaOH, 37°C, overnight and neutralized) or by adding an excess of unlabelled ZP3 or total zona pellucida glycoproteins. The background nonspecific cts/minute were similar in all instances and were subtracted from all assays containing 125I-ZP3.

**Egg penetration assay**

Sperm penetration through the zona pellucida was assayed as previously described (Miller et al., 1993a). Briefly, oviductal eggs were collected as above. Some were used without further treatment, others were heated for 75 minutes at 55°C to inactivate the cortical granule enzymes responsible for the zona block to polyspermy. Caudal epididymal sperm were collected, capacitated and 0.5-2×10⁶ sperm/ml were incubated in 200 μl droplets of dmKRBT with 30-50 eggs under mineral oil in 5% CO₂. Sperm and eggs were coincubated for 15 minutes at 37°C to allow binding to occur, after which the medium containing unbound sperm was aspirated and replaced with sperm-free medium. This was done three times. Penetration was allowed to occur for an additional 3-6 hours before the assay was stopped by addition of sodium azide (final concentration 0.01%).

Eggs were transferred to drops of sperm-free dmKRBT and a fine-pulled glass micropipette was used to remove sperm outside of the zona pellucida. Eggs were fixed in 4% paraformaldehyde in PBS, transferred onto a siliconized glass microscope slide and washed twice with 50 μl of 0.23% sodium citrate:ethanol (3:1, v/v, 0.75 mg/ml PVP). Extra medium was aspirated and 50 μl of stain [30 μg Hoechst 33258 per ml 0.23% sodium citrate:ethanol (3:1, v/v, no PVP)] was carefully added to eggs. The slide was placed on a 37°C slide warmer for 6 minutes. The staining solution was aspirated and the eggs on the slide were rinsed three times with 0.23% sodium citrate:ethanol (3:1) and covered with 12 μl of mounting solution (9:1 glycerol/0.23% sodium citrate, 0.4% n-propyl gallate). Coverslips were applied and sealed with nail polish, and sperm inside the zona pellucida and in the perivitelline space were counted by fluorescence microscopy at ×400.

**Lectin blotting**

Testes from each genotype were homogenized in 1 ml of Medium B. The homogenates were centrifuged at 16,000 g at 4°C for 5 minutes. 750 μl of supernatant was saved and mixed with 250 μl of 120 mM N-octyl glucopyranoside buffer. The mixture was incubated at 4°C for 2 hours and centrifuged at 16,000 g at 4°C for 5 minutes. The protein concentration in the supernatant was determined by BCA method. 70 μg protein of testis homogenate was fractionated on 7-15% gradient SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Schleicher & Schuell, Inc.). The membrane was blocked for 1 hour in blocking solution (1% gelatin in 100 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20) with gentle agitation, and then labeled.
epididymal sperm were incubated with rabbit antiserum raised against bacterially expressed murine GalTase (Nguyen et al., 1994), followed by biotinylated secondary antibody and FITC-avidin. GalTase is localized to the dorsal, anterior aspect of the sperm head in wild-type (+/+), but is not detectable in mutant sperm (gr<sup>-/-</sup>). No sperm were labeled with preimmune serum.

**RESULTS**

**Generation and fertility of GalTase-null males**

Mice were made deficient for both GalTase isoforms by homologous recombination as described previously (Lu et al., 1997). Approximately 85% of the GalTase-null (gr<sup>-/-</sup>) animals die during the neonatal period due to polyglandular endocrine insufficiency, but for unknown reasons, the remaining 15% recover normal endocrine function and grow into sexually mature adults (Lu et al., 1997). These adult males were used to analyze the effects of GalTase deficiency on sperm-egg interactions.

