A Role for Mouse Sperm Surface Galactosyltransferase in Sperm Binding to the Egg Zona Pellucida

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ABSTRACT Past studies have suggested that mouse sperm surface galactosyltransferase may participate during fertilization by binding *N*-acetylglucosamine (GlcNAc) residues in the zona pellucida. In this paper, we examined further the role of sperm surface galactosyltransferase in mouse fertilization. Two reagents that specifically perturb sperm surface galactosyltransferase activity both inhibit sperm-zona binding. The presence of the milk protein α-lactalbumin specifically modifies the substrate specificity of sperm galactosyltransferase away from GlcNAc and towards glucose and simultaneously inhibits sperm binding to the zona pellucida. Similarly, UDP-dialdehyde inhibits sperm binding to the zona pellucida and sperm surface galactosyltransferase activity to identical degrees. Of five other sperm enzymes assayed, four are unaffected by UDP-dialdehyde, and one is affected only slightly. Covalent linkage of UDP-dialdehyde to sperm dramatically inhibits binding to eggs, while treatment of eggs with UDP-dialdehyde has no effect on sperm binding. Heat-solubilized or pronase-digested zona pellucida inhibit sperm-zona binding, and they can be glycosylated by sperm with UDP-galactose. Sperm are also able to glycosylate intact zona pellucida with UDP-galactose. Thus, solubilized and intact zona pellucida act as substrates for sperm surface GlcNAc:galactosyltransferases. Finally, pretreatment of eggs with β-*N*-acetylglucosaminidase inhibits sperm binding by up to 86%, while under identical conditions, pretreatment with β-galactosidase increases sperm binding by 55%. These studies, in conjunction with those of the preceding paper dealing with surface galactosyltransferase changes during capacitation, directly suggest that galactosyltransferase is at least one of the components necessary for sperm binding to the zona pellucida.

This paper addresses the molecular mechanism of mouse sperm binding to the zona pellucida. It has been 70 years since Lillie first proposed that gamete recognition may involve interactions between complementary cell surface receptors (4). Only in the last 10 years have any specific molecular hypotheses been proposed to account for species-specific gamete recognition. Evidence suggests that sperm receptor proteins may bind specific carbohydrate residues on the egg surface (5–7). For example, sea urchin sperm acrosome vesicles contain "bindin", a lectinlike protein that is capable of agglutinating eggs of the homologous species (8), and a glycopeptide receptor has been isolated from sea urchin eggs by bindin-specific affinity chromatography (9).

Mammalian fertilization also likely involves specific complementary receptors on interacting gamete surfaces (10). A particular zona pellucida glycoprotein has been implicated as the receptor for mouse sperm (11). Recently, evidence has suggested that mouse sperm surface galactosyltransferase participates during fertilization by binding GlcNAc residues in the zona pellucida (1–3). Two types of results support this possibility. First, in the preceding paper (3), results showed that sperm capacitation is associated with the release of specific sperm bound galactosyltransferase substrates, thereby exposing the surface galactosyltransferase for binding to the zona pellucida. The released galactosyl acceptors serve as "decapacitation factors" (i.e., inhibit capacitated sperm binding to the zona pellucida) when added back to in vitro fertilization assays. Glycosides not recognized by sperm surface galactosyltransferases do not inhibit sperm binding to the zona pellucida. Second, sperm bearing mutant alleles of the T/t-complex, which show increased fertilizing ability, have a specific fourfold increase in surface galactosyltransferase activity. Eight other *t*-sperm enzyme activities are indistinguishable from normal (1). Sperm bearing recombinant *t*-chromosomes, or one of four

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 NOVEMBER 1982 574-579

dominant *T* mutations, none of which affect fertilization, have galactosyltransferase activity equal to normal (2).

