Distribution and Dynamics of Mouse Sperm Surface Galactosyltransferase: Implications for Mammalian Fertilization†

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ABSTRACT: It has been proposed that a mouse sperm surface β-1,4-galactosyltransferase functions as a receptor for the zona pellucida during fertilization. In this paper we used two monovalent fluorescent probes specific for galactosyltransferase: a trinitrophenylated derivative of UDP-galactose and rhodaminated α-lactalbumin. We found that galactosyltransferase was initially present over the posterior head of acrosome-intact sperm but became progressively localized to the plasma membrane overlying the acrosomal region after it was cross-linked with an anti-galactosyltransferase polyclonal antibody. Labeled mouse sperm that were treated with the calcium ionophore A23187 revealed that galactosyltransferase remained on the posterior head after acrosomal exocytosis. However, if galactosyltransferase was first cross-linked and redistributed with antibody and then acrosome reacted with A23187, all head fluorescence was lost. In addition, although anti-galactosyltransferase antibody induced a surface redistribution, it did not, by itself, lead to the release of acrosin, the endpoint of the acrosome reaction. Finally, using the technique of fluorescence recovery after photobleaching, we found that, in the absence of bivalent antibody, mouse sperm surface galactosyltransferase exhibited 40−50% recovery with a high diffusion coefficient on the anterior head ($5-8 \times 10^{-9}$ cm^2/s) approximately 2 times greater than on the posterior head ($2-4 \times 10^{-9}$ cm^2/s). When galactosyltransferase was cross-linked and redistributed to the anterior head using the bivalent antibody, the mobile fraction decreased to 20−30% with no significant change in the diffusion coefficient. These data demonstrate that mouse sperm surface galactosyltransferase has many important properties requisite for its function as a receptor for the zona pellucida, but bivalent cross-linking of mouse sperm surface galactosyltransferase is not sufficient to induce the final signal transduction events leading to acrosomal exocytosis.

At the site of fertilization, the sperm first encounters the zona pellucida, an acellular coat that surrounds the egg. The mouse zona pellucida consists of three glycoproteins ZP1, ZP2, and ZP3. ZP3 functions as both the adhesion ligand for sperm and as the agonist that induces the exocytotic event known as the acrosome reaction [for a review, see Wassarman (1989)]. The best chemically characterized candidates for zona pellucida receptors on mouse sperm are cell surface glycosyltransferases. In particular, two cell surface glycosyltransferases have been identified on the surface of mouse spermatozoa: a β-1,4-galactosyltransferase (GalTase)[1] (Shur & Hall, 1982a,b; Shur, 1984; Lopez *et al.*, 1985; Scully *et al.*, 1987; Shur & Neely, 1988; Miller *et al.*, 1990, 1992) and a fucosyltransferase (Cardullo *et al.*, 1989; Ram *et al.*, 1989). Within cells, glycosyltransferases create complex carbohydrates by enzymatically adding a specific donor sugar from a sugar−nucleotide to an acceptor. However, on the cell surface, glycosyltransferases are proposed to be adhesion molecules where they recognize complementary acceptor molecules but lack the necessary sugar−nucleotides and cofactors to complete the enzymatic cycle (Roseman, 1970; Shur, 1984). In support of this hypothesis, Miller *et al.* (1992) reported that mouse sperm surface β-1,4-GalTase can enzymatically transfer galactose from the sugar−nucleotide UDP-Gal to ZP3 but not to ZP1 or ZP2. Further, it was shown that sperm surface GalTase can galactosylate ~2.6 galactose residues per ZP3 molecule. Previous studies using bivalent polyclonal antibodies revealed that mouse sperm surface GalTase resides on the plasma membrane overlying the acrosomal vesicle on acrosome-intact sperm (Scully *et al.*, 1987), as well as a more extensive population of anti-GalTase sites on the lateral aspect of the sperm head following induction of the acrosome reaction (Lopez & Shur, 1987). In addition, using another fluorescently labeled antibody against a soluble acrosomal component, Macek *et al.* (1991) indicated that the cross-linking of antigenic sites on the mouse sperm surface using the anti-GalTase polyclonal antibody resulted in the induction of the acrosome reaction.

However, we have shown that, even under the mild fixation conditions required to maintain antigenicity, bivalent antibodies can induce redistribution of mammalian sperm surface antigens from the posterior to the anterior region of

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[1] Abbreviations: AES, acrosin extraction solution; BSA, bovine serum albumin; CTC, chlortetracycline; DMSO, dimethyl sulfoxide; FRAP, fluorescence recovery after photobleaching; GalTase, galactosyltransferase; GlcNAc, *N*-acetylglucosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MSCM, mouse sperm culture media; Rh-α-Lac, rhodamine-labeled α-lactalbumin; TUG, (2,4,6-trinitrophenyl)-2′(or 3′)-UDP-Galactose; UDP-Gal, uridine diphosphate galactose.

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the head (Wolf *et al.*, 1992). Accurate determination of surface distributions and diffusion rates requires the use of non-cross-linking monovalent probes.

In this paper, two monovalent probes that recognize galactosyltransferase, a trinitrophenylated UDP-galactose (TUG) (Cardullo *et al.*, 1990) and rhodaminated α-lactalbumin, were used to characterize the native distributions of mouse sperm surface GalTase under a variety of conditions. Using these probes we show that sperm surface GalTase resides on the posterior head on epididymal sperm and migrates to the anterior head after cross-linking with a bivalent rabbit anti-GalTase polyclonal IgG. Induction of the acrosome reaction with Ca^{2+} ionophore A23187 does not cause redistribution. However, if redistribution is first induced by cross-linking, subsequent addition of ionophore causes loss of the surface galactosyltransferase. We further demonstrate that this cross-linking and subsequent migration does not lead to acrosin release, the final event in the acrosome reaction. Finally, using these monovalent probes, we found that GalTase diffuses with a rapid diffusion coefficient despite its being confined to the posterior head of mouse sperm.

MATERIALS AND METHODS

Chemicals and Solutions. All chemicals were purchased from Mallinckrodt Chemical Works (St. Louis, MO) unless otherwise stated. BSA (fraction V), HEPES, Na_2 lactate, Na_2 pyruvate, streptomycin sulfate, trypsin (bovine pancreas, type III, E.C. 3.4.21.4), and polyvinyl alcohol were purchased from Sigma Chemical Company (St. Louis, MO). Penicillin G was purchased from the Eli Lilly Company (Indianapolis, IN), glutaraldehyde (70% EM grade) was from Polysciences (Warrington, PA), and fluorescein-labeled goat anti-rabbit IgG was from ICN (Costa Mesa, CA).