Previous studies have shown that gr<sup>-/-</sup> animals behave as null alleles as judged by Southern analysis, RT-PCR, GalTase enzymatic activity and analysis of GalTase reaction products (Lu et al., 1997). Elimination of GalTase from sperm was confirmed by the loss of enzyme activity and immunoreactivity. GalTase activity decreased to less than 10% that of wild-type (+/+) (Fig. 1A). This trace amount (<100 cts/minute 3H-Galβ1,4GlcNAc product/25 μg prot/hour) of enzyme activity was also detected in testis and shown to reflect GalTase-independent galactosylation (Lu et al., 1997). Immunofluorescence analysis using antibodies raised against bacterially expressed murine GalTase (Nguyen et al., 1994) demonstrated GalTase on wild-type sperm, but failed to detect GalTase on gr<sup>-/-</sup> sperm (Fig. 1B). As reported previously, sperm were not labeled by preimmune sera (Gong et al., 1995).

gr<sup>-/-</sup> males were fertile indicating that GalTase appears dispensable for fertility. There were some indications of reduced reproductive efficacy, since the gestation time of litters inseminated by gr<sup>-/-</sup> males appeared to be one day longer than litters inseminated by wild-type or heterozygous males. However, it was not clear whether these differences were significant and/or strain dependent. Given the possibility of reduced reproductive efficacy and the complexity of fertilization in vivo, we analyzed the behavior of gr<sup>-/-</sup> sperm with respect to capacitation, sperm binding to the zona pellucida, binding to radiolabeled ZP3 ligand, induction of the acrosome reaction and penetration of the zona pellucida.

**Fig. 1.** Sperm from gr<sup>-/-</sup> males have negligible levels of GalTase. (A) Cauda epididymal sperm was washed and assayed for GalTase activity by high voltage electrophoresis. Activity in gr<sup>-/-</sup> (○) sperm is less than 10% that of wild-type (●), which reflects GalTase-independent galactosylation (Lu et al., 1997). (B) Washed cauda epididymal sperm were incubated with rabbit antiserum raised against bacterially expressed murine GalTase (Nguyen et al., 1994), followed by biotinylated secondary antibody and FITC-avidin. GalTase is localized to the dorsal, anterior aspect of the sperm head in wild-type (+/+), but is not detectable in mutant sperm (gr<sup>-/-</sup>). No sperm were labeled with preimmune serum.

**Fig. 2.** gr<sup>-/-</sup> sperm are released as single, rapidly motile sperm (in many focal plans), unlike wild-type (+/+) sperm that are released from the epididymis in large aggregates that break apart during capacitation. Higher magnification photomicrographs (bottom row) illustrate that wild-type and gr<sup>-/-</sup> sperm are morphologically similar at the level of the light microscope.
from three independent experiments.

+C sperm, average 21 sperm bound to each egg. Three to five 2-cell embryos were included in each of the fertilization drops to determine nonspecific binding, which was defined as <4 sperm per embryo. Results represent the mean ± s.e.m. of at least 500 eggs from three independent experiments.

gt+ epididymal sperm fail to show normal agglutination

Cauda epididymal sperm were collected in dmKRBT buffer and immediately observed under the dissection microscope. Sperm from gt- males were released as single cells, rather than in large aggregates, as seen in wild-type (Fig. 2). Furthermore, the gt- sperm swam faster and more vigorously than wild type. However, these differences disappeared after capacitation for 1 hour; i.e., wild-type sperm separated from one another and showed the vigorous swimming characteristics seen in gt+ sperm prior to capacitation. These observations are consistent with previous studies indicating that prior to capacitation, sperm GalTase is masked by large molecular weight glycoconjugates. These competitive glycosides are shed from the sperm surface during capacitation, exposing GalTase for binding its ligand in the zona pellucida (Shur and Hall, 1982a). Presumably, the loss of GalTase from the sperm surface in gt- males prevents sperm from binding and agglutinating to large epididymal glycoconjugates, whose synthesis may also be compromised by the GalTase deficiency. Morphologically, wild-type and gt+ sperm were indistinguishable from one another at the level of the light microscope (Fig. 2).