Thus, two approaches have suggested that mouse sperm surface galactosyltransferase may participate during fertilization by binding GlcNAc residues in the zona pellucida. Both studies, however, were largely concerned with related issues, e.g., the nature of capacitation and *t*-sperm biochemistry. Therefore, the present analysis was undertaken to test more directly the involvement of sperm surface galactosyltransferase in zona pellucida binding. Results obtained with reagents that specifically perturb surface galactosyltransferase activity, and studies of glycosylation and glycosidase-digestion of the zona pellucida, directly support the notion that GlcNAc:galactosyltransferase is at least one of the sperm surface receptors required for zona pellucida binding.

MATERIALS AND METHODS

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO); pregnant mare serum (PMS), human chorionic gonadotropin (HCG), hyaluronidase, UDPGal, uridinediphosphate (UDP), Naperiodate, α-lactalbumin, Bovine Serum Albumin (BSA), β-*N*-acetylglucosaminidase, β-galactosidase, *p*-NO_2-phenyl-glycosides, and *p*-NO_2-phenyl-phosphate. Pronase was purchased from Calbiochem-Behring Corp. (San Diego, CA). One unit of glycosidase liberates 1 μmol reducing sugar/min.

Gametes and In Vitro Fertilization Assays

Viable cauda epididymal sperm were collected from CD1 (Charles River Breeding Laboratories, Inc., Wilmington, MA) males, and eggs collected from superovulated CD1 females. Sperm binding to the zona pellucida was assayed as described in the preceding paper (3).

Enzyme Assays

GALACTOSYLTRANSFERASE: Sperm surface galactosyltransferase activity was assayed under optimal conditions as described in the preceding paper (3). Incubations contained 0.5 × 10^6 sperm, 203 μM UDP[^{3}H]galactose (UDPGal) (197 mCi/mmol) (New England Nuclear, Boston, MA), and 10 mM $MnCl_2$ in 50 μl of Medium B (NaCl, 7.5 g/l; KCl, 0.4 g/l; HEPES buffer, 4.76 g/l; pH 7.2). Saturating levels of GlcNAc (30 mM final) were used to assay activity towards exogenous acceptors. The reaction was stopped with NaEDTA, and the reaction product isolated by high voltage borate electrophoresis (1–3). All enzyme assays have had background levels of radioactivity (0°C incubations) subtracted from them.

The galactosyltransferase inhibitor, UDP-dialdehyde was made by combining equal concentrations (30 mM each) of UDP and Na metaperiodate (12). After 2 h in the dark, at 4°C, unreacted periodate was consumed with glycerol (25 mM final), and the resulting UDP-dialdehyde was added to enzyme assays or in vitro fertilization assays at the indicated final concentration. For dose-dependency studies, 15 mM UDP-dialdehyde was diluted with glass-distilled water so that equal volumes of water (~0.1 final volume) were added across the entire concentration range. The concentrations of UDP-dialdehyde listed are the maximum possible, assuming that all of the UDP and periodate reacted within the 2-h incubation, producing 15 mM UDP-dialdehyde.

SPECTROPHOTOMETRIC ASSAYS: β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, β-galactosidase, α-mannosidase, and alkaline phosphatase were all assayed as previously described (3). 10 μl of sperm suspension (~1 × 10^6 sperm) in Medium B were added to 0.2 ml of either 0.1 M sodium citrate buffer, pH 5.0 (glycosidases) or 0.1 M glycine-NaOH buffer, pH 10.2 (alkaline phosphatase), which contained 3.5 mM of the appropriate *p*-NO_2-phenyl substrate, and 30 μl of either glass-distilled water or 1 mM UDP-dialdehyde (1.9 mM final). After 1 h at 37°C, the reaction was terminated with 2 ml of ice-cold 0.02 N NaOH, and the production of cleaved *p*-NO_2-phenol was determined spectrophotometrically at 400 nm. Assays without enzyme source, ±UDP-dialdehyde, or without substrate served as controls.