Mouse sperm culture media (MSCM) contained 94.66 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 4.15 mM Na_2HCO_3, 20.85 mM HEPES, 23.28 mM Na_2 lactate, 0.33 mM Na_2 pyruvate, 5.56 mM glucose, 60 μg/mL penicillin G, 50 μg/mL streptomycin sulfate, and 4 mg/mL polyvinyl alcohol adjusted to pH 7.4 at 37 °C. Reaction buffer contained 50 mM $CaCl_2$ and 50 mM Tris adjusted to pH 8.0. Acrosin extraction solution (AES) contained 1 M HCl and 10% (v/v) anhydrous glycerol. The calcium ionophore, A23187, was purchased from CalBiochem (San Diego, CA) and was dissolved at a final concentration of 10 mM in DMSO.

A rabbit polyclonal antibody against GalTase and a Fab fragment of that polyclonal were generously provided by Dr. Barry Shur (Department of Biochemistry, University of Texas, Houston). The intact rabbit serum derived anti-GalTase IgG was collected by protein A−Sepharose chromatography from rabbit serum having a titer of 1:160 000 as determined by ELISA. The eluted IgG was dialyzed against 1/2 strength 130 mM NaCl, 5 mM KCl, and 18 mM Na^+ HEPES, and 0.4-mL aliquots containing approximately 0.52 mg of IgG were lyophilized. The lyophilized powder was resuspended in 0.2 mL of deionized water and stored in 20-μL aliquots at −80 °C until it was used in labeling experiments. The total protein concentration was assayed using a Bio-Rad protein determination kit using BSA as the standard. The Fab was free of contaminating IgG or Fc as judged by SDS−PAGE. Prior to use, all antibody reagents were centrifuged in an airfuge for 60 min at 100000*g* to remove aggregates and particulate matter.

The fluorescent probe 2,4,6-trinitrophenyl-2′(or 3′)-UDP-galactose (TUG) was synthesized according to the procedure outlined by Cardullo *et al.* (1990). After the compound was purified by TLC (7:1:2 2-propanol:NH_4OH:H_2O) and reverse-phase HPLC, the product was lyophilized and stored at −80 °C until used for cell labeling studies.

Rhodamine-labeled α-lactalbumin (Rh-α-Lac) was purchased from Sigma Chemical Company (St. Louis, MO), dissolved at a concentration of 2 mg/mL in deionized water to a final volume of 5 mL, and then centrifuged at 10000*g* for 20 min to remove particulate matter. The supernatant was then placed in 6000−8000 MW cutoff dialysis tubing (SpectraPor) and dialyzed 3 times over 2 days against 1 L of deionized water at 4 °C. The resulting dialysate was lyophilized and resuspended in an equivalent volume of MSCM and stored at −4 °C until ready to be used for cell labeling studies. Just prior to cell labeling, 1 mL of the Rh-α-Lac was passed over a 7-mL P-10 size-exclusion column (Bio-Rad), and the first 1 mL of the void volume was collected.

Animals and Collection of Spermatozoa. Male CD-1 mice, 12−16 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and killed by cervical dislocation. The epididymides were dissected from the animal, and after the fat was removed and the blood drained away, the cauda epididymides were minced with scissors into approximately 10 mL of MSCM that had been warmed to 37 °C. The minced tissue suspension was then placed in a 5% CO_2/95% air incubator for 15 min at 37 °C and then centrifuged at 135*g* for 30 s to remove remaining tissue. The supernatant was then placed in another tube and centrifuged at 135*g* for 2 min. This supernatant was centrifuged at 135*g* for 10 min, and the pellet was suspended in 1 mL of MSCM. The cell concentration and percent motility were determined in a hemacytometer. Populations of sperm that exhibited less than 70% motility were discarded. The cell concentration was then adjusted to 2×10^6 sperm/mL in MSCM containing 1% BSA and placed in the incubator for at least 30 min prior to fluorescent labeling of the sperm. Under these conditions, sperm were capable of fertilizing zona-intact eggs in an *in vitro* fertilization assay with greater than 95% of the eggs being fertilized as determined using Hoechst and fluorescence microscopy to confirm that the sperm pronucleus was within the egg's cytoplasm. Typically more than 60% of these fertilized eggs progress to the two-cell stage.

Fluorescent Labeling of Sperm. Concentrations of stock solutions of TUG and Rh-α-Lac in MSCM were determined spectrophotometrically using $\epsilon_{260} = 26\,040\ M^{-1}\ cm^{-1}$ for TUG (Cardullo *et al.*, 1990) and $\epsilon_{280} = 10\,360\ M^{-1}\ cm^{-1}$ for α-lactalbumin. The concentrations of TUG and Rh-α-Lac in MSCM were adjusted to 20 μM and 200 nM, respectively. Spectrophotometric analysis of this material showed that the molar rhodamine:protein ratio was approximately 4.3 (for rhodamine, $\epsilon_{540} = 105\,000\ M^{-1}\ cm^{-1}$).

For fluorescence microscopy studies, 500 μL of the sperm suspension was added to 500 μL of the fluorophore stock solutions. When sperm were labeled with TUG, all solutions contained 3.5 mg/mL AMP to block pyrophosphatase activity and nonspecific nucleotide binding sites on the sperm surface (Cardullo *et al.*, 1989). The sperm suspensions were then

incubated for 30 min at 37 °C and then centrifuged once through 7 mL of a 5% BSA cushion in MSCM at 135*g*. The samples then either were placed on microscope slides or were further incubated with either 20 μg/mL of polyclonal anti-GalTase (approximately 130 nM), 20 μg/mL Fab (approximately 400 nM), or 10 μM A23187 for different times before viewing. Under these conditions, the sperm were in 1% BSA for at least 1 h (capacitating conditions), which led to over 40% hyperactivation of sperm flagella and was sufficient to achieve sperm adhesion to the zona pellucida in an *in vitro* fertilization assay.

For direct observation of anti-GalTase sites on mouse sperm using the anti-GalTase antibody, sperm were first labeled with 20 μg/mL anti-GalTase in MSCM and 0.4% BSA for 60 min at room temperature and then centrifuged through the 5% BSA cushion as outlined above. The resulting sperm pellet was then incubated with approximately 100 μg/mL fluorescein-labeled goat anti-rabbit IgG for 30 min and then centrifuged one more time through a 5% BSA cushion. The pellet was then resuspended in MSCM for viewing under the fluorescence microscope.

Binding Parameters of TUG and Rh-α-Lac on Mouse Sperm. For determination of IC_{50} of TUG, 2×10^7 mouse sperm were labeled with a mixture of 4 nM TUG and concentrations of UDP-Gal ranging from 0 to 100 μM. Similarly, to determine the IC_{50} of Rh-α-Lac, 2×10^7 mouse sperm were labeled with a mixture of 200 pM Rh-α-Lac and concentrations of α-lactalbumin ranging from 0 to 500 nM. The sperm were left in these solutions for 90 min and then centrifuged through a 7-mL, 5% BSA cushion in MSCM at 1500*g* for 5 min. The pellet was then collected, adjusted to a final concentration of 10^7 sperm/mL in MSCM, and placed in 3-mL quartz cuvettes (path length = 1 cm) for fluorescence intensity measurements in a fluorometer (α-SCAN, Photon Technology, Inc., South Brunswick, NJ). The excitation/emission pairs used were 450/530 nm for TUG and 540/590 nm for Rh-α-Lac.

