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-ß-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 (\(D = 2.5 \times 10^{-9} \text{cm}^2/\text{s}\)) 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-ß-D-glucopyranoside (OG). 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 30 mM n-octyl-ß-D-glucopyranoside (OG). A control 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-ß-D-glucopyranoside.
field diaphragm (31). Diffusion theory predicts an exponential decay of the metric scanning mirror placed between the Ronchi ruling and the illuminator of fluorescence scans with an attenuated laser pattern, produced by a galvanometric system. This distribution is calculated as a function of time after bleaching from a series of periodic distributions of unbleached fluorophore. The modulation amplitude of 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 sperm 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 $D = 2.5 \times 10^{-9} \text{ cm}^2/\text{s}$. 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 ($d_0$) we confirm the distance dependence of the diffusion coefficient measured for PT-1 is extremely large for a membrane protein. Compared to N-4-nitrobenzoxa-1,3-diazole phosphatidylethanolamine in the posterior tail region was determined to be $7.7 \times 10^{-9} \text{ cm}^2/\text{s}$.

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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 $^{125}\text{I}$ 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.

RESULTS AND DISCUSSION

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We observed a rapid decay of modulation amplitude in these experiments (Figs. 3 and 4), with decay rates corresponding to $D = 2.5 \times 10^{-9} \text{ cm}^2/\text{s}$. 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 ($d_0$), we confirm the distance dependence consistent with diffusion theory (Eq. 1). Scaling the time axes of the data by factors of $(2\pi d_0)^2$ 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 $7.7 \times 10^{-9} \text{ cm}^2/\text{s}$.

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 post-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 x = 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(t): computer calculated modulation amplitude for each post-bleach scan. Solid line is exponential fit to modulation decay.
in PT-1 lateral diffusion could account for the localization.

Lipid diffusion rates, however, though statistically different in re-

cognizable submembranous electron-dense ring and in freeze-frac-
ture as a band of small, intramembrane particles observed by
freeze-fracture on the P-face of the anterior tail plasma mem-
brane. 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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