

Receptor Function of Mouse Sperm Surface Galactosyltransferase during Fertilization

LINDA C. LOPEZ,* EVELYN M. BAYNA,*[§] DAVID LITOFF,[§] NANCY L. SHAPER,[‡] JOEL H. SHAPER,[‡] and BARRY D. SHUR*[§]

**Department of Biochemistry and Molecular Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030; [‡]The Oncology Center, and the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; [§]Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06032*

ABSTRACT Past studies from this laboratory have suggested that mouse sperm binding to the egg zona pellucida is mediated by a sperm galactosyltransferase (GalTase), which recognizes and binds to terminal *N*-acetylglucosamine (GlcNAc) residues in the zona pellucida (Shur, B. D., and N. G. Hall, 1982, *J. Cell Biol.* 95:567–573; 95:574–579). We now present evidence that directly supports this mechanism for gamete binding. GalTase was purified to homogeneity by sequential affinity-chromatography on GlcNAc-agarose and α -lactalbumin-agarose columns. The purified enzyme produced a dose-dependent inhibition of sperm binding to the zona pellucida, relative to controls. To inhibit sperm/zona binding, GalTase had to retain its native conformation, since neither heat-inactivated nor Mn⁺⁺-deficient GalTase inhibited sperm binding. GalTase inhibition of sperm/zona binding was not due to steric blocking of an adjacent sperm receptor on the zona, since GalTase could be released from the zona pellucida by forced galactosylation with UDPGal, and the resulting galactosylated zona was still incapable of binding sperm. In control experiments, when UDPGal was replaced with the inappropriate sugar nucleotide, UDPglucose, sperm binding to the zona pellucida remained normal after the adsorbed GalTase was washed away.

The addition of UDPGal produced a dose-dependent inhibition of sperm/zona binding, and also dissociated preformed sperm/zona adhesions by catalyzing the release of the sperm GalTase from its GlcNAc substrate in the zona pellucida. Under identical conditions, UDPglucose had no effect on sperm binding to the zona pellucida. The ability of UDPGal to dissociate sperm/zona adhesions was both time- and temperature-dependent. UDPGal produced nearly total inhibition of sperm/zona binding when the zonae pellucidae were first galactosylated to reduce the number of GalTase binding sites.

Finally, monospecific anti-GalTase IgG and its Fab fragments produced a dose-dependent inhibition of sperm/zona binding and concomitantly blocked sperm GalTase catalytic activity. Preimmune IgG or anti-mouse brain IgG, which also binds to the sperm surface, had no effect. The sperm GalTase was localized by indirect immunofluorescence to a discrete plasma membrane domain on the dorsal surface of the anterior head overlying the intact acrosome. These results, along with earlier studies, show clearly that sperm GalTase serves as a principal gamete receptor during fertilization.

Species-specific gamete interactions are thought to be mediated by their appropriate cell surface receptors (29). The nature of these gamete receptors has been best defined in studies of

sea urchins (24, 35) and of mice (3, 33, 34), in which gamete binding appears to be mediated by interactions between sperm surface proteins and their complementary glycoconjugates on

the egg surface. Studies with *Ciona* (23), *Fucus* (6) and *Limulus* (2), among other species, are consistent with this notion.

Past studies from this laboratory have proposed a specific molecular mechanism for mouse sperm binding to the egg zona pellucida (33, 34). Results suggest that gamete binding is mediated by a sperm surface galactosyltransferase (GalTase),¹ which recognizes and binds to terminal *N*-acetylglucosamine (GlcNAc) residues in the zona pellucida. This model for sperm binding to the zona pellucida is supported by multiple observations. For example, sperm that have a genetic predisposition for increased fertilizing ability have a specific elevation in surface GalTase activity (31). Recombinants that no longer show increased fertilizing ability no longer show elevated GalTase activity (32). Also, competitive GalTase substrates, a GalTase modifier protein (α -lactalbumin), and GalTase substrate analogues all produce a coincident, dose-dependent inhibition of sperm surface GalTase activity and inhibition of sperm binding to the zona pellucida (33, 34). Finally, enzymatic removal or unmasking of terminal GlcNAc residues on the zona pellucida produces a coincident inhibition or stimulation, respectively, of sperm binding (33, 34).

In light of these past results, we have designed experiments to more directly examine the receptor function of sperm surface GalTase during mouse gamete interactions. In this paper, we have tested four specific predictions of this model. (a) If sperm GalTase serves as a receptor during mouse fertilization, then purified, soluble GalTase should serve as a competitive receptor and produce a dose-dependent inhibition of sperm/zona binding. Furthermore, this inhibition should not result from steric blocking of adjacent sperm receptors in the zona pellucida. (b) Sperm binding to the zona pellucida should be inhibited by galactosylation of the zona with purified GalTase and UDPgalactose (UDPGal), which would eliminate potential sperm binding sites. (c) The addition of UDPGal should inhibit sperm/zona binding, as well as dissociate preformed sperm/zona adhesions, by catalytically dissociating the sperm GalTase from its GlcNAc substrate in the zona pellucida. Inappropriate sugar nucleotides should have no effect. (d) Anti-GalTase IgG should block sperm GalTase catalytic activity, inhibit sperm/zona binding, and define the topographical distribution of the surface GalTase. The results of these experiments, as well as others, are reported in this paper, and show that mouse sperm surface GalTase serves as a principal receptor during fertilization.

MATERIALS AND METHODS

Purification of Soluble GalTase

GLCNAc-AGAROSE AFFINITY CHROMATOGRAPHY: A 3.0-ml GlcNAc-agarose (Sigma Chemical Co., St. Louis, MO) affinity column was equilibrated in column buffer containing 0.12 M NaCl, 2 mM sodium cacodylate, 0.5 mM UDP, 25 mM MnCl₂, 1 mM dithiothreitol, and 0.02% NaN₃, adjusted to pH 7.5 with concentrated HCl. Bovine milk GalTase (Sigma Chemical Co.) was dissolved in column buffer and applied to the column at a flow rate of 10 ml/h. The column was washed with 7 vol, and the GalTase was eluted (1.0-ml fractions) at 10 ml/h with column buffer containing 23 mM GlcNAc. The fractions were assayed for GalTase activity by high voltage borate electrophoresis as previously described (33). Fractions 24–40 (Fig. 2) were pooled and passed over an α -lactalbumin-agarose affinity column.

α -LACTALBUMIN-AGAROSE AFFINITY CHROMATOGRAPHY: The GalTase preparation eluted from the GlcNAc-agarose column was applied at

¹ Abbreviations used in this paper: GalTase, galactosyltransferase; GlcNAc, *N*-acetylglucosamine; UDPGlc, UDPglucose; UDPGal, UDPgalactose.

8.0 ml/h to a 3.0-ml α -lactalbumin-agarose (Sigma Chemical Co.) affinity column previously equilibrated in buffer containing 0.12 M NaCl, 25 mM sodium cacodylate, 23 mM GlcNAc, 10 mM MnCl₂, 1 mM dithiothreitol, and 0.02% NaN₃, adjusted to pH 7.5 with concentrated HCl. The column was washed with 10 vol, and the GalTase was eluted (1.0-ml fractions) at 8.0 ml/h with column buffer minus GlcNAc. The fractions were assayed for GalTase activity, pooled, and the protein purity determined by SDS polyacrylamide electrophoresis according to Laemmli (15). Proteins were visualized using silver staining (18). All affinity chromatography was conducted at 4°C. If desired, the purified enzyme was dialyzed against 0.15 M NaCl, buffered with 10 mM HEPES, pH 7.2, before addition to sperm/zona binding assays. Unlabeled UDPGal was purchased from Sigma Chemical Co., and radiolabeled UDPGal was purchased from New England Nuclear (Boston, MA).