For determination of the dissociation rate (k_{off}) of TUG and Rh-α-Lac from the sperm surface, mouse sperm were labeled with either 20 μM TUG or 200 nM Rh-α-Lac for 30 min and then diluted 1:10 in 1000-fold excess UDP-Gal or α-lactalbumin. One-milliliter aliquots were retrieved at different time points over a 2-h period and centrifuged through a 7-mL, 5% BSA cushion in MSCM. The resulting pellet was then resuspended at a final concentration of 10^6 sperm/mL, and fluorescence intensities were read at the appropriate wavelengths in the fluorometer.

Video-Enhanced Microscopy. Cells were observed using a video-enhanced microscope system consisting of a Zeiss-Axiovert-35 microscope and a Photometrics-200 cooled CCD camera system (Photometrics Inc., Tucson, AZ). TUG fluorescence was observed using either a filter set that selected for emission wavelengths between 400 and 460 (BP/FT/LP = 365/395/420 nm) or a standard fluorescein filter set (BP/FT/LP = 465/490/515). Because of apparent higher stability, all photographs of TUG were taken using the latter pair. Rh-α-Lac fluorescence was observed using a rhodamine filter set (BP/FT/LP = 546/580/590 nm). To control for photobleaching, the 100-W Hg source was isolated from the microscope with a Uniblitz shutter that was controlled by the Photometrics camera controller. Typical exposures were between 0.1 and 1 s. Corresponding light images were taken under Nomarski differential interference contrast (DIC) after first subtracting a uniform background obtained by defocusing the image of the sperm. Pictures were generally displayed as fluorescence and DIC contrast pairs and were stored on a Panasonic optical memory disk recorder. Hard copies were generated using a Mitsubishi video printer.

Fluorometric Assay for Acrosin Release. Acrosin release was assayed in a spectrofluorometer using the fluorescent substrate carbobenzoxy arginyl-4-trifluoromethyl coumarin-7-amide (CBZ-Arg) (Enzyme Systems Products, Livermore, CA) by a modification of the procedure of McKinnon *et al.* (1991). Five hundred milliliters of mouse sperm at 2×10^6/mL in MSCM was incubated with either intact antibody, Fab, or calcium ionophore for 90 min and split into two 250-μL fractions. Ninety-minute incubations were needed to achieve maximum acrosin activity as determined using this fluorometric assay. To measure total extractable acrosin activity, 20 μL of AES was added to the first fraction and placed on ice for 15 min. The sperm were centrifuged at 1500*g* for 5 min, and the supernatant was adjusted to 270 μL in MSCM and diluted 10-fold in reaction buffer. Acrosin activity was determined spectrofluorometrically by measuring the rate of linear fluorescence emission intensity increase over a 5-min period at 25 °C using an excitation wavelength of 400 nm and an emission wavelength of 500 nm. To measure the amount of acrosin released into the supernatant in response to a particular treatment, the second 250-μL fraction was centrifuged and the supernatant was adjusted to approximately 250 μL of MSCM. Twenty microliters of extraction buffer was added, and the solution was then placed on ice for 15 min. After this was diluted 10-fold in reaction buffer, the activity of the released acrosin was measured spectrofluorometrically as outlined above. Activity was expressed as percent of the total extractable acrosin obtained in the first fraction.

The acrosin release assay was calibrated using the standard chlortetracycline (CTC) assay for determining the extent of acrosome reaction on mouse sperm (Saling & Storey, 1979; Ward & Storey, 1984). In addition, acrosomal status was monitored using a histological assay with Coomassie Brilliant Blue as outlined by Moller et al. (1990). Using both of these techniques, sperm incubated in the absence of any agents were scored microscopically to be greater than 80% acrosome intact after the initial incubation period and greater than 65% intact after 2 h in MSCM. However, after a 90-min incubation in 10 μM A23187 in MSCM, greater than 70% of the sperm were judged to be acrosome reacted (AR pattern), correlating with the large increase seen in the acrosin release assay (see Results).

Fluorescence Recovery after Photobleaching. The diffusion coefficient and percent mobility of Rh-α-Lac and TUG were determined using the technique of fluorescence recovery after photobleaching with equipment described by Wolf and co-workers (Wolf & Voglmayr, 1984; Wolf *et al.*, 1986a,b; Wolf, 1989). In these studies we employed a 63×/1.40 NA Planapo objective and bleached for 20 ms using an argon laser (Lexel Inc., Palo Alto, CA). Under these conditions, the $1/e^2$ laser beam radius was approximately 1 μm. Recovery was followed for times between 30 s and 2 min. Recovery curves showing atypical recovery patterns, reflecting cell movement or membrane flows, were not considered for analysis. Data were analyzed using nonlinear least square algorithms as outlined by Wolf (1989) and Cardullo *et al.* (1991) which are a modification of Bevington (1969).