gt+ sperm bind to unfertilized eggs better than wild-type sperm

Cauda epididymal sperm were collected and capacitated in vitro for 1 hour. Sperm were added to eggs and, after the desired incubation period, the loosely bound sperm were gently removed by repetitive washing and the remaining tightly bound sperm were counted. As shown in Fig. 3, three times the number of gt+ sperm remained adherent to the zona pellucida after washing, relative to wild-type sperm. Interestingly, the increased binding of gt+ sperm is reciprocal to that seen with sperm that overexpress surface GalTase, which bind less well to the zona pellucida than do wild-type sperm. Subsequent studies revealed that the elevated levels of GalTase render sperm hypersensitive to ZP3, leading to precocious acrosome reactions and low affinity binding to the zona (Youakim et al., 1994). We therefore tested the possibility that gt+ sperm bind to the zona pellucida greater than wild-type sperm due, at least in part, to a failure to undergo the zona-induced acrosome reaction.

gt+ sperm are unable to undergo acrosome reactions in response to either anti-GalTase antibody or zona pellucida glycoproteins

It has been demonstrated previously that ZP3 binding to sperm triggers the acrosome reaction (Bleil and Wassarman, 1983). This is thought to result from an aggregation of sperm-bound ZP3 receptors by multivalent oligosaccharide chains (Leyton and Saling, 1989b). In this regard, the requirement for ZP3 binding can be bypassed by anti-GalTase antibodies, but not by monovalent anti-GalTase Fab fragments, unless they are subsequently cross-linked by anti-Fab antibodies (Macek et al., 1991).

Antibodies raised against recombinant murine GalTase resulted in a time-dependent induction of the acrosome reaction in wild-type sperm, similar to that previously reported (Fig. 4). In contrast, the same GalTase antibodies were unable to induce acrosome reactions in gt+ sperm. Similar results were obtained for antibodies raised against two different sources of recombinant murine GalTase.

The inability of gt+ sperm to undergo an acrosome reaction in response to anti-GalTase IgG is not surprising, given that the
GalTase antigen has been eliminated from the sperm surface. More important is whether $g^{t/-}$ sperm are able to undergo an acrosome reaction in response to their natural ligand, ZP3. As expected, wild-type sperm showed a time-dependent induction of the acrosome reaction when incubated with solubilized zona pellucida glycoproteins (Fig. 4). In contrast, $g^{t/-}$ sperm were completely refractory to zona-induced acrosome reactions.

$g^{t/-}$ sperm undergo normal acrosome reactions in response to calcium ionophore

To determine if the GalTase mutation resulted in a generalized membrane defect preventing acrosomal exocytosis, we examined the response of sperm to the calcium ionophore A23187, which bypasses the ZP3 signaling pathway leading to the acrosome reaction. Both $g^{t/-}$ and wild-type sperm were equally responsive to calcium ionophore-induced acrosome reactions (Fig. 4). This indicates that $g^{t/-}$ sperm can undergo normal acrosomal exocytosis, showing a relatively specific defect in ZP3-dependent signaling.

$g^{t/-}$ sperm bind reduced levels of radiolabeled ZP3

$g^{t/-}$ sperm show prolonged binding to the zona pellucida and are refractory to zona-induced acrosome reactions. This phenotype is reciprocal to sperm that overexpress GalTase, which are hypersensitive to their ZP3 ligand producing a transient low-affinity binding to the zona pellucida (Youakim et al., 1994). Since elevating GalTase expression on sperm leads to increased binding of radiolabeled ZP3 ligand (Youakim et al., 1994), we determined whether the loss of GalTase on $g^{t/-}$ sperm was associated with reduced binding of radiolabeled ZP3.

ZP3 was purified and iodinated as previously described (Youakim et al., 1994). Radiolabeled ZP3 was added to sperm in the presence of PTx to inhibit the acrosome reaction, incubated for 30 minutes, and the sperm were pelleted and washed to remove unbound $^{125}$I-ZP3. Nonspecific binding was determined by substituting $^{125}$I-ZP3 with NaOH-treated $^{125}$I-ZP3, which no longer possesses sperm-binding activity due to release of the relevant oligosaccharides (Florman and Wassarman, 1985), or by a 20-fold excess of unlabeled ZP3. Both procedures resulted in similar levels of nonspecific, i.e., background, binding.