Gametes and Sperm/Zona Binding Assays: Sperm and eggs were collected from 8-wk-old CD-1 mice (Charles River Breeders) as previously described (33). Eggs were freed from their cumulus cells by hyaluronidase treatment, rinsed in fresh Brinster's complete medium containing 0.5% bovine serum albumin (BSA) to accommodate nonspecific protein adsorption to the zona surface, and repetitively rinsed in three, 1-ml droplets of protein-free medium. The last droplet contained any reagents that were being tested for effects on sperm/zona binding. The eggs were transferred in a 50- μ l pipet to a 150- μ l droplet of medium containing the test reagent, to which a 20- μ l aliquot of freshly capacitated sperm were added. In studies using IgGs, sperm were transferred to the incubation media immediately before the addition of eggs. Sperm were capacitated as previously described (33). After the desired incubation time, any loosely attached and unbound sperm were removed by three multiple transfers through sperm-free medium, and the sperm bound to the zona pellucida were quantitated. In all instances, the number of bound sperm was determined by counting sperm heads on the zona pellucida in all focal planes. The number of eggs counted in each experiment and the significance of the data determined using the Student's *t*-test is presented in the Results section.

Galactosylation of the Zona Pellucida: Eggs were isolated as described above, and incubated in the presence of 50 μ g/ml GalTase, 1 mM UDPGal, and 1 mM MnCl₂, in 500 μ l for 3 h at 37°C. The eggs in a 30- μ l pipet were transferred through three, 0.3-ml droplets of fresh medium, and assayed for sperm binding using freshly capacitated sperm. In parallel assays, eggs were treated under identical conditions except that UDPGal was substituted with UDPglucose (UDPGlc), which is not a substrate for GalTase. Control levels of sperm/zona binding were determined by using eggs that had been treated in parallel with medium containing 50 μ g/ml BSA and 1 mM MnCl₂, and then washed and assayed for sperm binding. In a separate experiment, 150 eggs were incubated as above with 50 μ g/ml of GalTase, 1 mM MnCl₂, and 0.1 mM UDPGal to which 10 μ Ci of UDP-³H-Gal was added. After the 3-h incubation, the eggs were washed by nine repetitive transfers to reduce the soluble radioactivity to background levels. The galactosylated zonae pellucidae were solubilized in acid Tyrode's solution and the radioactivity determined. The specific activity of the UDP-³H-Gal was used to calculate the picomoles transferred to each zona.

Effect of Anti-GalTase IgG on Sperm Surface GalTase Activity: Rabbits were immunized with affinity-purified bovine milk GalTase, and the anti-GalTase IgG was isolated and characterized as described (28). Monospecific anti-GalTase IgG was prepared by chromatography on an AffiGel-10 (BioRad Laboratories, Richmond, CA) column containing covalently bound, affinity-purified GalTase. Fab fragments were prepared by papain digestion and isolated by carboxymethylcellulose chromatography as described (11). The anti-GalTase IgG was shown to be cross-reactive with mouse serum GalTase using the following protocol (Fig. 1). Mouse serum (10 μ l) was incubated overnight at 37°C with antibody dilutions (10 μ l) containing 10 mg/ml BSA. PanAdsorbent (Calbiochem-Behring Corp., La Jolla, CA) was added, incubated for 1 h, and the antigen/antibody complexes were removed by centrifugation. The residual GalTase activity remaining in the supernatant was assayed as described (28).

Monospecific anti-GalTase IgG and preimmune rabbit IgG were heated at 60°C for 30 min to inactivate any contaminating GalTase activity. Sperm were collected from 8-wk-old CD1 mice as described (33), washed three times in medium B (NaCl, 7.5 g/liter; KCl, 0.4 g/liter; HEPES buffer, 4.76 g/liter; pH 7.2), and aliquots (4 \times 10⁶) were pelleted and resuspended in either anti-GalTase IgG (0.5 mg/ml), preimmune IgG (0.5 mg/ml), or medium B. After a 1-h preincubation at room temperature, the sperm/IgG mixtures were assayed for GalTase activity as described (33) using ovalbumin as substrate (2.0 mg/50 μ l assay). A protease inhibitor cocktail was included in all procedures, containing 0.1% aprotinin, 1 μ g/ml leupeptin, 2 μ g/ml antipain, 10 μ g/ml benzamide, 1 μ g/ml chymostatin, and 1 μ g/ml pepstatin.

Localization of Sperm Surface GalTase by Indirect Immuno-

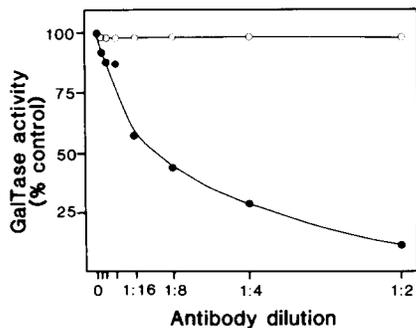


FIGURE 1 Anti-bovine milk GalTase IgG cross-reacts with mouse serum GalTase. Monospecific anti-GalTase IgG was incubated with mouse serum, the antibody/antigen complexes were removed by centrifugation, and the residual mouse serum GalTase was assayed. See Materials and Methods for details. Increasing concentrations of anti-GalTase IgG remove proportionately more mouse GalTase from the supernatant. ●, anti-GalTase IgG; ○, preimmune IgG.

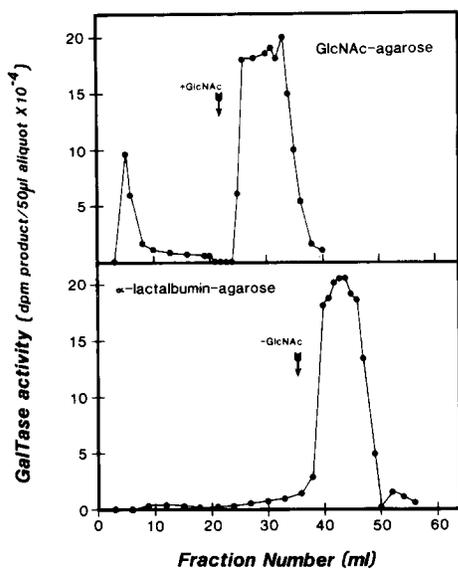


FIGURE 2 Affinity chromatography of GalTase on GlcNAc-agarose and α -lactalbumin-agarose columns. GalTase was dissolved in column buffer, applied to a GlcNAc-agarose column, eluted by the addition of 23 mM GlcNAc to the buffer, pooled, applied to an α -lactalbumin-agarose column, and eluted by the absence of GlcNAc from the buffer. The fractions with GalTase activity were pooled, dialyzed, and concentrated. For additional details, refer to Materials and Methods.

nofluorescence: Sperm were isolated, washed three times in medium B, pelleted, and resuspended in 0.2 ml medium B as described (33). All of the following procedures were conducted at room temperature in a moist atmosphere on sperm bound to glass microscope slides. To form wells, 7-mm holes were punched into Mylar Plastic sheets (Dynatech Laboratories, Inc., Alexandria, VA) and applied to glass slides. 20 μ l of sperm were added to the wells and allowed to adhere for 30 min. Loose sperm were washed away with medium B. To prevent nonspecific sticking of antibodies, 10 μ l of solution A (1% BSA, 0.05% NaN_3 in medium B) was added to the wells. After 10 min, 10 μ l of anti-GalTase IgG or preimmune rabbit IgG, all at 0.5 mg/ml, were added. After a 45-min incubation, the sperm were washed twice with medium B, fixed in 1% paraformaldehyde for 5 min, followed by 10-min incubations with 0.2 M glycine, pH 8.0, and then solution A. 10 μ l of biotinylated goat anti-rabbit IgG (5 μ g/ml in solution A) (Vector Laboratories, Inc., Burlingame, CA) were added and incubated for 30 min. The sperm were washed three times with solution A, and 10 μ l of fluorescein-conjugated avidin (62.5 μ g/ml in medium B) (Cappel Laboratories, Cochranville, PA) were added. After a 20-min incubation, the sperm were washed with solution A, and the slides were mounted and viewed with a Leitz fluorescence microscope using epilumination (E. Leitz,

Inc., Rockleigh, NJ).

