A Localized Surface Protein of Guinea Pig Sperm Exhibits Free Diffusion in Its Domain

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ABSTRACT Using the technique of fluorescence redistribution after photobleaching, we are studying the cellular mechanisms involved in localizing surface molecules to particular domains. A number of antigens localized to discrete surface regions have been identified with monoclonal antibodies on guinea pig sperm cells (Primakoff, P., and D. G. Myles, 1983, *Dev. Biol.*, 98:417–428). One of these monoclonal antibodies, PT-1, binds exclusively to the posterior tail region of the sperm cell surface. PT-1 recognizes an integral membrane protein that in complex with *n*-octyl-β-D-glucopyranoside has a sedimentation coefficient of 6.8S in sucrose density gradients. Fluorescence redistribution after photobleaching measurements reveal that within its surface domain the PT-1 antigen diffuses rapidly ($D = 2.5 \times 10^{-9}$ cm^2/s) and completely (>90% recovery after bleaching). These results rule out for this membrane protein all models that invoke immobilization as a mechanism for maintaining localization. We propose that the mechanism for localization of the PT-1 antigen may be a barrier to diffusion at the domain boundary.

The restriction of cell surface molecules into specific domains has been well established for a wide variety of mammalian cell types. These include acetylcholine receptors at the synapse on muscle cells (1), low density lipoprotein and asialoglycoprotein receptors in coated pits of fibroblasts (2) and hepatocytes (3) and more extensive localized domains on the surface of sperm (4, 5), intestinal epithelial cells (6, 7), and hepatocytes (8, 9). Although topographical localization of various surface receptors has been widely described, little is known concerning the mechanisms that maintain surface protein localizations. Immobilization in the membrane is a potential mechanism for maintaining a non-uniform distribution of surface molecules, and in the case of the acetylcholine receptor, it has been shown that clustered receptors are immobilized (10).

In the current study we have addressed the question whether there are mechanisms other than immobilization to localize surface molecules. We have used the guinea pig sperm cell. Previously, the existence of five different antigen domains on the surface of the guinea pig sperm was established with monoclonal antibodies directed to surface antigens. These antigens may be restricted to the anterior head, posterior head, whole head, whole tail, or posterior tail cell surface (11). In some cases, patching of localized sperm surface molecules can be induced, suggesting that these molecules may be mobile in the plane of the membrane (12–15). However, ability to patch may not be a reliable indicator of mobility of a receptor in the membrane (16–18) and gives no measure of the diffusion rate or percent mobile fraction. Therefore, we have directly measured these parameters using fluorescence redistribution after photobleaching of a localized sperm surface antigen that can be patched. This antigen, identified by the monoclonal antibody PT-1, is restricted to the posterior tail sperm surface. We have found that essentially all the PT-1 antigen is freely diffusing within its surface domain. This result shows that a mechanism other than immobilization can operate in maintaining the heterogeneous topography of a cell surface.

MATERIALS AND METHODS

Cells: Cauda epididymal sperm were obtained from mature, male Hartley guinea pigs.

Lipid Extracts: To test if chloroform/methanol extracts could inhibit PT-1 binding, we sonicated sperm and repeatedly extracted with 2:1 chloroform/methanol according to Robbins et al. (19) or 1:2:0.75 chloroform/methanol/water following the protocol used by Svennerholm and Fredman (20) and Magnani et al. (21). The final extracts from the two procedures were evaporated to dryness and resuspended in Mg-HEPES medium (22) containing 30 mM *n*-octyl-β-D-glucopyranoside (OG).[1] A control aliquot of sonicated sperm in Mg-HEPES was extracted with 30 mM OG.

Protease and Heat-treated OG Extracts: To test if OG extracts of sperm treated with protease or heat could inhibit PT-1 binding, we extracted freshly removed sperm, suspended in HEPES-buffered medium (22) containing 10 mM $CaCl_2$, with 30 mM (final) OG. One aliquot (control) of OG extract was incubated at 37°C for 24 h in the absence of added protease. After inactivation of contaminating glycosidases by incubation at 50°C for 30 min

[1] *Abbreviations used in this paper:* OG, *n*-octyl-β-D-glucopyranoside.

(23), Pronase E (Sigma Chemical Co., St. Louis, MO) was added to a second aliquot of OG extract at a final concentration of 125 μg/ml, and incubation was continued for 24 h at 37°C. To both aliquots, 3.6 mg/ml bovine α_2-macroglobulin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added to block further protease activity. From a second suspension of freshly removed sperm, a 30 mM OG extract was made and heated to 70°C for 10 min.