In all experiments, $g^{t/-}$ sperm consistently bound less $^{125}$I-ZP3 than an equivalent number of wild-type sperm, although the range varied considerably (Fig. 5). In two of five assays, $g^{t/-}$ sperm failed to show any detectable ZP3 binding above control levels; in the others, $g^{t/-}$ sperm bound 30-50% of wild-type levels. The reasons for this variability remain unclear and may simply reflect variations in the background level of binding between assays on different days with different ZP3 preparations. Alternatively, GalTase may cooperate with other surface components to form a multimeric ZP3-binding complex and the loss of GalTase from this hypothetical complex may lead to reduced affinity or avidity for ZP3. In any event, the results clearly show that $g^{t/-}$ sperm have reduced ability to bind ZP3 relative to wild-type sperm. As an additional test of specificity, the binding of $^{125}$I-ZP2 was determined, which does not bind to acrosome-intact sperm as does ZP3. As shown in Fig. 5, both $g^{t/-}$ and wild-type sperm bound the same level of $^{125}$I-ZP2.

$g^{t/-}$ sperm penetrate the zona pellucida poorly

The results thus far show that $g^{t/-}$ sperm are completely refractory to ZP3-induced acrosome reactions, which is correlated with decreased levels of ZP3 binding. Nevertheless, $g^{t/-}$ sperm are fertile. Thus, although GalTase is clearly required for ZP3-mediated acrosomal exocytosis, it is clearly not required for successful fertilization in vivo. We therefore considered the possibility that the integrity of the ZP3-GalTase-G-protein cascade gives sperm a physiological advantage over sperm with deficiencies in this pathway. To address this possibility, we compared the ability of wild-type and $g^{t/-}$ sperm to penetrate the zona pellucida and fertilize the oocyte, the ultimate outcome of successful acrosomal exocytosis.

Sperm were incubated with eggs for 15 minutes to allow sperm binding to the zona, after which the eggs with adherent sperm were transferred to sperm-free medium. This represented the start of the penetration assay. After 3 hours of incubation, sperm still adherent to the outside of the zona were removed by fine-bore pipets and the presence of sperm within or penetrated through the zona pellucida were detected following Hoechst staining.

After a 3 hour incubation, $g^{t/-}$ sperm had penetrated the zona only 21% as frequently as wild-type sperm (0.27 sperm/egg versus 1.23 sperm/egg) (Fig. 6). Longer incubation times resulted in similar levels of sperm penetration, since the fertilizing sperm initiated the zona-induced block to polyspermy thus terminating the assay at slightly greater than 1 sperm/egg.

This assay differs from the in vivo situation considerably in that ~1x10⁶ sperm are immediately available for binding to the zona pellucida. In the female reproductive tract, the insemi-
nated sperm are thought to be retained in storage crypts that release packets of sperm over an extended period, such that only a few sperm reach the zona surface at any one time. Consequently, fertilization of the entire egg population in vivo occurs over a much longer period of time than it does during the penetration assay in vitro, in which all of the eggs are immediately challenged with an excess number of sperm at time zero. Therefore, to better assess the physiological advantage of wild-type sperm without limiting the assay to the initial binding event, the zona-block to polyspermy was heat-inactivated as previously described (Miller et al., 1993a) to lengthen the window during which penetration could occur. Under these conditions, wild-type sperm continued to penetrate the zona matrix, whereas \( \text{gt}^- \) sperm were still relatively unable to penetrate, being only \(~7\%\) as efficient as wild-type sperm (0.48 sperm/egg versus 6.2 sperm/egg). Furthermore, using either source of eggs (untreated or heat-inactivated), male pronuclei were rarely, if ever, seen from \( \text{gt}^- \) sperm, although male pronuclei from wild-type sperm were readily detectable in the oocyte cytoplasm.