Parallel assays were conducted using identical levels of IgG raised against mouse brain (Cappel Laboratories), which was isolated by protein A-affinity chromatography (Sigma Chemical Co.).

RESULTS

Inhibition of Sperm/Zona Binding by Purified GalTase

A soluble form of GalTase was purified to homogeneity by sequential affinity chromatography on GlcNAc-agarose and α -lactalbumin-agarose absorbants (Fig. 2). The affinity-purified GalTase consisted of two molecular weight forms (45 and 54 kD) as analyzed on silver-stained polyacrylamide gels (Fig. 3). These results are similar to those previously reported for affinity-purified bovine milk GalTase (1). No other proteins were detectable after affinity chromatography, even on overloaded SDS polyacrylamide gels (Fig. 3). GalTase was purified from a soluble source, i.e., bovine milk, since membrane-bound GalTase requires the continued presence of detergent for its solubilization and activation, which would then interfere with the sperm/zona binding assay.

Freshly isolated, cumulus-free mouse eggs were transferred through droplets of medium containing 0.5% BSA to allow for nonspecific protein adsorption to the zona pellucida. After

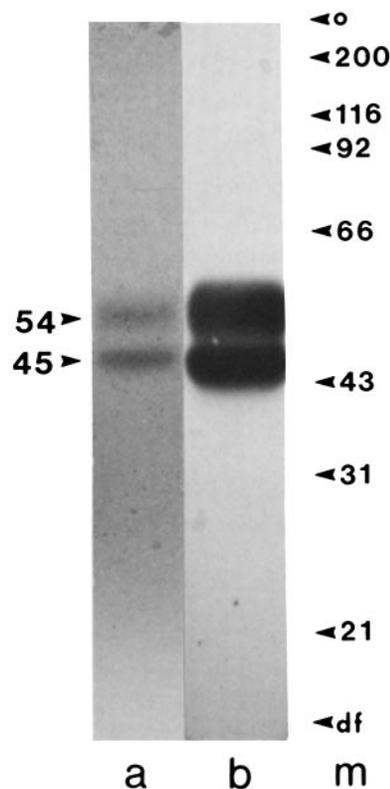


FIGURE 3 SDS-polyacrylamide gel electrophoresis of affinity-purified GalTase. 6.75 μ g of GalTase was electrophoresed in a 10% polyacrylamide gel containing 5% β -mercaptoethanol. Electrophoresis was for 19 h at 15 mA. The gel was stained with silver according to the method of Oakley et al. (18) which is sensitive down to 10 ng of protein. Two different gels are shown, one of which (lane b) was overloaded to detect trace protein contaminants. The mobilities of the molecular weight standards are shown in lane m (markers). The affinity purified GalTase has an estimated molecular weight of 45 kD and 54 kD, in agreement with previously published reports (1). o, origin; df, dye front.

three repetitive rinsings in protein-free medium, the eggs were then transferred through droplets containing various concentrations of GalTase and 20 μ M Mn⁺⁺. The eggs were then preincubated in the same GalTase/Mn⁺⁺-containing medium for 1 h. Control eggs were treated in parallel with identical concentrations of BSA and 20 μ M Mn⁺⁺. Aliquots of sperm were added, and the sperm/egg mixture was incubated for ~10 min. After washing and fixation, the number of bound sperm was determined.

The presence of purified GalTase produced a dose-dependent inhibition of sperm binding to the zona pellucida, relative to BSA-treated controls (Fig. 4). In contrast, heat denaturing the GalTase resulted in a complete loss of its inhibitory activity. In addition, GalTase, even at concentrations as high as 0.5 mg/ml, failed to inhibit sperm/zona binding in the absence of Mn⁺⁺. GalTase has a specific, high affinity (i.e., μ M) binding site for Mn⁺⁺, which is required for its native conformation (21). These results are representative of four

different GalTase preparations, all of which inhibited sperm/zona binding to similar degrees when normalized to the GalTase specific activity (cpm product/ μ g protein). Fig. 4 illustrates the comparative effects of 8 μ g/ml of purified GalTase and of BSA on sperm/zona binding.

Galactosylation of the Zona Pellucida Inhibits Subsequent Sperm Binding

The above experiments showed that purified GalTase inhibits sperm/zona binding. We determined whether adsorbed GalTase sterically prevents sperm from binding to an adjacent receptor, unrelated to the GalTase substrate. To test this possibility, bound GalTase was displaced from the zona pellucida by incubation with UDPGal. As diagrammed in Scheme I, the presence of UDPGal forces catalysis to occur, replacing the GalTase with a covalently linked low molecular weight galactose residue. All released GalTase and unused