Assay of Inhibition of Antibody Binding: To characterize the PT-1 antigen, we tested the lipid extracts and protease and heat-treated OG extracts for their ability to inhibit PT-1 antibody binding to a standard PT-1 antigen-containing extract bound to a solid phase. To prepare the standard PT-1 antigen-containing extract, we suspended sperm at 1×10^8/ml in Mg^{2+}-HEPES containing 10 μM leupeptin and 0.5 mM phenylmethylsulfonyl fluoride, and OG added to a final concentration of 25 mM. After 15 min incubation at 4°C the OG extract was centrifuged at 8,000 g_{av} for 10 min. The supernatant was removed, diluted 1/5 in Mg^2-HEPES containing 25 mM OG, 10 μM leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride, and incubated on a microtiter plate overnight at 37°C to allow the antigen to attach to a solid phase. The assay was then carried out as previously described (24). PT-1 supernatant was titered to determine a dilution (1/200), for which further dilution gives reduced antibody binding. 100 μl of test and control extracts from 2.5×10^6 cells were preincubated for 1 h with 100 μl PT-1 supernatant (diluted 1/100). Twofold serial dilutions of each extract were similarly preincubated with 1/100 diluted PT-1 supernatant. Binding of preincubated PT-1 was then tested in the solid phase radioactive binding assay, using ^{125}I goat anti-mouse Ig's (New England Nuclear, Boston, MA) as a second antibody.

Sedimentation Analysis of the PT-1 Antigen: An extract containing PT-1 antigen was made by suspending sperm at 2×10^8/ml in Mg^{2+}-HEPES containing 10 μM leupeptin and 0.5 mM phenylmethylsulfonyl fluoride and adding OG to a final concentration of 30 mM. After 15 min incubation at 4°C, the OG extract was centrifuged at 8,000 g_{av} for 10 min, and 100 μl of the supernatant was layered on top of a 5 ml 5–20% sucrose gradient in Mg^{2+}-HEPES containing 10 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 30 mM OG. After centrifugation at 40,000 rpm for 16 h at 4°C in an SW 50.1 rotor, fractions (~0.25 ml) were collected and assayed in a direct solid phase binding assay in which a twofold diluted aliquot from each fraction was bound to the surface of a microtiter plate. Two standard proteins, yeast alcohol dehydrogenase (7.6 S) and lysozyme (2.1 S), were centrifuged at the same time through identical sucrose gradients without OG (25–27).

Preparation of Cells for Fluorescence Redistribution after Photobleaching Measurements: Freshly removed sperm were labeled with PT-1 followed by a fluorescein isothiocyanate-Fab fragment of goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) as previously described (24). 10 μl of labeled sperm suspension was added to 100 μl of 0.66% agarose in Mg^{2+}-HEPES at 37°C, and briefly mixed. Small drops of the resulting suspension were put on microscope slides at 37°C and immediately covered with coverslips. The slides were then put at room temperature. As the agarose solidified, the sperm were trapped so that they no longer could swim, making it possible to monitor selected cells continuously. Cells exhibited the same regional binding patterns observed previously with a FITC-goat $F(ab')_2$ anti-mouse IgG (11), demonstrating the specificity of the Fab second antibody binding.

Fluorescence Redistribution after Photobleaching: Diffusion coefficients were determined using a new version of the periodic pattern bleaching technique (28–31). The cells were illuminated with laser light (λ = 488 nm) that has been masked to form a pattern of stripes. Ronchi rulings are imaged onto the field diaphragm of the microscope incident-light illuminator, and reimaged onto the sample through a 100× objective, giving a combined 60-fold linear reduction. A bleaching pulse of intense laser light produces a periodic distribution of unbleached fluorophore. The modulation amplitude of this distribution is calculated as a function of time after bleaching from a series of fluorescence scans with an attenuated laser pattern, produced by a galvanometric scanning mirror placed between the Ronchi ruling and the illuminator field diaphragm (31). Diffusion theory predicts an exponential decay of the modulation amplitude with a decay rate proportional to D, and inversely proportional to the square of the pattern repeat distance (ξ_0^{-1}):