RESULTS

α-Lactalbumin Effects on Sperm Galactosyltransferase Activity and Binding to the Zona Pellucida

Glycoprotein:galactosyltransferases normally transfer galactose from UDPGal to terminal GlcNAc residues, or to free GlcNAc, to produce *N*-acetyllactosaminyl linkages (i.e., Gal → GlcNAc) (13). However, the substrate specificity of galactosyltransferase can be modified by the addition of the milk protein α-lactalbumin (14). α-Lactalbumin specifically binds to galactosyltransferase and inhibits galactosylation of GlcNAc by raising its K_m, while simultaneously increasing galactosylation of glucose by lowering its K_m. Therefore, in the process of α-lactalbumin, glycoprotein:galactosyltransferase synthesizes lactose (i.e., milk sugar) rather than *N*-acetyllactosamine (14). Previous results have shown that BTBRTF/Nev sperm surface galactosyltransferase demonstrates lactose synthetase activity in the presence of α-lactalbumin, as do other membrane and soluble glycoprotein:galactosyltransferases (1). The lactose synthetase activity of CD1 sperm surface galactosyltransferase is shown in Fig. 1*A*, as well as the coincident inhibition of lactosamine synthesis. The concentrations of α-lactalbumin used are similar to the requirements of BTBRTF/Nev lactose synthetase activity, with 50% maximum activity requiring 0.1% α-lactalbumin, or 69 μM (1, 14).

When α-lactalbumin was added to in vitro fertilization assays containing capacitated sperm, sperm binding to the zona pellucida was inhibited in a dose-dependent manner (Fig. 1*B*). Half-maximal inhibition was seen at 0.1% α-lactalbumin (69 μM), similar to the concentration requirements for lactose synthetase activity and inhibition of *N*-acetyllactosamine synthesis. Two percent α-lactalbumin inhibited sperm binding by 92%. Glucose and GlcNAc (5 mM each) were present throughout these assays to insure efficient binding of the α-lactalbumin

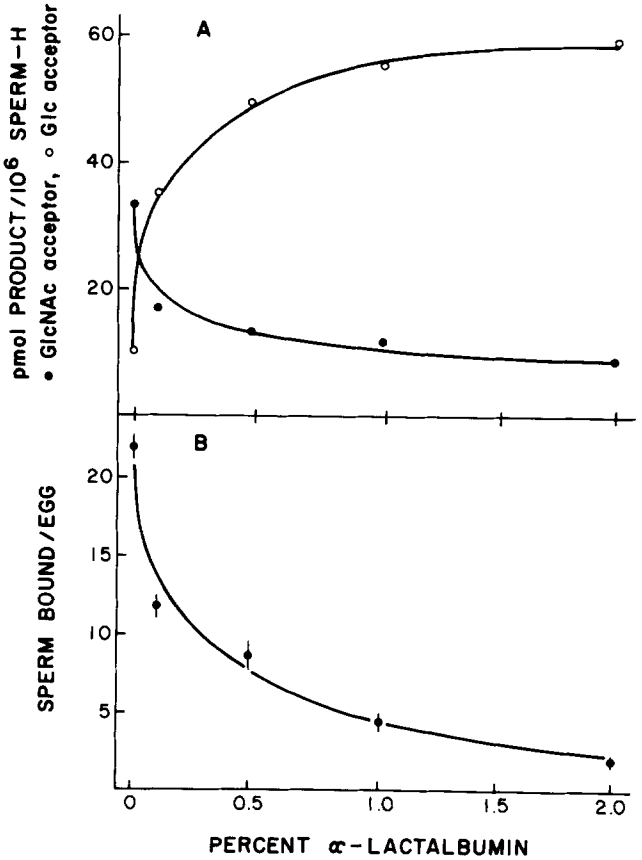

FIGURE 1 The effect of α-lactalbumin concentration on sperm surface galactosyltransferase activity (*A*) and sperm binding to the zona pellucida (*B*). In panel *A*, activity towards GlcNAc (●) and glucose (○) is shown, and is similar to results previously reported using another mouse strain (1). Error bars in *B*, ± SEM.