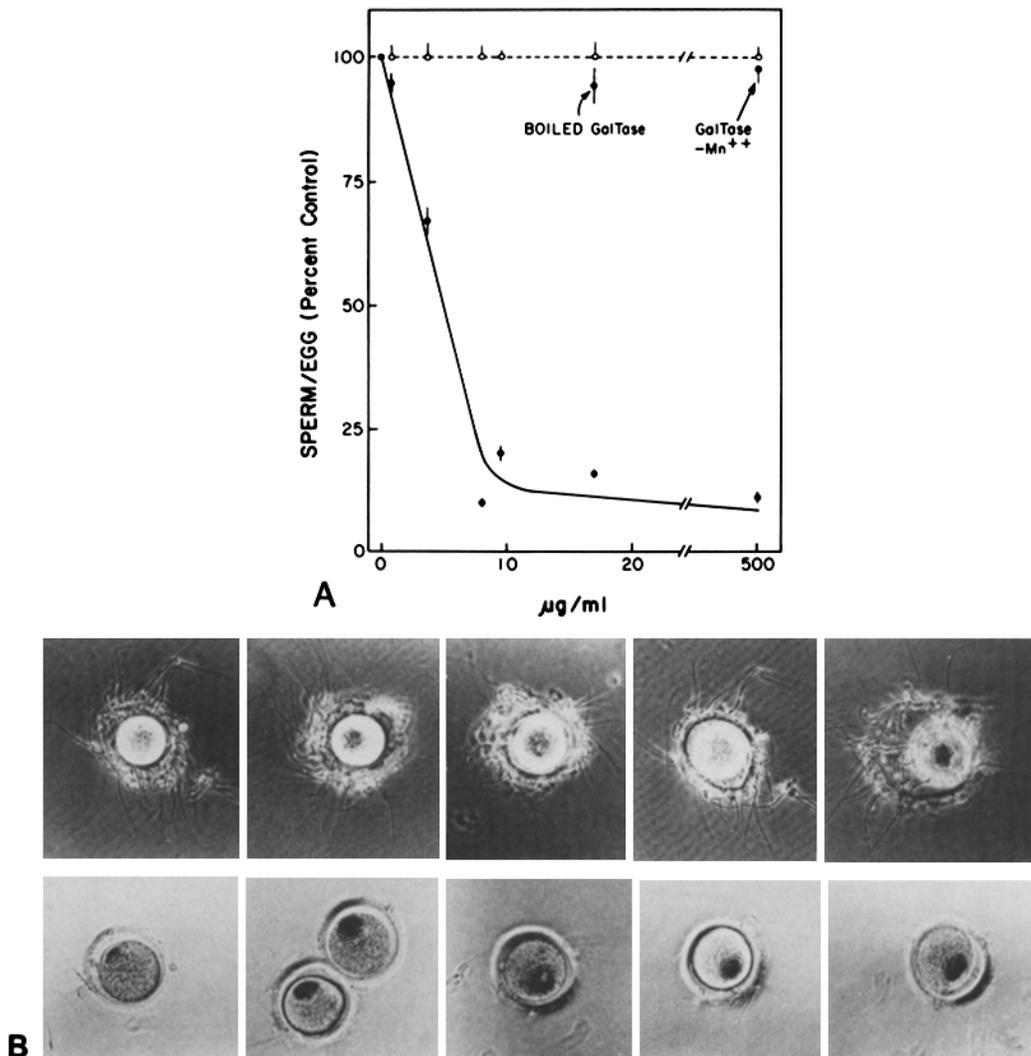


FIGURE 4 Inhibition of sperm binding to the zona pellucida with affinity purified GalTase. (A) Eggs were prerinse, and then incubated for 1 h at 37°C in 200 μ l of the indicated concentration of GalTase (●) (BSA in controls [O]) containing 20 μ M MnCl₂. 20 μ l of sperm were added, and sperm binding to the zona was assayed as described in Materials and Methods. GalTase was heat-inactivated by immersion into a boiling water bath for 2 min. Mn⁺⁺-deficient GalTase was prepared in complete medium minus Mn⁺⁺. (B) The effect of 8 μ g/ml of affinity purified GalTase on sperm zona binding is shown (lower panel) relative to incubations containing 8 μ g/ml BSA (upper panel). Representative eggs are shown from an experiment in which GalTase produced nearly total inhibition of sperm/zona binding. Sperm are bound to the zona pellucida in multiple focal planes.

UDPGal were then removed by exhaustive washing.

Eggs galactosylated with GalTase and UDPGal and then washed were less able to bind sperm than were control eggs (Table I). Control incubations were of two types. First, a similar number of eggs were incubated with standard levels of BSA (0.5%) and then washed to establish control levels of sperm binding to the zona pellucida. Second, eggs were incubated in parallel with identical amounts of GalTase and an inappropriate sugar nucleotide, UDPGlc, and then washed as above. This incubation controlled for both residual GalTase remaining after washing and for nonspecific sugar nucleotide effects on sperm/zona binding. Eggs incubated with GalTase and UDPGlc bound sperm similarly to eggs treated with BSA alone, showing that (a) the washing procedures were effective in removing all inhibiting GalTase and (b) the inhibition resulting from UDPGal incubation was not due to nonspecific sugar nucleotide effects. Washing less than 1,000-fold resulted in residual GalTase in the sperm/zona binding assays, as evidenced by an inhibition of sperm binding in both UDPGal- and UDPGlc-treated incubations. These experiments were conducted four times with similar results.

To determine the moles of galactose transferred to the zona pellucida under these incubation conditions, parallel incubations contained radiolabeled UDPGal and 50 µg/ml of purified GalTase. After washing, the zonae were solubilized in acid Tyrode's solution and the incorporated radioactivity determined. Radiolabeled galactose incorporation was proportional to the amount of GalTase added to the incubation. The incorporation of ~0.2 pmol of galactose onto each zona resulted in a 67% inhibition of sperm binding (Table I).

UDPGal Inhibits Sperm Binding to the Zona Pellucida

The effect of UDPGal on sperm/zona binding was examined. We reasoned that UDPGal may inhibit binding by catalyzing the dissociation of the sperm GalTase from its GlcNAc substrate in the zona pellucida. When present during the 10-min assay, 0.5 and 2.0 mM UDPGal produced a 32 and 60% inhibition, respectively, of sperm/zona binding (Fig. 5). Identical concentrations of UDPGlc had no effect. Three different lots of UDPGal produced similar results.

The ability of UDPGal to inhibit sperm/zona binding was

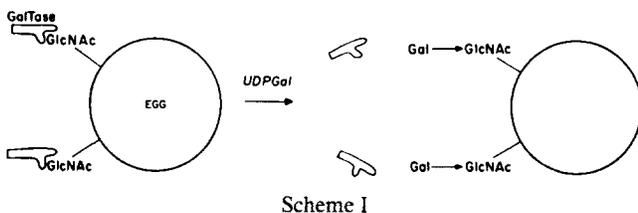


TABLE I. Inhibition of Sperm Binding by Zona Pellucida Galactosylation

Egg pretreatment	No. eggs	Sperm/egg <i>mean ± SEM</i>	% Control	Gal transferred/ zona <i>pmol</i>
BSA, washed	109	53.4 ± 3.1	100	—
GalTase + UDPGal, washed	193	17.6 ± 1.7	33 (<i>P</i> < 0.001)	0.196
GalTase + UDPGlc, washed	107	52.5 ± 3.1	98	—

Eggs were preincubated with purified GalTase and UDPGal in 500 µl for 3 h at 37°C, and washed 1,000-fold in fresh medium, by three repetitive 10-fold rinses. The resulting galactosylated zonae pellucidae were assayed for sperm binding ability. In control experiments, UDPGal was replaced with UDPGlc, and the level of sperm binding remained similar to that seen in BSA-treated incubations.

time-dependent (Table II). UDPGal was more effective in inhibiting sperm/zona adhesions during short (i.e., 5 min) incubations (59% inhibition), than during prolonged (i.e., 27 min) incubations (33% inhibition). As before, UDPGlc was used in control experiments, and had no effect on binding. These results suggest that UDPGal was being slowly degraded by sperm nucleotide pyrophosphatases (31), thus reducing its effective concentration.

Sequential Zona Galactosylation and UDPGal Dissociation of Sperm/Zona Binding

To maximize the effect of UDPGal on sperm/zona binding, zonae pellucidae were first galactosylated with purified GalTase and UDPGal, washed, and then incubated with sperm in the presence of UDPGal. In this experiment, galactosylation of the zona pellucida, or the presence of UDPGal, each produced approximately a 50% inhibition of sperm/zona binding when assayed individually (Scheme II, Table III). However, when galactosylated zonae pellucidae were then incubated with sperm in UDPGal-containing medium, sperm binding was inhibited to less than one sperm per zona pellucida.

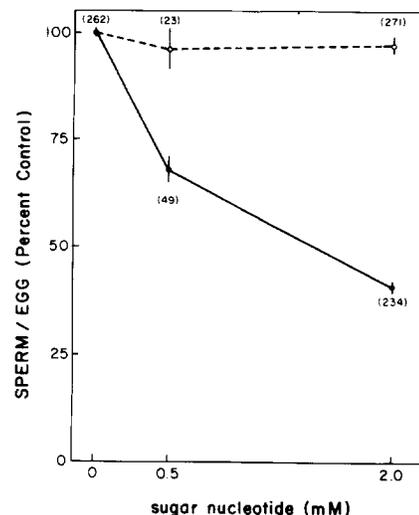


FIGURE 5. The effect of UDPGal on sperm binding to the zona pellucida. Eggs were prepared as described in Materials and Methods, and then rinsed and incubated in the indicated concentration of UDPGal (●). Sperm binding to the zona was assayed as described. Control incubations contained UDPGlc (○) to control for nonspecific sugar nucleotide effects. Control (i.e., 100%) levels of binding averaged 20.6 ± 0.31 sperm/zona pellucida. The number of eggs assayed under each condition is given in the parentheses. Error bars indicate \pm SEM.