$$A(\xi_0, t) = A(\xi_0, 0) \exp[-(2\pi\xi_0)^2 Dt]. \quad (1)$$

RESULTS AND DISCUSSION

The monoclonal antibody PT-1 (subclass IgG2b) recognizes a surface antigen that is restricted to the posterior tail region of guinea pig sperm freshly removed from the cauda epididymis (24). A series of experiments was performed to identify the antigen recognized by PT-1. Previous attempts to identify the PT-1 antigen by labeling sperm surface proteins with ^{125}I and immunoprecipitating from 1% Triton X-100 extracts with PT-1 antibody were unsuccessful (24). Reaction of nitrocellulose paper transfers from SDS polyacrylamide gels with PT-1 antibody also reveals no labeled bands. Both these techniques have been successful in identifying antigens recognized by other monoclonal antibodies in our collection. Since these standard protocols did not determine whether the PT-1 antigen is a protein or a glycolipid, we distinguished between these two possibilities by alternative methods. To test whether PT-1 antigenic activity is found in glycolipid fractions, we examined chloroform/methanol extracts of sperm for their ability to inhibit PT-1 binding in a solid phase assay. Sonicated sperm cells were extracted by two different methods, using 2:1 chloroform/methanol (19) or 1:2:0.75 chloroform/methanol/water (20, 21). Neither chloroform/methanol fraction showed inhibition of PT-1 binding (Fig. 1*a*). A second experiment tested the sensitivity of the PT-1 antigen in OG extracts to heat and pronase digestion. Inhibition of antibody binding was abolished both by heating for 10 min at 70°C and by protease treatment as shown in Fig. 1*b*. On the basis of these results, we infer that PT-1 recognizes a protein or the protein region of a glycoprotein.

To characterize this protein, we measured its rate of sedimentation on 5–20% sucrose gradients in the presence of 30 mM OG (26, 27). The PT-1 antigen sediments to form a single sharp peak on these gradients (Fig. 2) and has a sedimentation coefficient of 6.8S determined by comparison to known standards (25).

Measurements of lateral diffusion of the PT-1 antigen were made using fluorescence redistribution after photobleaching. Sperm tails were aligned along the scan axis, perpendicular to the pattern stripes, with a rotatable microscope stage. To enhance the fluorescence signal, we chose sperm that were associated in stacks (rouleaux) that allowed for several aligned tails to be bleached simultaneously. To insure that the sperm to be tested were viable, we selected cells embedded in agar in which a part of the tail, outside the area to be bleached, was in a nonsolid pocket of the agar and therefore continued to beat.

We observed a rapid decay of modulation amplitude in these experiments (Figs. 3 and 4), with decay rates corresponding to $D = 2.5 \times 10^{-9}$ cm^2/s. The decay is monophasic and complete (>90%) indicating that all the molecules binding PT-1 are rapidly diffusing. Moreover, by varying the pattern repeat distance (ξ_0^{-1}), we confirm the distance dependence consistent with diffusion theory (Eq. 1). Scaling the time axes of the data by factors of $(2\pi\xi_0)^2$ produces a universal curve with a decay rate D (Fig. 4). In similar experiments, the diffusion coefficient of the fluorescent lipid probe *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine in the posterior tail region was determined to be 7.7×10^{-9} cm^2/s.

The diffusion coefficient measured for PT-1 is extremely large for a membrane protein. Compared to *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine, it diffuses at a rate near the predicted theoretical limit for a freely diffusing membrane protein (32–34). Similar diffusion coefficients have been measured for rhodopsin in amphibian rod outer segment disks (35, 36), and for plasma membrane proteins in membranes in which an underlying cytoskeleton may be modified or is separated from the membrane (37–42). It is not known