**Defective sperm-egg interactions in \( \text{gt}^- \) sperm are not the secondary result of defective galactosylation during spermatogenesis**

Eliminating the GalTase gene leads to a loss of surface GalTase expression as well as to a loss of Golgi-based galactosylation. We therefore had to consider the possibility that the inability of \( \text{gt}^- \) sperm to bind ZP3, undergo an acrosomal reaction and penetrate the zona pellucida was the secondary result of defective galactosylation during spermatogenesis, rather than being the direct result of surface GalTase deficiency. To address this issue, mice were made deficient specifically in the GalTase isoform that is responsible for its ability to function as a signal transducing receptor (Gong et al., 1995).

The GalTase gene encodes two similar isoforms that differ in the length of their cytoplasmic domains (Shaper et al., 1988). It has been suggested that the two different cytoplasmic domains impact, at least to some degree, whether GalTase functions biosynthetically in the Golgi complex, or as a signal transducing receptor on the cell surface (Evans et al., 1995). In this regard, it has been shown that the ability of ZP3 or anti-GalTase IgG to induce the acrosome reaction in sperm is due to the binding of a PTx-sensitive G-protein complex to the cytoplasmic domain of the long GalTase isoform. The cytoplasmic domain of the short isoform is unable to bind and activate the G-protein complex (Gong et al., 1995).

An insertion-type targeting vector was designed to deliver a null point mutation into one allele of the long GalTase isoform. The targeted ES clones were microinjected into 3.5 day C57BL/6J blastocysts and developed to chimeras. Chimeric animals were mated to C57BL/6J to create heterozygous animals that were intercrossed to generate homozygous animals. The genotype of all animals was determined by Southern blot analysis (Fig. 7). Cells bearing this point mutation would still transcribe mRNAs encoding the long and short isoforms normally, but due to inactivation of the first in-

![Fig. 6. \( \text{gt}^- \) sperm are relatively unable to penetrate the zona pellucida. Eggs were incubated with sperm for 15 minutes, transferred to sperm-free medium and incubated for 3-6 hours. After incubation to allow sperm penetration of the zona pellucida, sperm still attached to the outer zona surface were removed with fine-bore pipets. The penetrated sperm were stained with Hoechst and visualized by fluorescence microscopy. Eggs were used either untreated or after heating to 55°C for 75 minutes to destroy the block to polyspermy (Miller et al., 1993a). Results represent the mean ± s.e.m. of 4 independent experiments, totaling at least 500 eggs in each treatment.](image1)

![Fig. 7. Southern blot analysis of mice bearing targeted mutations in the long GalTase isoform. (A) Restriction maps of wild-type (+/+ ) and mutant (long \( \text{gt}^- \)) alleles. An internal probe was used to assess the point mutation in the first ATG, which generated a new HindIII site (HindIII*). (B) Tail DNA was digested with HindIII and labeled with probe. The probe identifies a 9.8 kb wild-type band as well as two mutated bands of 7.0 kb and 2.8 kb, as shown in the diagram. H, HindIII.](image2)
frame translation initiation codon, only the short isoform would be translated from both long and short transcripts.

Long isoform-null mice appeared to develop normally, and importantly, did not suffer from the endocrine insufficiency phenotype characteristic of \( \text{gt}^{-/-} \) mice that is caused by incomplete galactosylation of the anterior pituitary hormones (Lu et al., 1997). Sperm from long isoform-null males expressed the short isoform due to translation from the remaining in-frame initiation codon and the short isoform functioned biosynthetically during spermatogenesis.

Sperm from long isoform-null animals were examined for their ability to undergo an acrosome reaction in response to anti-GalTase IgG, zona pellucida glycoproteins and calcium ionophore. Although galactosylation appeared normal, the sperm were unresponsive to either anti-GalTase IgG or zona pellucida glycoproteins (Fig. 9). However, they responded normally to calcium ionophore. Similarly, long isoform-null males were fertile and their sperm bound to the zona with much greater efficacy than did wild-type sperm (77.0 sperm/egg versus 18.6 sperm/egg). Thus, in every respect assayed, except for glycoprotein galactosylation sperm from long isoform-null and from \( \text{gt}^{-/-} \) males were indistinguishable from one another.