to the galactosyltransferase (14). (These levels of free sugar had little inhibitory effect by themselves on sperm-zona binding). To accurately assess the effect of α-lactalbumin, these assays were conducted in the absence of BSA, which was normally present during the assay (2% final concentration). The inhibition of sperm binding to the zona by α-lactalbumin was not the result of simply adding protein, since BSA additions (including free sugar) over the same dose range actually enhanced binding, but by insignificant levels (0% BSA, 18.8 ± 1.8 sperm/egg; 0.1% BSA, 19.5 ± 1.6 sperm/egg; 2% BSA, 21.8 ± 1.5 sperm/egg). Also, the effects of α-lactalbumin could not be attributable to any changes in sperm motility. Thus, α-lactalbumin apparently acted by modifying the sperm surface galactosyltransferase so as to inhibit binding to GlcNAc residues, and simultaneously inhibit sperm-zona binding.

UDP-Dialdehyde Effects on Sperm Galactosyltransferase Activity and Binding to the Zona Pellucida

A number of laboratories have shown that galactosyltransferases are readily inhibitable by UDP-dialdehyde (12, 15, 16). UDP-dialdehyde is made by periodate oxidation, which opens the uridine ribose ring, and forms Schiff's bases within the galactosyltransferase UDPGal binding site (12). Therefore, we examined the effect of UDP-dialdehyde on sperm surface galactosyltransferase activity and sperm-zona binding.

UDP-dialdehyde inhibited sperm surface galactosyltransferase activity towards saturating levels of exogenous GlcNAc, with 50% inhibition seen at 0.5 mM UDP-dialdehyde (Fig. 2). Five other sperm enzymes were also examined; four glycosidases and alkaline phosphatase. None of the glycosidases were affected by UDP-dialdehyde, while alkaline phosphatase was slightly inhibited (23%) at 1.9 mM UDP-dialdehyde (Fig. 2). These findings are consistent with the binding properties of these six enzymes, i.e., only galactosyltransferase and alkaline phosphatase are UDP binding enzymes, showing that UDP-dialdehyde did not cause nonspecific enzyme inhibition.

UDP-dialdehyde also inhibited sperm-zona binding. The inhibition of sperm binding and sperm surface galactosyltransferase activity was identical (Fig. 2). As was the case with α-lactalbumin, UDP-dialdehyde apparently did not affect sperm motility, relative to controls.

Sperm surface galactosyltransferases can be covalently bound with UDP-dialdehyde by borohydride reduction of Schiff's bases (12). Consequently, sperm were pretreated with 2 mM UDP-dialdehyde, treated with 5 mM potassium borohydride, pelleted by centrifugation, and resuspended in fresh CM. Control sperm without UDP-dialdehyde were treated with borohydride, centrifuged, and resuspended in parallel. Fig. 3 shows that sperm whose surface galactosyltransferases were covalently occupied by UDP (Fig. 3 *B*), failed to bind the zona pellucida relative to control sperm (Fig. 3 *A*). When eggs were pretreated with UDP-dialdehyde, Schiff's bases reduced by borohydride, and resuspended in fresh CM, they bound sperm indistinguishable from control eggs (as in Fig. 3 *A*).

Sperm Galactosylation of Zona Pellucida Substrates

If sperm surface galactosyltransferases mediate binding to the zona pellucida, then the zona should have galactosyl acceptors for this sperm enzymatic activity. We tested for this possibility in three ways, using high voltage borate electrophoresis to isolate the glycosylated zona acceptor(s) (see Materials and Methods).