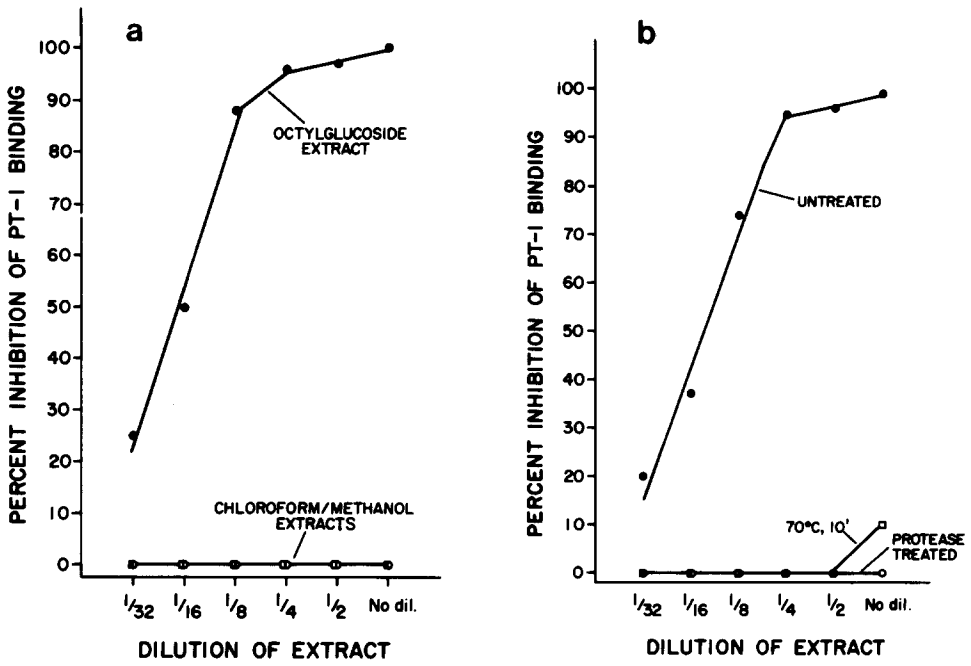

FIGURE 1 Inhibition of PT-1 antibody binding by sperm extracts. (a) PT-1 antibody was pre-incubated for 1 h with an OG extract, a 2:1 chloroform/methanol extract, or a 1:2:0.75 chloroform/methanol/water extract prepared as described in Materials and Methods. Binding of preincubated PT-1 was then tested in the solid phase assay. (●) Preincubation with OG extract; (□) preincubation with 2:1 chloroform/methanol extract; (○) pre-incubation with 1:2:0.75 chloroform/methanol/water extract. (*b*) PT-1 antibody was preincubated for 1 h with an OG extract, an OG extract heated at 70°C for 10 min, or an OG extract treated with Pronase E as described in Materials and Methods. Binding of preincubated PT-1 was then tested in the solid phase assay. (●) Pre-incubation with control extract; (○) preincubation with protease-treated extract; (□) preincubation with 70°C-treated extract.

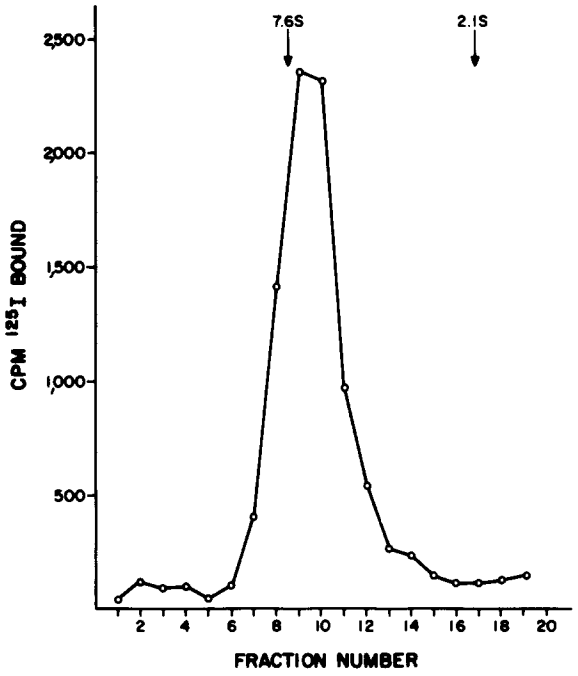

FIGURE 2 PT-1 antibody binding activity of fractions collected from a 5–20% sucrose gradient containing 30 mM OG. An OG extract of sperm was layered on a 5–20% sucrose gradient and centrifuged at 40,000 rpm for 16 h at 4°C in an SW 50.1 rotor. PT-1 antibody binding of the gradient fractions is shown. The S value of the single peak of antibody-binding activity was calculated relative to the standards, yeast alcohol dehydrogenase (7.6 S) and lysozyme (2.1 S).

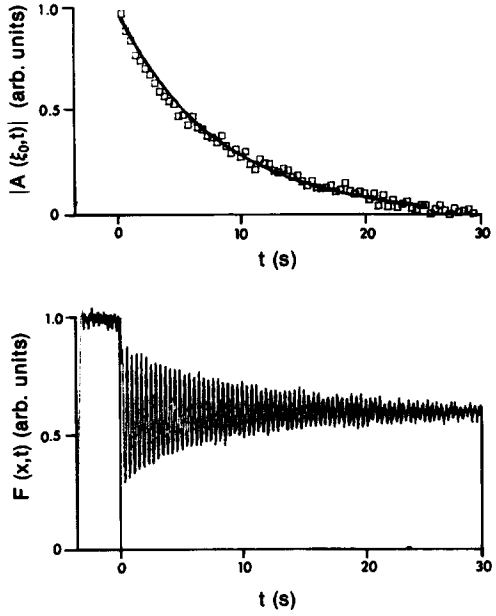

FIGURE 3 Fluorescence redistribution after photobleaching of posterior tail region labeled with PT-1 followed by FITC-conjugated Fab fragments of goat anti-mouse IgG. The sample was bleached (t = 0) with a pulse of laser light in the form of a pattern of stripes with repeat distance ξ_0^{-1} = 9.5 μm. Fluorescence scans with the attenuated striped pattern were taken every 384 msec before and after bleaching. $F(x, t)$: full display of recorded fluorescence intensity for 84 consecutive scans. A clear sinusoidal modulation is observed after bleaching. $A(\xi_0, t)$: computer calculated modulation amplitude for each post-bleach scan. Solid line is exponential fit to modulation decay.