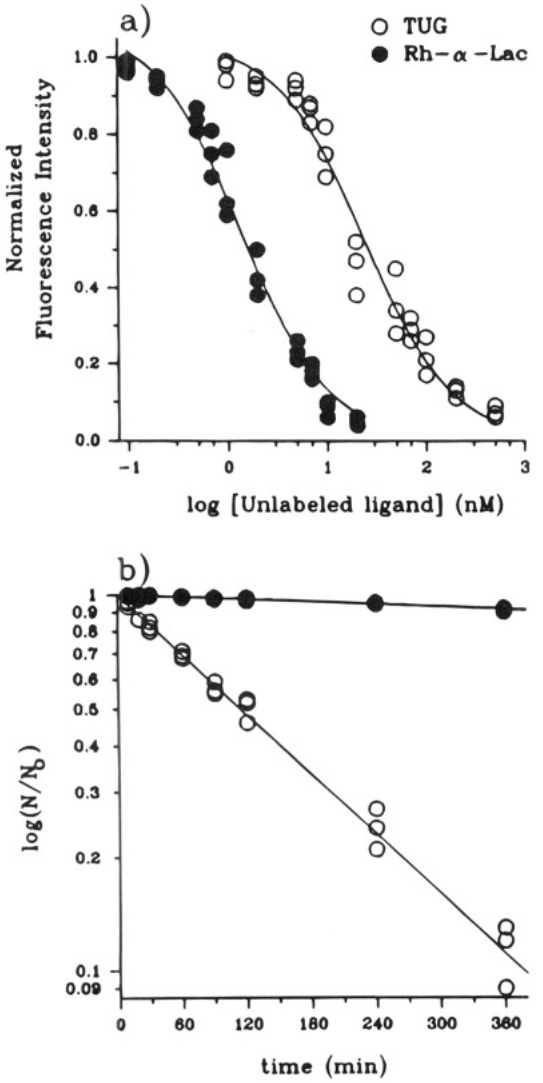

FIGURE 1: Binding properties of TUG (○) and Rh-α-Lac (●) on mouse sperm. (a) Both Rh-α-Lac and TUG exhibit saturable binding on mouse sperm. Data were fit to the equation $FI = FI_{max} \times (1 - [\text{unlabeled ligand}])/(IC_{50} + [\text{unlabeled ligand}])$. The data were fit using a nonlinear least squares analysis, and FI_{max} and IC_{50} were calculated. TUG was competed off with UDP-Gal at an IC_{50} of 24.61 ± 1.04 nM, while Rh-α-Lac was competed off with α-Lac at an IC_{50} of 1.39 ± 0.17 nM. (b) Dissociation of TUG and Rh-α-Lac from the sperm surface. Data were fit to the equation $N = N_o \exp(-k_{off}t)$, and N_o and k_{off} were determined ($k_{off} = \ln(2)/\tau_{1/2}$). TUG dissociated from the mouse sperm surface with $k_{off} = (1.02 \pm 0.32) \times 10^{-4}$ s^{-1} ($\tau_{1/2} = 114$ min), while Rh-α-Lac dissociated with $k_{off} = (0.04 \pm 0.01) \times 10^{-4}$ s^{-1} ($\tau_{1/2} = 2843$ min). Each point represents the mean of three determinations from a single animal.

RESULTS

Characterization of Rh-α-Lac and TUG Binding Properties on Mouse Sperm. It has been previously shown that when TUG binds to GalTase in solution, its fluorescence properties are modulated and it acts as a competitive substrate with UDP-Gal for the donor site in both binding and enzymatic assays (Cardullo *et al.*, 1990). The other molecule used in these studies, α-lactalbumin (M_r = 14 500), binds to a separate site on GalTase and changes the specificity of the enzymatic acceptor site away from GlcNAc and toward glucose (Yadav & Brew, 1991). Figure 1 shows the binding isotherms (Figure 1a) and dissociation kinetics (Figure 1b) of the fluorescent homologs of UDP-Gal and α-lactalbumin (TUG and Rh-α-Lac) on mouse sperm under conditions used for cell labeling and diffusion studies.

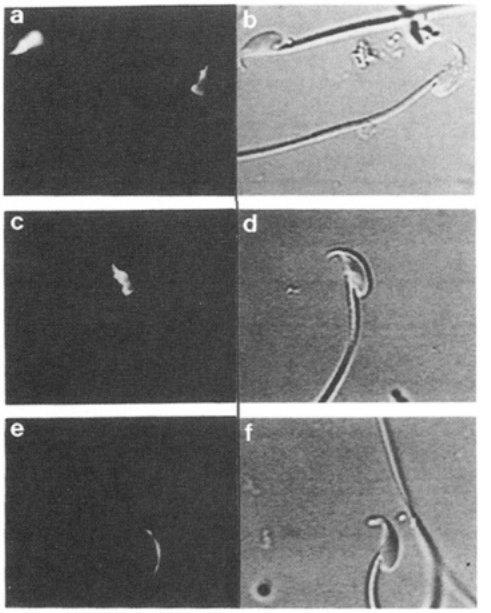

FIGURE 2: Paired fluorescence and DIC micrographs of mouse sperm labeled with different probes against mouse sperm surface GalTase. Both TUG (a, b) and Rh-α-Lac (c, d) labeled mouse sperm on the posterior head. In contrast, a polyclonal rabbit anti-GalTase IgG with a fluorescein-labeled goat anti-rabbit IgG as the secondary (e, f) labeled mouse sperm on the anterior head overlying the acrosome. All fluorescence micrographs represent a 1-s exposure, and DIC photographs are 0.1-s exposures. All micrographs were optimized for fluorescence and not for DIC, making determination of acrosomal status using DIC impossible.

Both Rh-α-Lac and TUG were displaced on mouse sperm by excess concentrations of α-Lac and UDP-Gal, respectively (Figure 1a). TUG bound to sperm with relatively low affinity and was displaced by UDP-Gal at an IC_{50} of 24.61 nM. By comparison, Rh-α-Lac displayed a higher affinity for GalTase with α-Lac displacement at an IC_{50} of 1.39 nM. Figure 1b illustrates that this difference in IC_{50} is due to the difference in dissociation rates of TUG and Rh-α-Lac from the mouse sperm surface. The dissociation rate, k_{off}, for TUG on mouse sperm was 1.02×10^{-4} s^{-1} ($\tau_{1/2} = 114$ min); this was approximately 25 times higher than the dissociation rate for Rh-α-Lac ($k_{off} = .04 \times 10^{-4}$ s^{-1}; $\tau_{1/2} = 2843$ min). These binding studies demonstrated that Rh-α-Lac was the better GalTase probe for long-term physical and redistribution studies.

TUG and Rh-α-Lac Bind to the Posterior Head of Epididymal Mouse Spermatozoa. The two monovalent probes for GalTase, TUG and Rh-α-Lac, were used to identify the location of GalTase on living mouse sperm using fluorescence microscopy (Figure 2). Both TUG (Figure 2a,b) and Rh-α-Lac (Figure 2c,d) were localized to the posterior head of mouse spermatozoa. Further, the specificity of these probes for GalTase was tested using a number of criteria. As shown in Table 1, TUG fluorescence was virtually abolished in the presence of saturating concentrations of UDP-Gal or by initiating the transfer of galactose from TUG to the acceptor GlcNAc (Cardullo *et al.*, 1990; Ram *et al.*, 1988). Rh-α-Lac fluorescence was abolished using excess concentrations of α-lactalbumin (Table 1). Hence, both TUG and Rh-α-Lac labeled the same morphological regions on acrosome-intact mouse sperm, and both reagents were

Table 1: Summary of Predominant TUG and Rh-α-Lac Distributions on Mouse Sperm[a]

treatment	sperm distribution with TUG	sperm distribution with Rh-α-Lac
none	PH	PH
10 μM A23187 (90 min)	PH	PH
anti-GalTase IgG (90 min)	AH	AH
anti-GalTase IgG (90 min) + 10 μM A23187 (90 min)	none	none
anti-GalTase Fab (90 min)	PH	PH
anti-GalTase Fab (90 min) + 10 μM A23187 (90 min)	PH	PH
100 mM GlcNAc + 10 mM $MnCl_2$	none	PH
10 mM UDP-Gal	none	PH
10 mM α-Lac	PH	none
1 mg/mL trypsin (10 min)	none	none
1% Glutaraldehyde (15 min)	none	PH

[a] Distributions reflect >75% distribution observed. PH = posterior head; AH = anterior head. Numbers in parentheses represent incubation time of each of the reagents.

competed by substrates consistent with the complementary receptor being GalTase.

Previous immunofluorescence localization studies using a polyclonal antibody against a bovine milk GalTase revealed that the antigen is on the anterior portion of the mouse sperm head plasma membrane overlying the acrosome (Lopez & Shur, 1987; Scully *et al.*, 1987). Indeed, we confirmed this localization using an intact rabbit anti-GalTase IgG (Figure 2e,f). The observation that TUG and Rh-α-Lac labeled mouse sperm surfaces differently than the anti-GalTase antibody suggested to us that anti-GalTase IgG polyclonal antibodies might have been cross-linking the antigen, thereby causing it to redistribute from the posterior to the anterior head.