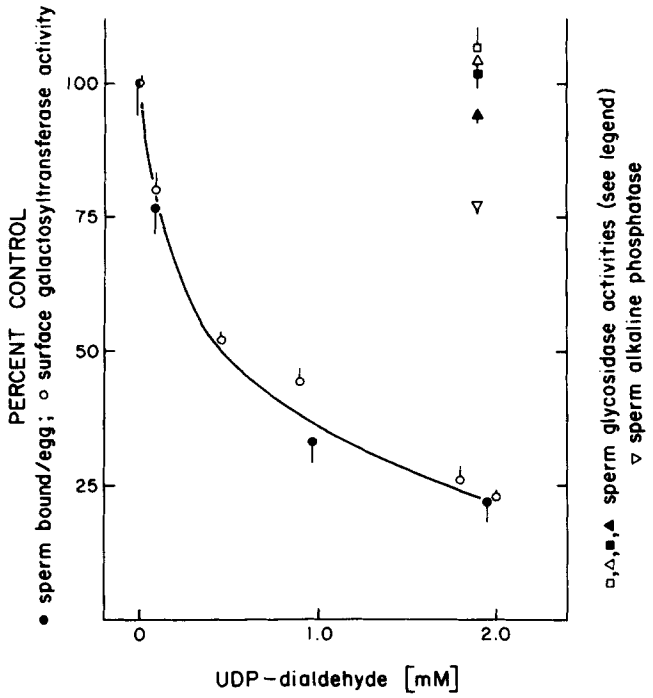

FIGURE 2 The effect of UDP-dialdehyde concentration on sperm binding to the zona pellucida (●), and on six sperm enzymatic activities. Control (i.e., 100%) values for each assay were as follows: sperm/egg (●); 24; surface galactosyltransferase (○), 26.4 pmol/10^6 sperm-h; β-*N*-acetylglucosaminidase (▲), 2.14 O.D./10^6 sperm-h; α-mannosidase (△), 1.32 O.D./10^6 sperm-h; β-*N*-acetylgalactosaminidase (□), 0.228 O.D./10^6 sperm-h; β-galactosidase (■), 0.135 O.D./10^6 sperm-h; alkaline phosphatase (▽), 0.281 O.D./10^6 sperm-h. Error bars, ±SEM.

We determined whether sperm surface galactosyltransferase was capable of glycosylating intact zona pellucida. Galactosyltransferase activity endogenous to 98 eggs was heat-inactivated (56–60°C, 30 min), without heat-solubilizing their zona pellucida. Extensively washed sperm were then added to these heated, zona-intact eggs in the presence of UDP[^{3}H]Gal. As seen in Table I, washed sperm, incubated alone, possessed negligible levels of endogenous acceptors for their own galactosyltransferases, in agreement with previous results (1, 3). Similarly, 98 heat-inactivated eggs assayed alone also lacked galactosyltransferase activity. However, when washed sperm and heated eggs were incubated together, the sperm bound to the zona (7.5 ± 0.9 sperm/egg) and showed dramatic glycosylation of zona pellucida glycosides (Table I).

We next determined whether sperm surface galactosyltransferases could glycosylate solubilized zona pellucida. As seen in Table I, washed sperm glycosylated both pronase-digested (1,455 eggs, 0.5% pronase, 10 min, 23°C) and heat-solubilized (1,465 eggs, 80°C, 30 min) zona pellucida glycosides. Pronase-digested zona had to be boiled for 10 min to denature the protease, since pronase solutions (0.5%) boiled for <10 min still were able to degrade sperm GlcNAc:galactosyltransferases. Glycosylation of solubilized zona pellucida glycosides was linear within the range assayed (0.5–3.3 zona equivalents/μl). The moles of galactose transferred/10^6 sperm-h to each zona equivalent were similar for both heat-solubilized and pronase-digested zona pellucida, and ~20 times more active than intact