UDPGal Dissociates Preformed Sperm/ Zona Adhesions

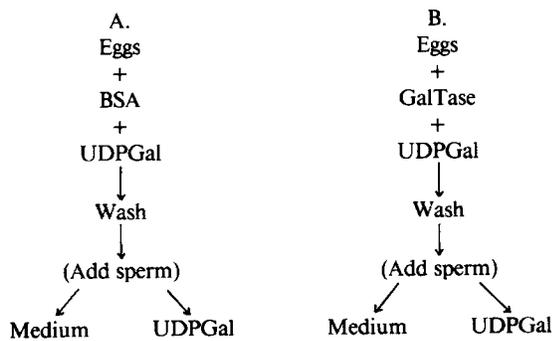
To define more precisely the mode of action of UDPGal on sperm/zona binding, we determined whether UDPGal could catalytically dissociate preformed sperm/zona adhesions, as diagrammed in Scheme III. Sperm and eggs were incubated together under standard conditions for 5 min, and the eggs with adhering sperm were transferred into sperm-free, UDPGal-containing medium. The presence of UDPGal caused visible dissociation of adherent sperm from the zona pellucida. After a 45-min incubation in UDPGal, the eggs were fixed with glutaraldehyde to quantitate the degree of dissociation. Table IV shows that UDPGal produced 71% dissociation, while under identical conditions, UDPGlc had no effect on sperm/zona adhesions.

TABLE II. Temporal Decrease in UDPGal-Mediated Inhibition of Sperm/Zona Binding

Incuba- tion time min	Additions	No. eggs	Sperm/egg mean ± SEM	% Control
5	Medium	30	15.9 ± 0.75	100
5	UDPGal	37	6.5 ± 0.52	41* (<i>P</i> < 0.001)
5	UDPGlc	35	17.6 ± 1.0	111
27	Medium	26	26.8 ± 1.5	00
27	UDPGal	32	18.0 ± 1.5	67* (<i>P</i> < 0.001)
27	UDPGlc	18	24.2 ± 2.4	90

Sperm and eggs were isolated and prepared for assay as described in Materials and Methods. Assays contained either control medium or 2 mM sugar nucleotide. After the indicated incubation time, the eggs were repetitively rinsed and fixed with glutaraldehyde.

* *P* < 0.001 between these two data points.



Scheme II

TABLE III. Sequential Zona Galactosylation and UDPGal Inhibition of Sperm/Zona Binding

	Medium*	UDPGal*	Medium [‡]	UDPGal [‡]
Sperm/egg (±SEM)	6.5 ± 0.56	3.2 ± 0.35	3.4 ± 0.37	0.83 ± 0.16
% Control	100%	49%	52%	13%
		(<i>P</i> < 0.001) [§]	(<i>P</i> < 0.001) [§]	(<i>P</i> < 0.001) [‡]
No. eggs	28	29	30	32

Eggs were isolated as described in Materials and Methods. One-half of the eggs were incubated with 50 µg/ml of GalTase, 100 µM UDPGal, and 1 mM MnCl₂ for 3 h at 37°C. The remaining eggs were incubated with 50 µg/ml BSA, 100 µM UDPGal, and 1 mM MnCl₂ under the same conditions. After washing 100-fold in fresh medium, each egg preparation was split in half and assayed for sperm binding ability. One-half of each preparation was incubated in 2 mM UDPGal to inhibit sperm/zona binding, while the remaining eggs were incubated in parallel in plain medium. Galactosylation of the zona pellucida followed by inclusion of UDPGal in the incubation medium produces nearly total inhibition of sperm/zona binding.

* Refers to data from Scheme 2A.

[‡] Refers to data from Scheme 2B.

[§] Relative to 100% control.

[‡] Relative to all other incubations.

The ability of UDPGal to dissociate sperm/zona adhesions was both time- and temperature-dependent. First, preformed sperm/zona adhesions were resistant to UDPGal dissociation when the sperm were immotile, as was the case when transferred from warm into cool (i.e., 22°C), slightly alkaline, UDPGal-containing medium (Table IV). Also, preformed sperm/zona adhesions became resistant to UDPGal dissociation if the adhering sperm were allowed to bind for an additional 25 min before UDPGal exposure.

The Effect of Competitive NAc-Glucosaminidase Substrates on Sperm/Zona Binding

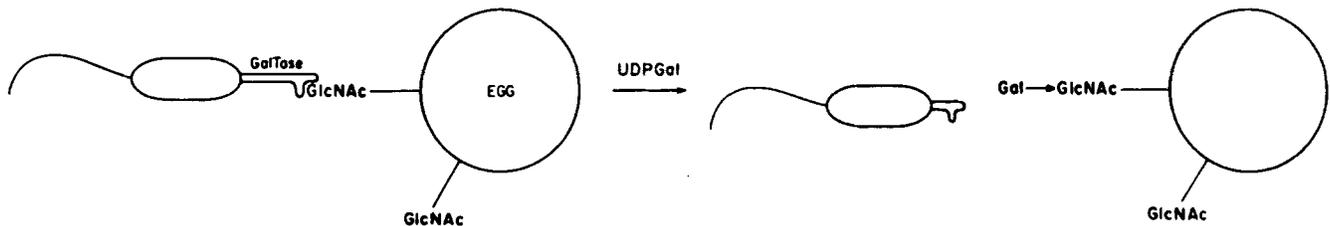
We tested the possibility that a sperm surface NAc-glucosaminidase could be participating in sperm/zona adhesions by binding to exposed GlcNAc residues on the zona pellucida under noncatalytically active conditions. The presence of saturating levels (8, 31) of competitive NAc-glucosaminidase substrates (3 mM *p*-NO₂-phenyl-NAc-glucosaminide) did not inhibit sperm binding to the zona pellucida (27.6 ± 1.2 sperm/egg vs 23.2 ± 1.2 sperm/egg in controls). In fact, the presence of competitive NAc-glucosaminidase substrates produced a slight stimulation in sperm binding (119% of control, *p* < 0.01). The competitive galactosidase substrate, *p*-NO₂-phenyl-galactoside, was used as an additional control (104% of control; 24.1 ± 3.1 sperm/egg), since sperm do not possess detectable galactosidase activity (7, 8; Shur, B. D., unpublished observations).

Inhibition of Sperm/Zona Binding and GalTase Activity by Monospecific Anti-GalTase IgG

Rabbits were immunized with affinity-purified GalTase. The IgG was isolated and made monospecific by repurification on a GalTase-AffiGel affinity column (28). The anti-GalTase IgG was shown to cross-react with mouse GalTase (Fig. 1). We examined the effect of this monospecific anti-GalTase IgG on sperm binding to the zona pellucida and on sperm GalTase activity.

Appropriate dilutions of anti-GalTase IgG were made in medium containing 0.5% BSA. Sperm were added to the IgG solutions, followed by the addition of eggs in a measured volume of medium. After 10 min of incubation, the sperm/egg mixture was washed, fixed, and the number of bound sperm determined.