Anti-GalTase Redistributes GalTase on Epididymal Mouse Sperm. Table 1 reports the final distribution of TUG and Rh-α-Lac under a variety of conditions. Using either probe, we found that although sperm surface GalTase appeared initially on the posterior head, its distribution progressively changed to the anterior head in the presence of antibody (Figures 3 and 4). Three distributions were always seen during the course of an experiment: an initial distribution where sperm were labeled only on the posterior head (Figure 3a,b), an intermediate pattern where sperm showed both posterior and anterior head labeling (Figure 3c,d), and a final pattern where sperm were labeled only on the anterior head (Figure 3e,f). In all experiments, approximately 20% of the sperm were labeled on the anterior head (Figure 4c), while only about 25% of the sperm remained in the intermediate pattern (Figure 4b). When monovalent anti-GalTase Fab was used instead of bivalent IgG, the predominant pattern was on the posterior head (Figure 3g,h). Moreover, no significant redistribution occurred from the posterior to the anterior head, although there was a gradual increase in the porportion of sperm in the intermediate pattern after 90 min.

When the acrosome reaction was induced using the calcium ionophore A23187, only posterior head labeling was seen, and both the final and intermediate patterns were abolished (Table 1). When sperm were first treated with label, incubated with bivalent antibody for 90 min, and then treated with ionophore, no detectable fluorescence was observed over the head of the mouse sperm (data not shown),

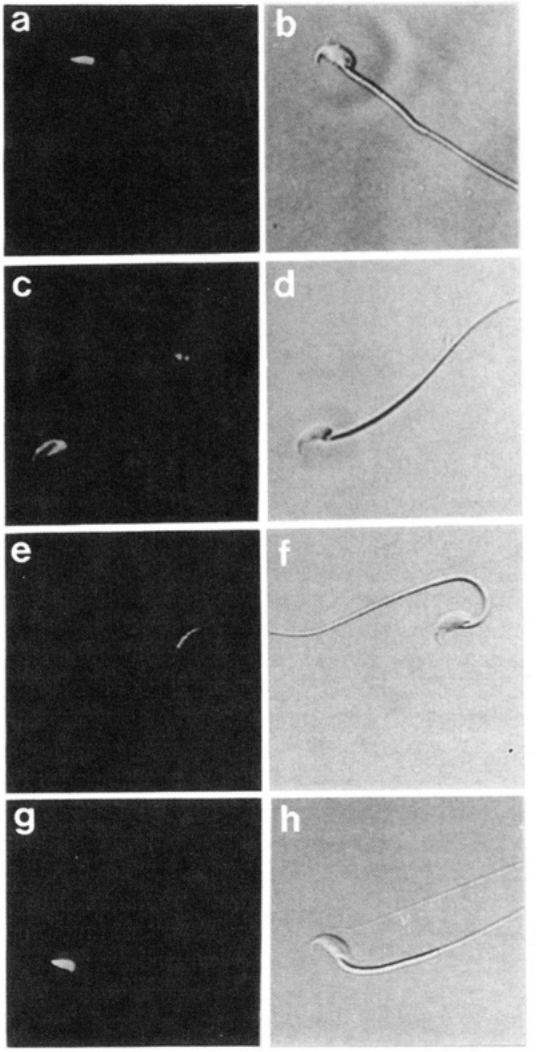

FIGURE 3: Paired fluorescence and DIC micrographs of Rh-α-Lac binding sites on mouse spermatozoa in the presence and absence of anti-GalTase IgG or Fab. Rh-α-Lac labeling in the presence of IgG showing (a, b) the initial distribution at $t = 0$ min, (c, d) the intermediate distribution at $t = 20$ min, and (e, f) the final distribution at $t = 90$ min. When Fab was used instead of IgG (g, h) the initial pattern was predominant at $t = 90$ min. Exposures were identical to those described in Figure 2.

suggesting that α-lactalbumin and GalTase binding sites were either removed from the sperm surface or proteolytically destroyed during the acrosome reaction. Indeed, when sperm were exposed to 1 mg/mL trypsin for 10 min, no detectable TUG or Rh-α-Lac was seen (Table 1). Sperm that were fixed with 1% glutaraldehyde for 15 min exhibited no TUG fluorescence but retained Rh-α-Lac fluorescence (Table 1), indicating that fixation destroyed the UDP-Gal binding site but not the α-Lac site on GalTase.

Aggregation of Sperm Surface GalTase Does Not Result in Acrosin Release. Using the fluorescent membrane probe chlortetracycline (CTC), Leyton and Saling (1989a) demonstrated that bivalent immuno-cross-linking of ZP3 glycopeptides bound to the mouse sperm surface was sufficient to induce the acrosome reaction. We were interested in determining whether the antibody-induced redistribution of sperm surface GalTase led to the release of acrosin, which we correlated with the acrosome-reacted pattern using the CTC assay (see Materials and Methods). Figure 5 shows the results of experiments where acrosin activity was assayed in the absence and presence of bivalent antibody, Fab, or

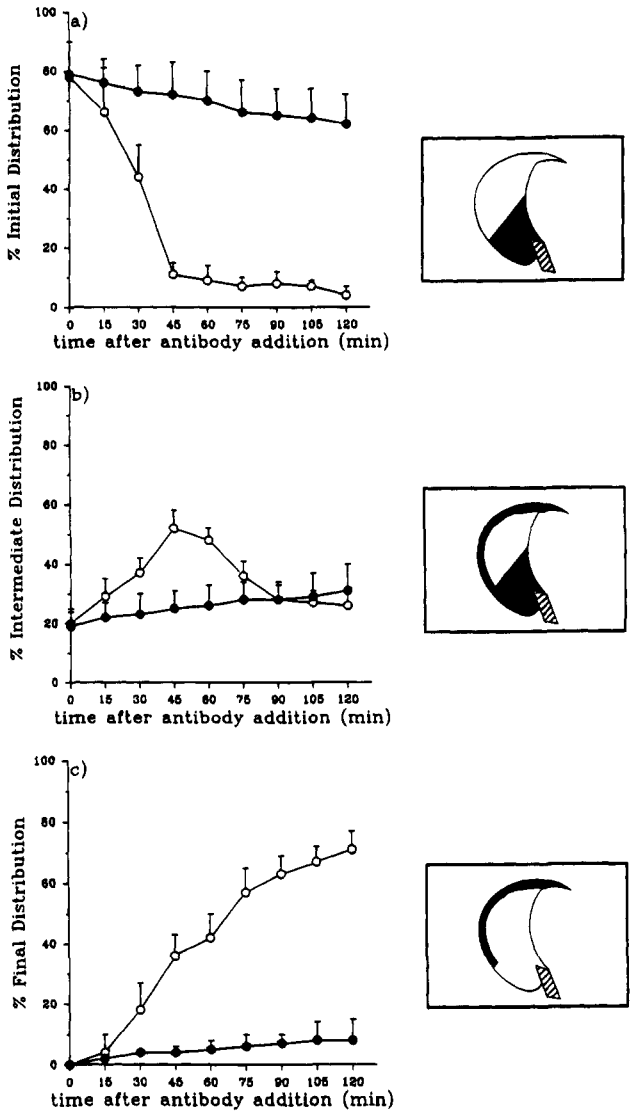

FIGURE 4: Time course of redistribution of Rh-α-Lac showing fraction of sperm exhibiting (a) the initial distribution (posterior head labeling only), (b) the intermediate distribution (anterior head + posterior head labeling), and (c) the final distribution (anterior head labeling only) in the presence of either anti-GalTase IgG (○) or Fab (●). For each experiment, distributions from 100 sperm were tabulated. Values represent the mean ± σ_{n-1} from five different animals. Black shading in the line drawings to the right of each panel illustrates the actual fluorescence patterns observed.