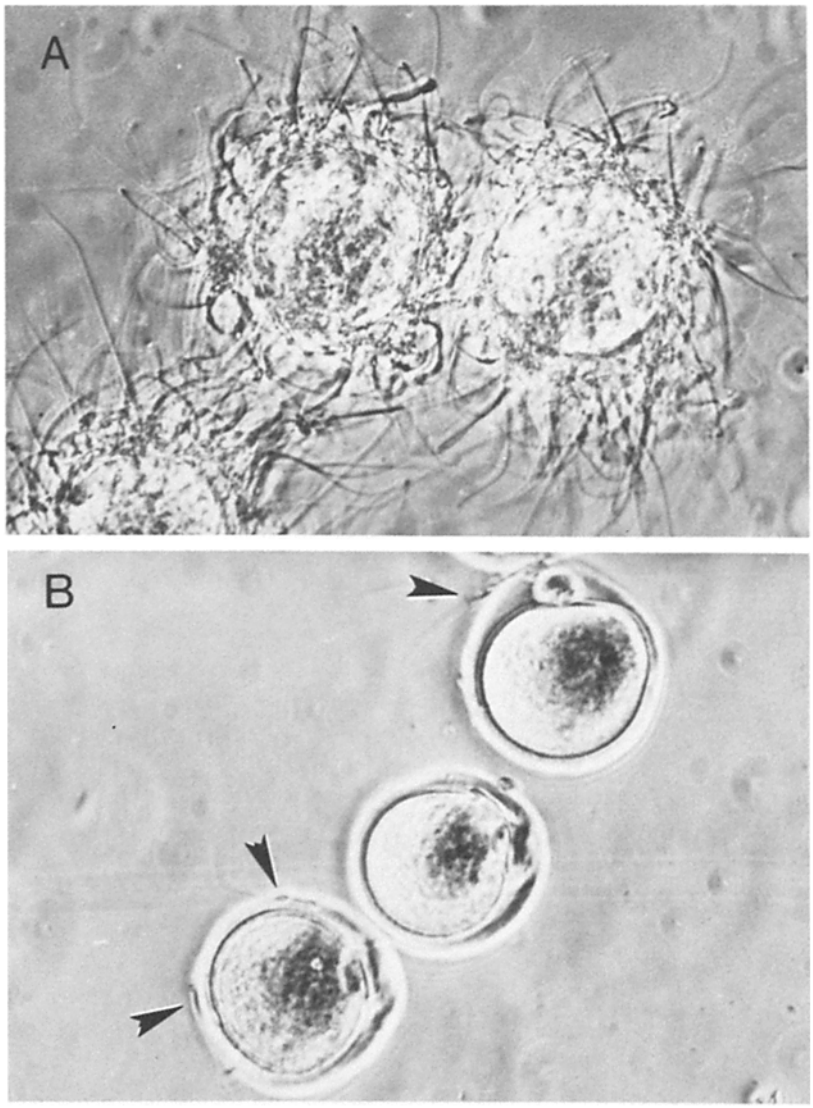

FIGURE 3 Phase-contrast photographs of sperm binding to the zona pellucida. In *B*, sperm were pretreated with 2 mM UDP-dialdehyde, Schiff's bases reduced with borohydride, and washed with CM. Arrows point to the few adhering sperm (~1 per egg). In *A*, sperm were pretreated in parallel with water, rather than UDP-dialdehyde. Eggs pretreated with UDP-dialdehyde, reduced with borohydride, and resuspended in fresh CM, bind sperm similar to controls (*A*). This figure illustrates the large number of bound sperm we occasionally encounter, and which prevent quantitation. Original magnification, × 200.

zona pellucida. This probably was due to the ability of the entire sperm population to glycosylate solubilized zona substrates, rather than only the few sperm that actually bound to the intact zona pellucida. Alternatively, solubilizing the zona may have exposed galactosyl acceptors not normally accessible to the sperm when bound to intact zona pellucida.

We examined whether pronase-digested zona pellucida (boiled for 10 min) would inhibit sperm binding to untreated, intact zona pellucida. This was found to be the case, and the inhibition produced agrees well with the extent of inhibition reported by other workers (see reference 11) using NaH_2PO_4-solubilized zonae pellucidae (70% inhibition of binding at 1.5 pronase-digested zona equivalents/μl, control and experimental assays each contained 20 eggs in 100 μl of medium).

