Lateral Regionalization and Diffusion of a Maturation-dependent Antigen in the Ram Sperm Plasma Membrane

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Abstract. We have used a monoclonal antibody ESA152 in fluorescence recovery after photobleaching (FPR) studies of a maturation-dependent surface antigen of ram sperm. The antibody is an immunoglobulin G secreted by a hybridoma derived from NS1 mouse myeloma cells. The ESA152 antigen is not detectable in testicular sperm. It is localized on the surface of ejaculated sperm where it is present on all regions of the surface, but tends to be concentrated on the posterior region of the head. The ESA152 antigen can be extracted by detergents or chloroform-methanol. The extracted antigen is sensitive to proteases and migrates with an apparent $M_r \sim 30{,}000$ in SDS-containing 10–20% polyacrylamide gradient gels. FPR measurements of ESA152 lateral mobility in the membrane yield diffusion coefficients in the range 10^{-9}–10^{-8} cm^2/s, values typical of lipids but observed for proteins only at the fluid dynamic limit where diffusion is controlled by lipid fluidity. Immobile fractions, typical of membrane proteins, are observed on all regions. When the antigen is stained by a fluoresceinated F_{ab} fragment of the ESA152 antibody, the diffusibility is highly regionalized, with particularly low, but rapid, recovery on the midpiece. Cross-linking of the antigen with the intact ESA152 antibody induces a redistribution in which the antigen is excluded from the posterior head region. This cross-linking is accompanied by increases in ESA152 diffusibility on both the anterior head and the midpiece.

SINCE the experiments of Frye and Edidin (16), which demonstrated the ability of surface antigens to intermix upon heterokaryon fusion, it has been clear that cell plasma membrane components are generally free to diffuse within the plane of the membrane. The development of fluorescence recovery after photobleaching (FPR)[1] has enabled us to quantitate diffusion of membrane proteins and lipids in a number of cell systems (for reviews see references 8, 11, and 35). Diffusion is a random process (12). Yet, during processes of cellular differentiation, such as sperm maturation and capacitation (4), preimplantation embryogenesis (22, 52, 55, 58), erythropoiesis (37), epithelial tight junction formation (29, 43), and myotube development (2), cells overcome the randomizing effect of diffusion and regionalize the distribution of certain membrane components. The ubiquity of surface regionalizations during cellular differentiation suggests a close relationship between these two phenomena, and demonstrates the need to understand how cells restrain the free diffusion of their surface components if one hopes to understand the processes of differentiation.

Some of the most dramatic examples of surface component regionalization and its relationship to cellular differentiation are exhibited by mammalian sperm. Sperm localize a number of surface components: antigens (13, 20, 23, 25, 30, 38, 39, 45, 46), enzymes (for review see reference 23), lectin receptors (23, 30, 32, 33), charged lipids (4, 15), and cholesterol (4, 15) to specific surface regions. Some of these patterns of regionalization are transformed during the differentiative processes of epididymal maturation (19, 32) in the male tract and capacitation (4, 34) in the female tract. These processes result in the functional differentiations that lead to the acquisition of motility, the acrosome reaction, and fertilization competence.

Recent FPR studies have begun to investigate the nature of the restraints to random mixing by diffusion of surface components in mammalian sperm (31, 53, 59). Myles et al. (31) have shown that a membrane protein antigen that is localized to the posterior region of the guinea pig sperm tail is completely free to diffuse within this region and diffuses at or near its fluid dynamic limit. Such a result is consistent with the presence of a barrier to interregional diffusion or with lateral segregation of antigen due to its insolubility in regions other than the posterior region of the tail. Wolf and Voglmayr (59) have measured the regionalization and diffusion of an exogenously added fluorescent lipid analogue on testicular and ejaculated ram spermatozoa. While this analogue stained all

1. *Abbreviations used in this paper:* D, diffusion; FPR, fluorescence recovery after photobleaching; %R, percent recovery.

regions of the sperm, both its distribution and diffusion were regionalized. At least some of the analogue was free to exchange between regions, and both distribution and diffusion changed with epididymal maturation. These experiments demonstrate that the sperm plasma membrane cannot be treated as having a single bulk membrane fluidity, but rather is laterally segregated into large scale interactive domains.