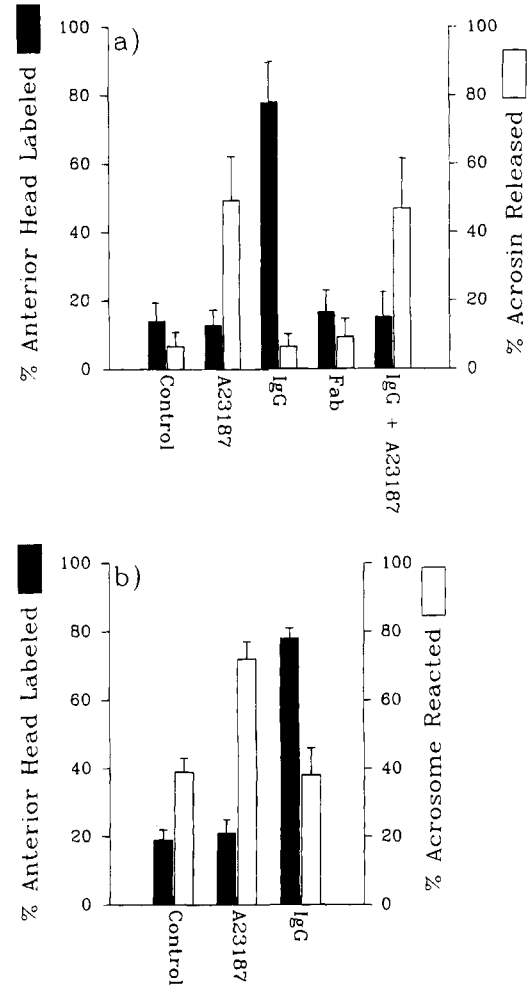

FIGURE 5: (a) Acrosin release (open bars) and final distribution (filled bars) of Rh-α-Lac in response to A23187, intact IgG, Fab, or IgG and A23187. Although 10 μM A23187 released acrosin, it did not induce a redistribution of GalTase. In addition, IgG resulted in a redistribution of GalTase from the posterior to the anterior head, but did not result in acrosin release. Monovalent Fab had no effect on either acrosin release or the distribution of GalTase on the mouse sperm surface. Data represent the mean ± σ_{n-1} from three different animals. (b) Percent of acrosome reaction as scored using a Coomassie Blue histological assay (open bars) and final distribution (filled bars) of Rh-α-Lac in response to A23187 or intact IgG. As with the acrosin release assay, antibody induced the redistribution of GalTase from the posterior to the anterior head but not the acrosome reaction. Data represent the mean ± σ_{n-1} from three different animals.

ionophore. In the absence of any additions, a small amount of acrosin was released over a 90-min period, which was approximately 11% of the maximum releasable acrosin using the calcium ionophore A23187. At a concentration of 20 μg/mL of Fab, which was in 3-fold molar excess of the concentration of intact IgG used to cause the redistribution of GalTase from the posterior to the anterior head, no detectable increase in acrosin activity was observed (Figure 5). Therefore, although bivalent cross-linking was sufficient to cause the redistribution over the mouse sperm surface (Figures 2 and 5 and Table 1), it did not induce the acrosome reaction as measured by the release of acrosin into the supernatant.

Diffusion Properties of Sperm Surface GalTase. It has been suggested that the zona pellucida receptor on mouse sperm must freely diffuse in order to ensure its association with ZP3 (Baltz & Cardullo, 1989). Using both TUG and Rh-α-Lac, we measured the diffusion coefficient and percent mobility of sperm surface GalTase on both the anterior head and the posterior head prior to cross-linking, and on the anterior head after cross-linking with bivalent polyclonal anti-GalTase. Table 2 shows the results of these FRAP experiments, and Figure 6 shows typical FRAP curves for TUG. TUG consistently demonstrated a slightly higher diffusion coefficient and percent recovery than Rh-α-Lac, reflecting either differences in their binding affinities for sperm surface GalTase (Figure 1) or structural changes in GalTase upon binding to either the UDP-Gal or the Rh-α-Lac sites.

In the absence of anti-GalTase IgG, both TUG and Rh-α-Lac binding sites demonstrated 40−50% recovery with diffusion coefficients of $2-9 \times 10^{-9}$ cm^2/s, which are rapid compared to those of proteins on somatic cell plasma membranes (Peters, 1981). When sperm were observed in the intermediate pattern in the absence of antibody, we observed an approximately 2-fold higher diffusion coefficient

Table 2: Mobility Parameters of Native and Redistributed Populations of Mouse Sperm Surface GalTase Using TUG and Rh-α-Lac[a]

	no addition			+ anti GalTase IgG			+ Fab			+ A23187		
	D(×10⁹ s/cm²)	%R	n	D(×10⁹ s/cm²)	%R[b]	n	D(×10⁹ s/cm²)	%R[b]	n	D(×10⁹ s/cm²)	%R[b]	n
TUG												
posterior head	3.2 ± 1.8	53 ± 7	52	2.8 ± 1.4	52 ± 6[d]	33	3.8 ± 1.6	49 ± 9[d]	36	2.9 ± 1.3	56 ± 11[f]	44
anterior head	8.5 ± 2.7	56 ± 10[c]	31	7.1 ± 1.6	27 ± 4[e]	47	8.1 ± 2.4	53 ± 6[c,e]	32	NP[g]		
Rh-α-Lac												
posterior head	2.6 ± 0.9	53 ± 5	87	1.9 ± 0.7	48 ± 5[d]	42	2.4 ± 1.1	51 ± 7[d]	37	2.6 ± 0.8	47 ± 5[f]	67
anterior head	5.6 ± 1.3	48 ± 4[c]	34	5.4 ± 1.2	21 ± 4[e]	51	4.9 ± 0.8	53 ± 5[c,e]	31	NP[g]		

[a] These data represent pooled measurements from three different animals. Values represent the mean ± σ_n, where n is the number of measurements. [b] The area of the crescent-shaped mouse sperm head can be approximated as a cone (Cardullo & Wolf, 1990). The area of a cone is given by $\pi\omega l/2$ where ω is the width of the base and l is the height. For mouse sperm, $\omega = 3.2$ μm and $l = 8$ μm; thus the area is approximately 13 π μm². If we assume that the anterior and posterior heads are approximately equal in area at approximately 6.5 $\pi\mu$m² and that we are bleaching an area of about $\pi\mu$m², then we are bleaching about 15% of the surface in a FRAP measurement. This means that the mobile fractions are overestimated by approximately 15% $f(0)$, where $f(0)$ is the fractional intensity immediately after the bleach. In these experiments $f(0)$ is approximately 0.5; therefore, we are underestimating the mobile fraction by about 8% of the value given. [c] Measurements on anterior head were taken from cells which showed both anterior and posterior head labeling. [d] Measured after 15 min of incubation with anti-GalTase IgG or Fab. [e] Measured after 2 h of incubation with anti-GalTase IgG or Fab. [f] Measured after 2 h of incubation in A23187. [g] Not present.