Anti-GalTase IgG produced a dose-dependent inhibition of sperm binding to the zona pellucida, relative to controls (Fig. 6). Five different anti-GalTase IgG preparations were



Scheme III

TABLE IV. UDPGal-mediated Dissociation of Sperm/Zona Binding

Treatment	Incubation conditions	No. eggs	Sperm/egg	% Control
Sperm + eggs (5-min incubation) transferred into:				
Medium	36°C, motile sperm	66	7.6 ± 0.44	100
UDPGal		93	2.2 ± 0.25	29 (<i>P</i> < 0.001)
UDPGlc		85	7.3 ± 0.32	96
Medium	22°C, immotile sperm	29	8.8 ± 0.51	100
UDPGal		31	8.9 ± 0.50	101
UDPGlc		32	9.2 ± 0.62	105
Complete medium (25-min incubation) transferred into:				
Medium	36°C, motile sperm	34	5.4 ± 0.46	100
UDPGal		28	5.8 ± 0.48	107
UDPGlc		25	5.2 ± 0.63	96

Eggs were isolated as described in Materials and Methods, and incubated with sperm under standard conditions for 5 min at 37°C. The eggs with adhering sperm were transferred to sperm-free medium containing 2 mM sugar nucleotide. After a 45-min incubation at the indicated temperature, the eggs were fixed, and the number of bound sperm determined. In parallel experiments, eggs with adhering sperm were transferred after 5 min of incubation to fresh sperm-free medium for an additional 25-min incubation at 37°C. This second incubation enables the bound sperm to undergo subsequent binding events before being transferred to UDPGal-containing medium. Only the early (i.e., 5 min) interactions between the sperm and egg can be dissociated by UDPGal.

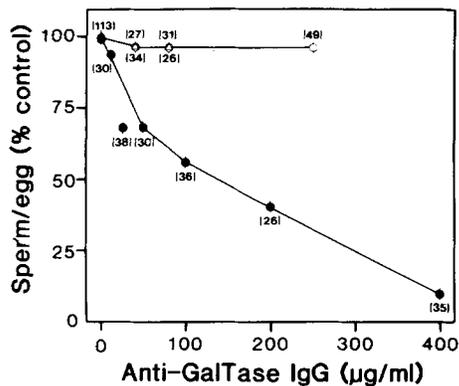


FIGURE 6. Inhibition of sperm binding to the zona pellucida by monospecific anti-GalTase IgG. Eggs and sperm were prepared as described in Materials and Methods. IgG was added at the indicated final concentration, and sperm binding to the zona pellucida was assayed as described. Only anti-GalTase IgG inhibited sperm/zona binding. Control level of binding averaged 20.6 ± 0.11 sperm/zona pellucida. The number of eggs assayed at each concentration of antibody is given in parentheses. Error bars indicate \pm SEM. ●, anti-GalTase IgG; ○, preimmune rabbit IgG; △, anti-mouse brain IgG.

used in these experiments and produced similar results. There was no noticeable inhibition of sperm motility. Control incubations were of three types: no IgG, preimmune rabbit IgG, and anti-mouse brain IgG. Anti-mouse brain IgG was used to control for nonspecific effects of IgG binding to the sperm surface, since this antibody also binds to the sperm plasma membrane (see below). Neither the preimmune nor the anti-mouse brain IgG had any effect on sperm/zona binding at the concentrations shown (Fig. 6). High concentrations of anti-mouse brain IgG (>0.3 mg/ml) produced partial inhi-

TABLE V. Univalent Anti-GalTase Antibody Inhibits Sperm/Zona Binding

Addition	No. eggs	Sperm/egg	% Control
mg/ml		mean \pm SEM	
None	34	121.4 \pm 1.19	100
Preimmune Fab			
3.2	16	100.8 \pm 1.27	83
1.6	17	105.6 \pm 0.75	87
Anti-GalTase Fab			
3.2	17	29.1 \pm 0.46	24*
1.6	25	49.8 \pm 0.52	41*
0.8	22	69.2 \pm 0.29	57*
0.4	18	92.3 \pm 0.41	76*
0.2	17	114.1 \pm 0.57	94

Fab fragments were prepared as described (11) and added to sperm/zona binding assay at the indicated concentration. After 10 min of incubation, the eggs with adhering sperm were rinsed as described in Materials and Methods, and the number of bound sperm was determined. * *P* < 0.001.

bition of sperm/zona binding due to an inhibition of sperm motility. Anti-GalTase IgG was cleaved with papain as described in Materials and Methods to generate univalent Fab fragments. These Fab fragments could still block sperm/zona binding (Table V).

We examined the ability of the anti-GalTase IgG to inhibit sperm GalTase activity. Sperm that had been preincubated with anti-GalTase IgG showed a 79% inhibition of GalTase activity (Table VI). Similar levels of preimmune IgG had no effect on enzyme activity. The inhibition of sperm GalTase activity by anti-GalTase IgG was of similar degree to the

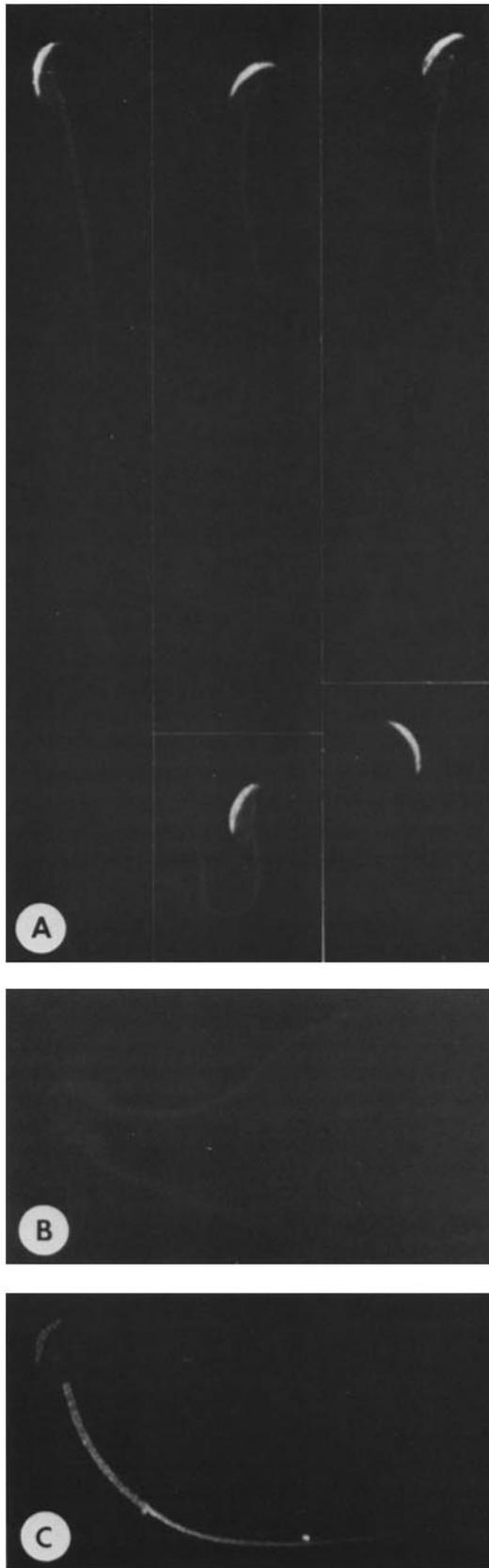


FIGURE 7 Immunofluorescent localization of surface GalTase. Sperm were washed and stained for surface GalTase using biotinylated goat anti-rabbit IgG/fluoresceinated avidin as described in Materials and Methods. The GalTase is localized exclusively to the plasma membrane overlying the intact acrosome on the dorsal

TABLE VI. Effect of Anti-GalTase IgG on Sperm GalTase Activity

Addition	pmol Product/10 ⁶ sperm per h <i>mean ± SEM</i>	% Control
None (medium)	7.99 ± 0.16	100
Preimmune IgG	8.78 ± 0.17	109
Anti-GalTase IgG	1.68 ± 0.03	21

Sperm surface GalTase activity was assayed as described in the Materials and Methods. IgGs were heat-inactivated to destroy any contaminating GalTase activity and preincubated with washed sperm for 1 h at room temperature before assay.

inhibition of sperm/zona binding (89%) seen at this concentration of IgG (0.4 mg/ml).