Glycosidase Digestion of the Egg Zona Pellucida

If sperm surface galactosyltransferases bind zona pellucida GlcNAc residues, then β-*N*-acetylglucosaminidase digestion of the zona might expect to decrease sperm binding. To determine this, eggs were prepared as described in Materials and Methods and incubated with 2 U of β-*N*-acetylglucosaminidase (previously dialyzed against CM) for 2 h, in a 37°C tissue culture CO_2 incubator. Control eggs were incubated in parallel using the dialysate rather than the retentate. The eggs were then washed once with fresh CM and assayed for sperm binding. As seen in Table II, β-*N*-acetylglucosaminidase pretreatment inhibited sperm binding by 63–86% under these conditions. When eggs were pretreated with 2 U of β-galactosidase (previously dialyzed against CM), the reciprocal effect was observed; i.e., binding to zona increased by 55% (Table II), relative to controls. This suggests that some of the zona pellucida GlcNAc residues were masked by galactose, suggestive of conventional *N*-acetyllactosaminyl linkages (Gal → GlcNAc) (12).

DISCUSSION

Collectively, the results in this paper strengthen the suggestion

TABLE I

Sperm Surface Galactosyltransferase Substrates in the Zona Pellucida

Zona pellucida treatment	Assay additions	Product/10^6 sperm-h – zona equivalent
Intact zona pellucida* (heat-inactivated eggs)	Sperm alone	<5.0 fmol
	Eggs alone	<5.0 fmol
	Sperm + eggs	82.1 fmol
Solubilized zona pellucida	Sperm + pronase-digested zona pellucida	1.7 ± 0.2‡ pmol
	Sperm + heat-solubilized zona pellucida	1.5 ± 0.2 pmol

Sperm surface galactosyltransferases were assayed as described in Materials and Methods. Zonae pellucidae were prepared as detailed in the text. All sperm were capacitated in CM and washed by centrifugation before assay. Solubilized zona pellucida acceptors are ~20 times more active than intact zona pellucida, probably due to increased accessibility to the entire sperm population.

* Data presented are representative of three experiments.

‡ ±SEM.

TABLE II

Sperm Binding to Glycosidase Pretreated Eggs

Experiment	Egg pretreatment	Sperm bound/egg	Control
			%
1	Medium (control)	52.0 ± 3.6	100
	β-*N*-acetylglucosaminidase	7.3 ± 2.7 ($p < 0.05$)	14
2	Medium (control)	4.6 ± 1.3	100
	β-*N*-acetylglucosaminidase	1.7 ± 0.5 ($p < 0.05$)	37
3	Medium (control)	4.5 ± 0.6	100
	β-galactosidase	7.0 ± 0.8 ($p < 0.01$)	155

Eggs were pretreated with either glycosidase or dialysis buffer (controls) for 2 h as described in the text. After rinsing, capacitated sperm were added to the eggs, and sperm binding was assayed. Experiments *1* and *2* show the extreme variability in sperm from males sacrificed on different days, yet, the same relative effect of β-*N*-acetylglucosaminidase pretreatment is seen. Sperm bound/egg values are given ±SEM.

that sperm surface galactosyltransferases bind zona pellucida GlcNAc residues during fertilization. In all instances, the sperm galactosyltransferase was assayed under optimal conditions and was shown previously to be cell surface localized and not the result of epididymal fluid contamination (1, 3).