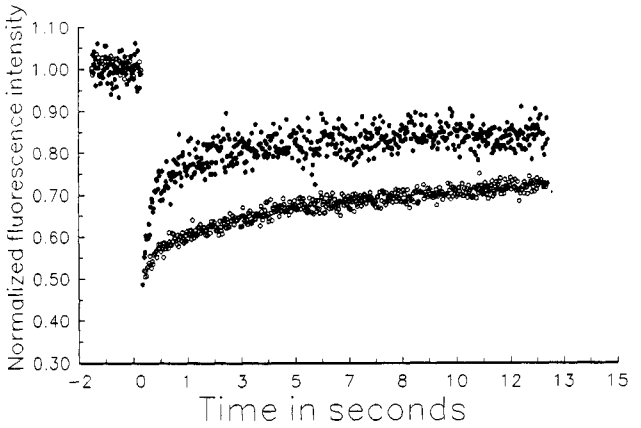

FIGURE 6: Typical FRAP recovery curves for TUG on the anterior (●) and posterior (○) head regions of the same sperm. For this particular sperm the anterior region shows $D = 6.5 \times 10^{-9}$ cm²/s and $\%R = 66$, while the posterior region shows $D = 0.83 \times 10^{-9}$ cm²/s and $\%R = 52$.

on the anterior head than on the posterior head using either probe (Table 2). In the presence of 20 μg/mL anti-GalTase IgG, there was no significant change in the diffusion parameters of GalTase on the posterior head. However, redistributed GalTase on the anterior head exhibited a reduction in percent recovery to a final value between 20 and 30% (approximately 50% reduction) with no significant difference in the diffusion coefficient from values obtained prior to antibody treatment (Table 2). These results demonstrate that concentrations of bivalent anti-GalTase that cause redistribution of sperm surface GalTase from the posterior head to the anterior head lead to a detectable decrease in the fractional mobility of GalTase but do not affect the diffusion coefficient of that mobile fraction.

DISCUSSION

In this paper we have used two fluorescent probes which exhibit chemical specificity toward GalTase: TUG and Rh-α-Lac. It was shown previously that TUG is a competitive substrate for UDP-galactose and exhibits a characteristic blue shift upon binding GalTase in solution (Cardullo *et al.*, 1990). In this study we have provided further evidence that TUG is a competitive substrate for UDP-galactose and specifically targets a β-1,4-GalTase on the mouse sperm surface (Table 1). We have demonstrated that TUG binds specifically to the posterior head of acrosome-intact sperm since this labeling is competed away with saturating concentrations of UDP-galactose (Table 1). In addition, this labeling was also abolished by initiating enzymatic activity with GlcNAc and $MnCl_2$ (Table 1). Taken together, these experiments reveal that the only significant UDP-galactose binding protein on the mouse sperm surface is the β-1,4-GalTase previously described by Shur and colleagues (Shur & Hall, 1982a,b; Shur, 1984; Lopez *et al.*, 1985; Scully *et al.*, 1987; Shur & Neely, 1988; Miller *et al.*, 1990, 1992). However, although TUG is useful for identifying GalTase sites on cell surfaces, its relatively weak affinity for its complementary receptor (Figure 1) made it of lesser value for the dynamic studies outlined in this paper.

In contrast, the rhodaminated derivative of α-lactalbumin (Rh-α-Lac) was extremely useful for studying the distribution and dynamics of the β-1,4-GalTase. It was shown previously that α-lactalbumin could be used to purify the 60-kDa cell surface GalTase from mouse sperm (Shur & Neely, 1988). In our studies, we demonstrated that, in contrast to TUG, Rh-α-Lac had a higher affinity for its complementary receptor, which made it a useful probe for both redistribution and FRAP studies. Under all cases, the distribution observed with Rh-α-Lac was identical to those seen with TUG (Table 1; Figure 2). These observations, along with the previous chemical characterizations of TUG (Cardullo *et al.*, 1990) and α-Lac (Shur & Neely, 1988), demonstrate that both TUG and Rh-α-Lac are recognizing sperm surface GalTase.

Earlier reports on the distribution of mouse sperm surface GalTase using a primary bivalent polyclonal anti-GalTase IgG and a fluorescently labeled secondary antibody indicated that GalTase was present only on the anterior head overlying the acrosome (Lopez & Shur, 1987; Scully *et al.*, 1987; Shur & Neely, 1988). In addition, on the basis of both immunofluorescence and enzymatic assays, Lopez and Shur (1987) observed a population of GalTase on the inner acrosomal membrane and equatorial segment after induction of the acrosome reaction. These authors argued that GalTase on the anterior head could interact with the zona pellucida and function as an adhesion molecule. Subsequent to the induction of the acrosome reaction, GalTase could redistribute from the cell surface overlying the acrosome to the equatorial segment and inner acrosomal membrane where it would be available for other cell adhesion events with either the zona pellucida or the egg plasma membrane itself. Our

findings, using TUG and Rh-α-Lac as monovalent probes against GalTase, demonstrate that these distributions represent an artifact of antibody-induced cross-linking. Similar results have been obtained comparing mono- and bivalent labeling for mouse sperm antigens M5 and M42 (Wolf *et al.*, 1992) and for ram sperm antigen ESA152 (McKinnon *et al.*, 1991). Our inability to locate a population of GalTase on the inner acrosomal membrane after ionophore-induced acrosin release suggests that the binding sites for TUG and Rh-α-Lac may have been proteolytically destroyed, while antigenicity for the anti-GalTase polyclonal is retained. In this regard, it is possible that the inner acrosomal membrane population of GalTase reported by Lopez and Shur (1987) represents a preexisting population similar to the PH-20 population reported by Phelps and Myles (1987). This would not be surprising, given that the acrosome derives from the Golgi apparatus, which is the primary site for glycosyltransferases.