These data show that the inability of GalTase-null sperm to undergo a ZP3-induced acrosome reaction is a direct result of the lack of the long GalTase isoform on the sperm surface and is not the secondary result of defective galactosylation during spermatogenesis. Furthermore, the cytoplasmic domain unique to the long GalTase isoform that is responsible for association of the G-protein complex (Gong et al., 1995) appears to be absolutely required for ZP3-dependent signal transduction.

**DISCUSSION**

Despite much attention, the identity of the gamete receptors that facilitate sperm-egg binding has remained unresolved and actively debated. On one hand, the relatively simple structure of the mammalian egg coat has enabled the rapid identification...
of the biologically relevant sperm-binding glycoprotein, ZP3. In marked contrast, the complementary receptor on the sperm surface that binds ZP3 remains an area of active controversy. A number of sperm surface proteins have been suggested to function as the ZP3-binding component (Snell and White, 1996). Among numerous candidates, three sperm membrane proteins are frequently mentioned: sp56 (Bleil and Wassarman, 1990; Cheng et al., 1994; Bookbinder et al., 1995), p95 (Leyton and Saling, 1989a; Leyton et al., 1992) and GalTase (Shur and Hall, 1982a;b; Lopez et al., 1985; Miller et al., 1992).

sp56 is a sperm peripheral membrane protein that was first identified by ZP3-photoaffinity labeling (Bleil and Wassarman, 1990) and claimed to confer sperm ZP3-binding activity and species specificity of sperm-egg recognition in mice (Cheng et al., 1994; Bookbinder et al., 1995). Protein immunoblot analysis of egg or embryo extracts show that purified sp56 binds to unfertilized eggs but not to fertilized embryos and preincubation of eggs with purified sp56 blocks sperm-egg zona binding (Bookbinder et al., 1995). These data are consistent with sp56 having zona-binding activity. However, the physiological role of sp56 in zona-binding remains unclear, since it can only be removed from ZP3 affinity columns by strong denaturants (i.e., urea) and it is a peripheral membrane protein. Recently, sp56 has been shown to be a soluble constituent of the acrosomal matrix and not a cell surface component, which argues that sp56 does not function during initial gamete recognition (Foster et al., 1997).

Another mouse sperm protein claimed to physically associate with ZP3 is p95, a phosphotyrosine-containing protein of M:\text{r} 95,000 (Leyton and Saling, 1989a; Leyton et al., 1992). This protein has recently been demonstrated to be a testis-specific tyrosine phosphorylated form of hexokinase (Kalab et al., 1994). A putative human analog, hu9, has been isolated and shown to have protein kinase activity, and peptides deduced from its sequence inhibit sperm-egg binding (Burks et al., 1995). How these peptides interfere with recognition of sperm-binding oligosaccharides on ZP3 and whether the human clone is, in fact, the mouse homolog rather than the previously identified c-mer proto-oncogene (Bork, 1996; Tsai and Silver, 1996), is a matter of active debate.

Sperm surface GalTase is the best studied of the candidate receptors. It is found on the dorsal, anterior aspect of the sperm surface in several species, where it behaves as an integral membrane protein (Shur and Neely, 1988; Fayer-Hosken et al., 1991; Larsen and Miller, 1997). In mouse, sperm surface GalTase participates during fertilization by binding GlcNAc residues in the zona pellucida (Shur and Hall, 1982b). Inhibition of sperm surface GalTase activity by a variety of GalTase-specific reagents blocks sperm-egg binding, as does glycosidase digestion of the GalTase-binding site on the zona pellucida (Shur and Hall, 1982a;b; Lopez et al., 1985). Sperm GalTase specifically recognizes ZP3 and not ZP1 or ZP2, even though all three zona pellucida glycoproteins are recognized by Golgi-derived, nonsperm GalTase (Miller et al., 1992). Thus, the presence of a terminal GlcNAc residue is not, in itself, sufficient for recognition by sperm GalTase. Importantly, the sperm-binding activity of ZP3 is dependent upon the integrity of its GalTase-binding site (Miller et al., 1992).