In our opinion, the most suggestive experiment in this study involved the use of α-lactalbumin to modify the substrate specificity of the sperm surface galactosyltransferase away from GlcNAc and towards glucose, and simultaneously inhibit binding to zona pellucida GlcNAc residues. The value of this experiment lies in the exquisite specificity of α-lactalbumin for glycoprotein:galactosyltransferase as detailed by the studies of Ebner, Hill, Brew, and their co-workers (see reference 14 for review). While α-lactalbumin has always been thought to be a product exclusively of lactation, low levels of α-lactalbumin-like activity have been reported recently in specific rat epididymal extracts (17). If so, then epididymal α-lactalbumin activity may influence sperm surface and/or epididymal fluid galactosyltransferases, in ways not yet known.

The simultaneous inhibition of sperm-zona binding and sperm surface galactosyltransferase activity by UDP-dialdehyde also speaks for a role of this enzyme during fertilization. Of six enzymes examined, only galactosyltransferase activity was inhibited coincident with inhibition of sperm-zona binding. While we do not yet fully understand the mode of UDP-dialdehyde inhibition of sperm-zona binding, three possibilities can be considered. First, our assay for sperm-zona binding involves the washing away, by centrifugation or pipetting, of loosely adherent sperm, such that the final sperm bound/egg have withstood this shearing force. Catalysis of sperm galactosyltransferase:GlcNAc complexes at the zona surface may help "drive" the sperm head deeper into the zona matrix. Inhibiting this catalysis with UDP-dialdehyde may simply keep sperm adhesion to the zona superficial, making the adhering sperm more readily removable by washing. Indeed, sperm adhesion to the zona has been dissected into a loose initial "attachment", dislodged by pipetting, and a subsequent sperm "binding", which is more resistant to washing (18). The second possibility is that UDP-dialdehyde may induce a conformational change in the enzyme, thus preventing it from binding to its GlcNAc substrate. A third possibility is that blocking the catalytic site with UDP-dialdehyde sterically inhibits binding of the GlcNAc substrate. In either case, it is clear that specific perturbation of sperm surface galactosyltransferase with UDP-dialdehyde is correlated with coincident inhibition of sperm binding to the zona pellucida.

Three types of experiments all demonstrated that intact, and solubilized zona pellucida can serve as substrate for sperm surface galactosyltransferase. Although these experiments do *not* show the zona galactosyl acceptor to be the receptor for sperm, they demonstrate that sperm galactosyltransferase can bind and glycosylate the zona pellucida, which is an essential finding if sperm surface galactosyltransferases are to participate during fertilization in the manner suggested. While the carbohydrate composition of mouse zona pellucida has not been reported, GlcNAc is the most prevalent sugar (11.7% by weight) in porcine zona pellucida (19).

The involvement of zona pellucida GlcNAc residues in sperm binding was shown by the dramatic (up to 86%) inhibition of binding after pretreatment with purified β-*N*-acetylglucosaminidase. On the other hand, exposing galactosyltransferase binding sites (i.e., GlcNAc) with β-galactosidase pretreatment, elevated binding by 55%. This relatively small effect of β-galactosidase treatment, in comparison to that seen with β-*N*-acetylglucosaminidase, probably indicates that most of the GlcNAc residues are exposed, available for sperm binding, and only a limited number are masked by β-galactosyl moieties. The reciprocal effects of these two enzymes, assayed at identical levels, eliminates nonspecific enzyme effects on the zona's ability to bind sperm.

The results presented in this paper, along with those of the preceding paper (3) can now be added to past studies (1, 2, 6), all of which focus our attention on sperm surface galactosyltransferase binding to the mouse zona pellucida during fertilization. We believe the simplest explanation compatible with all of the biochemical and genetic results, is that galactosyltransferase is at least one of the sperm surface receptors necessary for binding the zona pellucida, and does so by recognizing zona GlcNAc residues. It will be of interest to determine whether a particular zona pellucida protein (ZP3), implicated as a possible sperm receptor (11), serves as a sperm galactosyltransferase substrate. To rule out, or establish more firmly, sperm surface galactosyltransferase participation during mouse fertilization, we will require purified sperm enzyme, zona sub-

strate, and specific antisera raised against them, for the appropriate inhibition studies. Such experiments are in progress.

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