The localization of GalTase to the posterior region of the sperm head raises the issue of whether the zona pellucida initially interacts with this region. Autoradiographs of ^{125}I-labeled ZP3 binding to the sperm indicates labeling over the entire head (Bleil & Wassarman, 1986). Drobnis *et al.* (1988) showed, using video microscopy, that hamster sperm first contact the zona pellucida in cumulus-intact sperm by the sides of their heads, consistent with a posterior head localization. Saling *et al.* (1979) have shown by an electron micrographic time study that initial contact between mouse sperm and the zona pellucida is random. If, as our data indicate, the initial binding contact is indeed at the posterior head, then this would reflect the favorable condition of zona binding to the region of maximum surface area and minimum curvature (Baltz & Cardullo, 1989).

The redistribution of GalTase from the posterior to the anterior region of the head of mouse sperm after cross-linking may be physiologically relevant in light of reports that cross-linking zona pellucida glycopeptides on the mouse sperm surface leads to the induction of the acrosome reaction as measured by the CTC assay (Leyton & Saling, 1989a) and may resemble well-known signal transduction paradigms seen in other receptor systems (e.g., insulin, epidermal growth factor, platelet derived growth factor, etc.). In addition, Macek *et al.* (1991) reported that cross-linking mouse sperm surface GalTase with identical antibodies used in this study led to a loss of HS-21 immunoreactivity which has been correlated with acrosomal loss in human sperm using transmission electron microscopy (Wolf *et al.*, 1985). This finding agrees favorably with our report that anti-GalTase IgG results in a redistribution of GalTase on the sperm surface but does not correlate with our finding that this cross-linking does not complete the acrosome reaction. Since intracellular domains of HS-21 have never been identified, it is possible that loss of HS-21 immunoreactivity may precede acrosin release. In support of this hypothesis is the report that for HS-21 "target antigen loss may actually precede complete acrosomal loss depending on the antigen's subcellular localization" (Wolf *et al.*, 1985). In this regard, loss of HS-21 immunoreactivity, as reported by Macek *et al.* (1991), may be coincident with membrane redistributions of sperm surface GalTase reported here, but may not correlate with acrosin release, the endpoint of the acrosome reaction.

Our finding that cross-linking GalTase does not, by itself, induce the acrosome reaction suggests that GalTase does not act alone as both an adhesion molecule and a receptor which induces exocytosis. Indeed, it is probable that sperm adhesion to the zona and the ZP3-induced acrosome reaction reflect heterofunctional interactions between ZP3 and multiple receptors on the sperm surface. The abundance of putative ZP3 receptors other than GalTase that have recently been identified by a number of investigators [e.g., mannosidase (Tulsiani *et al.*, 1989, 1990), fucosyltransferase (Cardullo *et al.*, 1989; Ram *et al.*, 1989; Apter *et al.*, 1989), a trypsin inhibitor insensitive site (Saling, 1981; Benau & Storey, 1987, 1988), a 95-kDa tyrosine kinase (Leyton & Saling, 1989b; Leyton *et al.*, 1990), a 56-kDa protein (Bleil & Wassarman, 1990), and PH20 on guinea pig sperm (Myles *et al.*, 1984; Cowan *et al.*, 1986; Phelps & Myles, 1987; Phelps *et al.*, 1988)] supports this hypothesis.

As described above, antibody-induced redistributions on mammalian sperm are common [see, for instance, Wolf *et al.* (1992) and McKinnon *et al.* (1991)]. The mechanism for such regionalization relates to the issue of what localizes their distribution in the first place. It is tempting to suggest that a physical barrier to interregional diffusion is responsible for regionalization. However, it is difficult to see how a physical fence will constrain a monomer but not an antibody-induced aggregate. A more likely scenario in light of phase separations (Wolf *et al.*, 1990) and lipid regionalizations (Wolf & Voglmayr, 1984; Wolf *et al.* 1986c) reported on sperm is that protein sequestration reflects preferential solubility in a given lipid phase. In this context antibody-induced redistribution is the result of precipitation in two dimensions. The precipitates either are preferentially soluble in the second phase or form their own phase and migrate as a result of surface tension.

While cross-linking of glycopeptides proteolytically derived from ZP3 will induce the acrosome reaction (Leyton & Saling, 1989a), neither these glycopeptides nor antibodies have been fully characterized. Therefore, one cannot rule out the possibility that signal transduction requires the cross-linking of different receptors or ligand-induced conformational changes in the receptor(s), or both. It is conceivable that these redistributions are related but physiologically separate from the induction of the acrosome reaction *in vivo*. Hence, redistributions of receptors (like GalTase) may represent an early event in the initiation of the acrosome reaction, but only cross-linking between correct pairs will result in its final stages (i.e., acrosin release).

The final question addressed in this study was Is GalTase moving at a sufficient rate on the mouse sperm surface to ensure adhesion with ZP3? In our experiments using TUG and Rh-α-Lac we found that cross-linking the antigen with bivalent antibody led to large-scale redistributions of GalTase on the cell surface but did not greatly affect the rapid diffusion coefficient of GalTase within the bleached spots. At the time of first contact with the sperm, the zona pellucida presents a rigid structure and the rate of ligand−receptor bond formation is dependent on the surface density of ZP3, ZP3 receptor density on the sperm surface, and the mobility of the receptor within the plane of the membrane (Baltz & Cardullo, 1989). In general, the rate constant for association between a receptor and an immobile ligand is proportional to the diffusion coefficient of the receptor (e.g., $k_{on} = 4\pi D_m$). During fertilization in the mouse, the bond formation rate (BFR) between ZP3 and its complementary receptor (ZP3r) on the mouse sperm surface is BFR $= 4\pi D_m A$[ZP3][ZP3r],

where A is the contact area between the sperm and the zona pellucida. On the mouse egg zona pellucida the surface density of ZP3 has been calculated to be approximately 300 molecules/μm^2 (Bleil & Wassarman, 1986; Baltz & Cardullo, 1989). Using enzymatic data, the surface density of GalTase on the anterior head (after cross-linking with antibody) is approximately 400 molecules/μm^2 (Baltz & Cardullo, 1989). Using these numbers, along with a median diffusion coefficient measured for GalTase of 6×10^{-9} cm^2/s (Table 2), the bond formation rate per contact area is calculated to be on the order of 10^7 bonds formed μm^{-2} s^{-1}. Finally, given that the initial contact area between the curved surfaces of the mouse sperm surface and the zona pellucida is most likely between 10^{-6} and 10^{-5} μm^2 (Baltz & Cardullo, 1989), the *maximum* bond formation rate is only about 100 bonds formed per second between the sperm surface and the zona pellucida. Hence, at densities of GalTase calculated on the mouse sperm surface, association with the zona pellucida is ensured only if GalTase possesses a rapid diffusion coefficient. Our finding that mouse sperm surface GalTase diffuses close to the fluid dynamic limit ($>10^{-9}$ cm^2/s) suggests that it has this necessary property of a zona adhesion molecule. However, whether GalTase interacts with other sperm surface molecules in the signal transduction cascade leading to acrosomal exocytosis is still unclear and an area of active investigation in our laboratory.

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