Localization of Sperm Surface GalTase by Indirect Immunofluorescence

The above experiments show that anti-GalTase IgG inhibits sperm/zona binding and GalTase enzymatic activity. The monospecific anti-GalTase IgG was used to localize the sperm surface GalTase by indirect immunofluorescence using biotinylated goat anti-rabbit IgG and fluoresceinated avidin as described in Materials and Methods.

Live sperm were stained with anti-GalTase IgG, without prior fixation, and under conditions in which the acrosomal membranes remained intact. (Inducing the acrosome reaction with Ca⁺⁺, ionophore, and solubilized zonae, changes the GalTase distribution; manuscript in preparation.) Fig. 7a shows that sperm surface GalTase is localized exclusively to a discrete patch of the plasma membrane on the dorsal surface of the anterior head overlying the acrosome. Rabbit anti-mouse brain IgG also binds to the sperm surface, being localized over the acrosome and along the midpiece and proximal tail (Fig. 7c). Under identical conditions, preimmune rabbit IgG used as first antibody produced background levels of fluorescence (Fig. 7b).

DISCUSSION

The data presented in this paper show that GalTase, purified to homogeneity, inhibits mouse sperm binding to the zona pellucida, and that this inhibition is due to direct binding of the GalTase to the sperm receptor located within the zona. This inhibition is not due to nonspecific protein effects on sperm/zona binding, since identical levels of BSA had no effect on binding. In addition, the GalTase must retain its appropriate conformation for it to inhibit sperm/zona adhesion, since neither heat-inactivated nor Mn⁺⁺-deficient GalTase inhibit sperm binding. Results show that GalTase inhibition is not due to contaminating proteases or glycosidases that might not be seen on polyacrylamide gel electrophoresis (Fig. 3), since the purified GalTase can be washed away from the eggs leaving no residual inhibition. The data also show that GalTase inhibition is not due to steric blocking of sperm binding to the zona, since catalytically removing the GalTase off of the zona with UDPGal, resulting in a galactosylated zona pellucida, still produces an inhibition of sperm binding.

surface of the anterior head. At similar concentrations, anti-mouse brain IgG also stains the dorsal aspect of the anterior head, as well as the midpiece and proximal tail. (A) anti-GalTase IgG, (B) preimmune rabbit IgG, and (C) anti-mouse brain IgG.

Furthermore, the addition of the GalTase substrate, UDPGal, produces a dose-dependent inhibition of sperm binding to the zona pellucida. We believe that the ability of UDPGal to inhibit sperm/zona binding is being compromised by the continued binding, dissociation, and rebinding of excess sperm present in the incubation medium. In this regard, UDPGal dissociates over 70% of preformed sperm/zona adhesions. Under identical conditions, UDPGlc has no effect on sperm/zona binding, demonstrating the appropriate substrate specificity for GalTase. Sperm/zona adhesions can no longer be dissociated by UDPGal after prolonged sperm/zona binding, or when the sperm become immotile. The possible involvement of sperm NAc-glucosaminidases as receptors in mouse sperm binding to the zona appears unlikely, since saturating levels of competitive NAc-glucosaminidase substrates fail to inhibit sperm/zona binding. Rather, their presence actually elevates sperm binding to the zona. This elevation is probably due to the competitive inhibition of sperm acrosomal NAc-glucosaminidases, which are hydrolyzing terminal GlcNAc residues on the zona pellucida, thus eliminating potential binding sites for the sperm surface GalTase.

Finally, monospecific anti-GalTase IgG produces a dose-dependent inhibition of sperm/zona binding, and coincidentally blocks sperm surface GalTase activity. The anti-GalTase IgG was used to localize the GalTase by indirect immunofluorescence to the plasma membrane overlying the intact acrosome. This distribution of GalTase is consistent with its receptor function, since acrosome-reacted sperm can no longer bind to the zona pellucida (9, 25), suggesting that the receptor is localized over the intact acrosome.

Under identical conditions, preimmune rabbit IgG has no inhibitory effect on any activity assayed. In addition, IgG raised against mouse brain also binds to the sperm surface overlying the acrosome, in addition to the tail, but has no effect on sperm/zona binding at concentrations that inhibit binding with anti-GalTase IgG. (At higher concentrations, anti-mouse brain IgG inhibits sperm motility, unlike anti-GalTase IgG, and partially inhibits sperm/zona binding.) More significantly, univalent Fab fragments of anti-GalTase IgG still inhibit GalTase activity and sperm/zona binding.

These observations, when coupled with our past studies (32-34) on the effects of sperm GalTase competitive substrates, a GalTase modifier protein (α -lactalbumin), GalTase substrate analogues, and glycosidase and pronase digestion of the zona pellucida, strongly suggest that sperm plasma membrane GalTase is the principal receptor for sperm binding to the zona pellucida during gamete recognition.

Other workers have shown that sperm/zona adhesions can be temporally dissected into at least two component parts (5, 9, 12, 13, 25). Initially, sperm attachment to the zona is of low affinity, and sperm can be easily removed from the zona surface by washing. The attached sperm become progressively more adherent to the zona, and these bound sperm can no longer be dislodged by simple washing. It is not clear at present what the biochemical distinction is, if any, between sperm attachment and subsequent binding to the zona pellucida. Results reported in this paper show that GalTase functions at least during the high affinity binding between the sperm and zona pellucida, since UDPGal dissociates sperm/zona adhesions that have resisted washing in excess sperm-free medium to dislodge loosely attached sperm. Whether sperm GalTase

participates during other stages of gamete binding is not clear.

The identity of the GalTase substrate in the zona pellucida to which the sperm GalTase binds is of obvious interest to us. Studies from the laboratories of Wassarman (3, 4) and Dean (30) show the mouse zona to be composed of three principal glycoprotein families, one of which, ZP3, has many properties expected of the receptor for sperm. Whether or not ZP3 is the substrate for sperm GalTase upon sperm binding is now under investigation. Consistent with this possibility is the recent finding that the sperm receptor activity of ZP3 resides in its carbohydrate, rather than protein residues (10). Our studies predict that the terminal glucose moiety on ZP3, which possesses sperm receptor activity, serves as a GalTase substrate and is likely to be an *N*-acetylhexosamine.

After sperm bind to the zona pellucida, the acrosome reaction is triggered, and the sperm penetrates the zona matrix and eventually fuses with the egg plasma membrane (5, 9, 25). Fusion of the acrosome-reacted sperm with the egg plasma membrane initiates the cortical reaction, during which the contents of the cortical granules are released into the zona pellucida. This results in a chemical modification of the zona matrix eliminating sperm receptor activity and leading to the zona block to polyspermy (12). Cortical granules contain both glycosidases and lectins, which can remove or mask, respectively, terminal carbohydrate residues (27). It is therefore possible that polyspermy prevention results in part from the hydrolytic removal of sperm GalTase binding sites, i.e., GlcNAc residues, by glucosaminidases released from the cortical granules. This would predict that the zona receptor for sperm would lose its terminal GlcNAc residue after fertilization. Such an enzymatic modification of the zona receptor would not be detectable on SDS polyacrylamide gels, consistent with the lack of any changes in ZP3 after fertilization that are detectable on polyacrylamide gels (3).