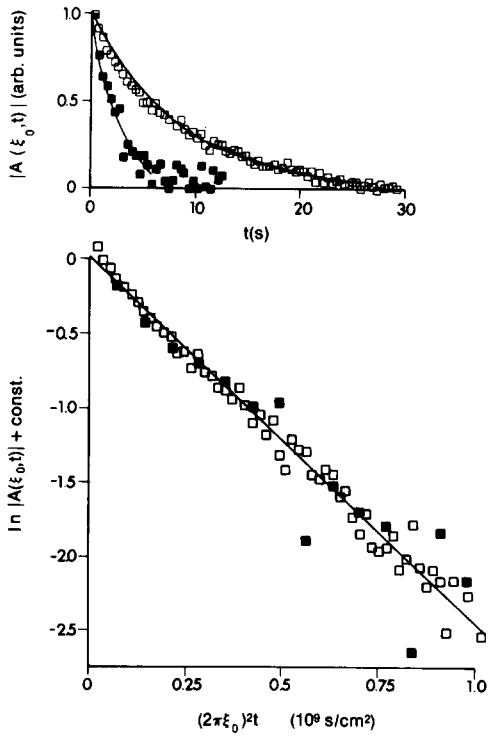

FIGURE 4 (*Top*) Modulation amplitudes as function of time after bleaching recorded with ξ_0^{-1} = 9.5 μm (□) and 4.25 μm (■). Solid lines are least-squares exponential fits. (*Bottom*) Semi-log plots of same sets of data with time axes scaled to compensate for the difference in characteristic distance (ξ_0^{-1}). Solid line is least-squares straight line fit with a slope equal to a diffusion coefficient of 2.5 × 10^{-9} cm^2/s.

whether a membrane-associated cytoskeleton is present in the posterior tail of guinea pig sperm.

Rapid fluorescence redistribution after photobleaching is not observed on cells fixed with glutaraldehyde (1.25% for 30 min) before antibody labeling. Under the same conditions as those of Fig. 3, modulation amplitude decay with fixed cells is barely detectable, being at least 200 times slower than with unfixed cells. This immobilization verifies that the rapid fluorescence redistribution observed with unfixed cells corresponds to surface antigen mobility and not some form of antibody surface "hopping."

There are several possible schemes by which a localized distribution of surface molecules might be maintained. The molecules could be constrained to a surface domain by being immobilized. Alternatively, molecular segregation could be maintained by thermodynamic partitioning into a physicochemically distinct membrane domain. If such partitioning is a consequence of favorable interactions with stable localized structures, segregation would necessarily be accompanied by a sharp reduction of lateral mobility. Molecules could remain mobile, however, if partitioning comes through favored associations with other mobile molecules, such as lipids in a fluid lipid domain. A third possibility for maintaining a localized distribution is that the molecules be constrained to their region by a barrier to diffusion at the domain boundary.

Our finding of rapid PT-1 antigen diffusion rules out for this membrane protein all schemes that involve immobilization or any significant reductions of lateral mobility. Thermodynamic partitioning schemes that do not posit a reduction in PT-1 lateral diffusion could account for the localization. Lipid diffusion rates, however, though statistically different in the various surface regions of ram sperm (43), do not indicate the dramatic differences in physical state that one might suppose would be necessary for a substantial partitioning effect of this kind.

The model of a barrier at the anterior tail-posterior tail junction is compatible with the finding of a freely diffusing localized antigen. In this case, the barrier would need to selectively block the diffusion of specific surface molecules, since it has been shown that lipids are free to diffuse between regions (43). One candidate for such a barrier would be the annulus observed at this junction. The annulus appears in thin sections observed by transmission electron microscopy as a submembranous electron-dense ring and in freeze-fracture as a band of small, intramembrane particles on the P-face (protoplasmic face) of the plasma membrane (44, 45). A second possibility for a diffusion barrier is the closely spaced circumferential rows of intramembrane particles observed by freeze-fracture on the P-face of the anterior tail plasma membrane (44). This array of molecules along the length of the anterior tail could be impenetrable by PT-1. Other types of barriers to diffusion can be imagined that would not have been detected morphologically.

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