Sperm surface GalTase has also been shown to be essential for the zona-induced acrosome reaction, which can be mimicked by anti-GalTase IgG, but not by monovalent Fab fragments, unless they are cross-linked with a secondary IgG (Macek et al., 1991). These results suggest that the acrosome reaction is induced by aggregating GalTase on the sperm surface, consistent with the observation that ZP3 has multiple binding sites for sperm GalTase (Miller et al., 1992). Aggregation of GalTase, by either ZP3 oligosaccharides or by anti-GalTase IgG, activates a PTx-sensitive heterotrimeric G-protein complex that associates with the cytoplasmic domain of the long GalTase isoform, but not the short isoform (Ward et al., 1992; Gong et al., 1995). Activation of the G-protein cascade culminates in acrosomal exocytosis, which releases among other enzymes, N-acetylglucosaminidase, which facilitates sperm penetration through the zona, presumably by removing potential binding sites for sperm GalTase in the vicinity of the penetrating sperm (Miller et al., 1993a). In contrast, the release of N-acetylglucosaminidase from egg cortical granules following egg activation is responsible for the global elimination of GalTase-binding sites from ZP3, thus preventing polyspermic binding (Miller et al., 1993b).

In light of the suggestions that other sperm surface proteins function as receptors for ZP3 (Bookbinder et al., 1995; Leyton and Saling, 1989a), we felt it imperative to examine the consequences of GalTase null mutations on sperm-egg interactions. It is striking that the phenotype of gr–/– sperm is reciprocal in virtually every manner to sperm that overexpress surface GalTase (Youakim et al., 1994). Overexpressors bind more radiolabeled ZP3 ligand, and undergo accelerated G-protein activation and precocious acrosome reactions. The ultimate result of this is a low affinity, transient adhesion to the zona pellucida. In contrast, gr–/– sperm bind low levels of ZP3, are refractory to ZP3-induced acrosome reactions and remain adherent to the zona pellucida long after wild-type sperm have triggered their acrosome reaction, facilitating their easy removal from the zona pellucida.

A defect in the ability to undergo an acrosome reaction can be caused by several reasons. First, the loss of GalTase from the sperm surface may make them refractory to ZP3 and thus fail to activate the signal transduction pathway. Second, the GalTase deficiency may cause a defect in sperm membranes that makes these sperm unable to undergo a normal acrosome reaction. Alternatively, a defect in galactosylation during spermatogenesis of a critical, but unrelated cell surface glycoprotein may prevent the sperm from being responsive to ZP3 binding. Results from this study favor the first possibility; two experiments support this conclusion. First, gr–/– sperm can undergo normal acrosome reactions in response to calcium ionophore, which bypasses the ZP3-induced signal transduction pathway. Second, even when GalTase enzyme activity and galactosylation appear normal, sperm are refractory to ZP3-induced acrosome reactions as long as they do not make the long GalTase isoform. These data exclude the possibility that defective acrosome reactions in gr–/– sperm are caused by incomplete galactosylation of sperm glycoproteins. These data also show that the cytoplasmic domain of the long GalTase isoform is required to confer ZP3-mediated signal transduction.