Biochemical and antibody approaches are being applied to reach an understanding of sperm/zona interactions in many species, ranging from algae, to invertebrates, to mammals (for review, see references 16 and 29). Whether sperm surface GalTase participates in gamete interactions in any other species is of course unknown, as is the relationship of GalTase, if any, to membrane components thought to function during fertilization in other species (14, 17, 19, 20, 22, 26). GalTase has been identified biochemically on the surface of guinea pig (Primakoff, P., D. G. Myles, and B. D. Shur, unpublished observations) and human (Cross, N., and Shur, B. D., unpublished observations) sperm, but its role during gamete interactions in these species awaits to be determined.

This work was supported by grant PCM 8400817 from the National Science Foundation and by grant CA 30471 from the National Institutes of Health.

Received for publication 1 July 1985.

REFERENCES

1. Barker, R., K. W. Olsen, J. H. Shaper, and R. L. Hill. 1972. Agarose derivatives of uridine diphosphate and *N*-acetylglucosamine for the purification of galactosyltransferase. *J. Biol. Chem.* 247:7135-7147.
2. Barnum, S. R., and G. G. Brown. 1983. Effect of lectins and sugars on primary sperm attachment in the horseshoe crab, *Limulus polyphemus* L. *Dev. Biol.* 95:352-359.
3. Bliel, J. D., and P. M. Wassarman. 1980. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell.* 20:873-882.
4. Bliel, J. D., and P. M. Wassarman. 1980. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida.

- Dev. Biol.* 76:185-203.
5. Bliel, J. D., and P. M. Wassarman. 1983. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* 95:317-324.
 6. Bolwell, G. P., J. A. Callow, and L. V. Evans. 1980. Fertilization in brown algae. III. Preliminary characterization of putative gamete receptors from eggs and sperm of *Fucus serratus*. *J. Cell Sci.* 43:209-224.
 7. Conchie, J., and T. Mann. 1957. Glycosidases in mammalian sperm and seminal plasma. *Nature (Lond.)*. 179:1190-1191.
 8. Conchie, J., J. Findlay, and L. A. Levvy. 1959. Mammalian glycosidases: distribution in the body. *Biochemistry*. 71:318-325.
 9. Florman, H. M., and B. T. Storey. 1982. Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization *in vitro*. *Dev. Biol.* 91:121-130.
 10. Florman, H. M., K. B. Bechtol, and P. M. Wassarman. 1984. Enzymatic dissection of the functions of the mouse egg's receptor for sperm. *Dev. Biol.* 106:243-255.
 11. Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. *Methods in Immunology*. Third ed. W. A. Benjamin, Inc., Reading, MA. 256.
 12. Gwatkin, R. B. L. 1976. Fertilization. In *The Cell Surface in Animal Embryogenesis and Development*. G. Poste and G. L. Nicolson, editors. Elsevier/North Holland Biomedical Press, Amsterdam. 1-43.
 13. Hartmann, J. F., R. B. L. Gwatkin, and C. F. Hutchinson. 1972. Early contact interactions between mammalian gametes *in vitro*: evidence that the vitellus influences adherence between sperm and the zona pellucida. *Proc. Natl. Acad. Sci. USA*. 69:2767-2769.
 14. Huang, T. T. F., Jr., E. Ohzu, and R. Yanagimachi. 1982. Evidence suggesting that L-fucose is part of a recognition signal for sperm-zona pellucida attachment in mammals. *Gamete Res.* 5:355-361.
 15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
 16. Monroy, A., and F. Rosati. 1983. Review article: a comparative analysis of sperm-egg interaction. *Gamete Res.* 7:85-102.
 17. Naz, R. K., N. J. Alexander, M. Isahakia, and M. S. Hamilton. 1984. Monoclonal antibody to a human germ cell membrane glycoprotein that inhibits fertilization. *Science (Wash. DC)*. 225:342-344.
 18. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361-363.
 19. O'Rand, M. G., J. J. Emery, J. E. Welch, and S. J. Fisher. 1984. Zona binding proteins of mammalian spermatozoa. *J. Cell Biol.* 99 (4, Pt. 2): 395a. (Abstr.)
 20. Peterson, R. N., L. D. Russell, D. Bundman, M. Conway, and M. Freund. 1981. The interaction of living boar sperm and sperm plasma membrane vesicles with the porcine zona pellucida. *Dev. Biol.* 84:144-156.
 21. Powell, J. T., and K. Brew. 1976. Metal ion activation of galactosyltransferase. *J. Biol. Chem.* 251:3645-3652.
 22. Primakoff, P., and D. G. Myles. 1983. A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* 98:417-428.
 23. Rosati, F., and R. DeSantis. 1980. Role of the surface carbohydrates in sperm-egg interaction in *Ciona intestinalis*. *Nature (Lond.)*. 283:762-764.
 24. Rossignol, D. P., B. J. Earles, G. L. Decker, and W. J. Lennarz. 1984. Characterization of the sperm receptor on the surface of the eggs of *Strongylocentrotus purpuratus*. *Dev. Biol.* 104:308-321.
 25. Saling, P. M., J. Sowinsky, and B. T. Storey. 1979. An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae *in vitro*: sequential relationship to the acrosome reaction. *J. Exp. Zool.* 209:229-238.
 26. Saling, P. M. 1981. Involvement of trypsin-like activity in binding of mouse spermatozoa to zonae pellucidae. *Proc. Natl. Acad. Sci. USA*. 78:6231-6235.
 27. Schmeil, E. D., B. J. Gulyas, and J. L. Hedrick. 1983. Egg surface changes during fertilization and the molecular mechanism of the block to polyspermy. In *Mechanisms and Control of Animal Fertilization*. J. F. Hartmann, editor. Academic Press, Inc., New York. 365-413.
 28. Shaper, N. L., P. L. Mann, and J. H. Shaper. 1985. The demonstration of a cell surface UDP-galactosyltransferase on mammalian cells by indirect immunofluorescence. *J. Cell. Biochem.* 28:229-239.
 29. Shapiro, B. M., and E. M. Eddy. 1980. When sperm meets egg: biochemical mechanisms of gamete interaction. *Int. Rev. Cytol.* 66:257-302.
 30. Shimizu, S., M. Tsuji, and J. Dean. 1983. *In vitro* biosynthesis of three sulfated glycoproteins of murine zonae pellucidae by oocytes grown in follicle culture. *J. Biol. Chem.* 258:5858-5863.
 31. Shur, B. D., and D. Bennett. 1979. A specific defect in galactosyltransferase regulation on sperm bearing mutant alleles of the T/t locus. *Dev. Biol.* 71:243-259.
 32. Shur, B. D. 1981. Galactosyltransferase activities on sperm bearing multiple *t^{lethal}* and *t^{viable}* haplotypes of the T/t-complex. *Genet. Res.* 38:225-236.
 33. Shur, B. D., and N. G. Hall. 1982. Sperm surface galactosyltransferase activities during *in vitro* capacitation. *J. Cell Biol.* 95:567-573.
 34. Shur, B. D., and N. G. Hall. 1982. A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *J. Cell Biol.* 95:574-579.
 35. Vacquier, V. D., and G. Moy. 1977. Isolation of bindin: the protein responsible for adhesion of sperm to sea urchin eggs. *Proc. Natl. Acad. Sci. USA*. 74:2456-2460.