The acrosome reaction has traditionally been viewed as a prerequisite for sperm penetration through the zona pellucida. However, gr–/– sperm are fertile, even though they cannot undergo ZP3-induced acrosome reactions. Similarly, sperm made null for the acrosomal protease, acrosin, are also fertile
(Baba et al., 1994; Adham et al., 1997). Both of these observations suggest that the entire ZP3-GalTase-G-protein activated release of acrosomal exocytosis is dispensable for fertilization. This then begs the question as to whether the presence of this receptor-ligand complex offers some physiological or competitive advantage to sperm. Consistent with this possibility, we found that gr−/− sperm were much less efficient (i.e., ~7%) in penetrating the zona pellucida and fertilizing the oocyte than wild-type sperm. Thus, in a non-competitive environment where all sperm are phenotypically similar, as in the gr−/− male, a few sperm eventually find their way through the zona pellucida, by either spontaneous acrosome reactions, cracks in the zona pellucida or other scenarios, and fertilize the egg. However, when compared to wild-type sperm, this process is extremely inefficient, suggesting that, in a competitive environment where not all sperm may be functionally equivalent, the integrity of the GalTase receptor complex is advantageous for successful fertilization. In this regard, it is noteworthy that when acrosin-null sperm have been directly challenged with wild-type sperm, by either mixed inseminations or in chimeric testes, the wild-type sperm effectively competed out the acrosin-null sperm (Adham et al., 1997). It is likely that the fertility of the gr−/− male would be more compromised if backcrossed to the isogenic 129/Sv background as recently shown for mice null for the POU protein sperm-1 (Pearse et al., 1997). Presumably, the C57BL/6J hybrid background introduces compensatory gene products that reduce the phenotype penetrance.

It is particularly interesting that gr−/− sperm still bind to the zona pellucida, even though they show low levels of binding ZP3 ligand in solution. This implies that other sperm components, either specific or nonspecific, facilitate the adhesion of sperm to the egg coat. Since the zonae of fertilized embryos have been modified by the action of the cortical granule enzymes, they may not be as ‘sticky’ as are unfertilized zonae pellucidae and, as such, the binding to gr−/− sperm to unfertilized eggs may simply reflect a nonspecific adhesion. Alternatively, the reduced binding of ZP3 to gr−/− sperm may reflect a lower affinity or avidity for ZP3 (as assayed by a reduced level of stable binding), rather than reflecting the complete absence of a ZP3 ‘receptor’. In the latter scenario, other sperm surface proteins may cooperate in some way with GalTase to increase the affinity or avidity of ZP3 binding.

In any event, gr−/− sperm still adhere to the zona pellucida. We presently favor a model in which GalTase sees ZP3 oligosaccharides in the context of another sperm surface component that is responsible for the initial docking of sperm to the egg coat, similar to the concerted action of selectins and integrins in lymphocyte adhesion to the endothelium (Lasky, 1992). Such a model would account for the apparent specificity of sperm surface GalTase to selectively bind ZP3 oligosaccharides, even though ZP1 and ZP2 are recognized by soluble, nonsperm GalTase (Miller et al., 1992). This model would also help explain the apparent paradox of why a variety of GalTase-specific perturbants, such as anti-GalTase antibodies, inhibit sperm-egg binding, although gr−/− sperm still bind to the zona. Presumably, blocking GalTase with competitive reagents sterically blocks adjacent residues in this hypothetical multimeric receptor complex, thereby inactivating sperm-egg binding. The loss of GalTase in gr−/− mice still permits adherence to the zona, but prevents recognition of ZP3 and subsequent induction of the acrosome reaction.

With this in mind, it would be of interest to determine if other sperm surface components thought to facilitate adhesion to the zona are responsible for the binding of gr−/− sperm, such as PH-20 (Primakoff et al., 1985), zonaadhesin (Hardy and Garbers, 1995) and/or those surface components that require the chaperone, calmodulin, for proper folding and expression (Ikawa et al., 1997). From the studies reported here, it appears that GalTase’s function is as a signal transducing receptor for ZP3 oligosaccharides, activating the heterotrimeric G-protein cascade to elicit the acrosome reaction (Ward et al., 1992; Gong et al., 1995). These studies also show that the ZP3-GalTase-receptor-ligand complex is dispensable for fertility, but its presence offers a physiological advantage to the fertilizing sperm.

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