In the present paper, we consider the distribution and diffusion of a maturation-dependent membrane antigen on ram sperm. This antigen, which is a protein or glycoprotein with an $M_r \simeq 30{,}000$, has been probed using a monoclonal antibody denoted ESA152. The antigen is not detectable on the surface of immature testicular sperm. It is present on all regions of the surface of ejaculated sperm, but tends to concentrate on the posterior region of the head.

The unusual distribution of ESA152 over the entire sperm surface provides us with the opportunity to extend the work of Wolf and Voglmayr (59) to a membrane protein, and to compare results with those of Myles et al. (31) for PT-1, which is highly restricted in its distribution. Like PT-1, ESA152 shows lipid-like diffusion coefficients on all regions. Unlike PT-1, significant immobile fractions are observed on all regions. Both diffusion coefficient and mobile fraction vary significantly over the surface. Exposure to bivalent antibody induces a redistribution of the antigen which excludes it from the posterior region of the head. This redistribution is associated with striking and distinct shifts in the diffusibilities of the antigen on the midpiece and head.

Materials and Methods

Sperm

Sperm were collected as described below from Shropshire rams aged 4–6 yr. These animals were maintained in a controlled environment (12 h of light/day at 15°C). Ejaculated spermatozoa and seminal plasma were obtained by electrical stimulation using the bipolar electrode described by Blackshaw (6). To avoid temperature shock, semen was collected and stored in a receptacle maintained at 34°C. Testicular sperm and rete testis fluid were collected through a catheter inserted into the rete testis as described by Voglmayr et al. (49). The free end of the catheter was placed into a receptacle attached to the anterior surface of the scrotum.

Immediately after collection, sperm were washed three times in phosphate-buffered saline (PBS) pH 7.4 that contained 5 mM glucose (PBS) by centrifugation at 500 g_{max} for 10 min. Spermatozoa can be maintained under these conditions at 34°C for ~3 h without significant diminution of glycolytic activity (41).

Production of Hybridoma ESA152

Immunization. Ejaculated spermatozoa were washed three times in Krebs-Ringer-phosphate buffer and resuspended in 20 vol of the same buffer. The suspension was mixed with an equal volume of Freund's complete adjuvant, and a portion of the emulsion that contained ~5 × 10^7 spermatozoa was injected subcutaneously into a male BALB/c mouse (Charles River Breeding Laboratories, Inc., Wilmington, MA). A similar subcutaneous injection was given 2 wk later. 2 mo after the initial immunization, the mouse was given an intraperitoneal booster injection that consisted of ~2 × 10^7 spermatozoa in the adjuvant.

Fusion. NS1 mouse myeloma cells (P3-NS1/1Ag4-1 [24], obtained from Dr. George S. Bloom, University of Texas, Dallas) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (HyClone Laboratories, Hogan, UT) and 0.25% glucose, 1 mM sodium pyruvate, 1% 100× non-essential amino acids, and 100 μg/ml gentamicin (complete culture medium) (see references 17 and 61). Spleen cells prepared from the hyperimmunized mouse 5 d after the final booster injection were mixed with NS1 cells at a spleen cell/myeloma cell ratio of 4:1. The cells were pelleted and induced to fuse by addition of 42% (wt/vol) polyethylene glycol 3350 (Cat. No. U221, lot 238504, J. T. Baker Co., Sanford, ME) in 15% (vol/vol) dimethyl sulfoxide (spectrophotometric grade).

Selection and Screening. The fusion products were dispensed to 2.0-cm^2 wells of cluster plates to which mouse peritoneal macrophages had been allowed to attach during an overnight preincubation (14). Hybridomas were selected as the survivors of growth for 9 d in HAT medium (complete culture medium that contained 0.136 mg/ml hypoxanthine, 0.19 μg/ml aminopterin, and 3.8 μg/ml thymidine). The hybridoma cells were then propagated in HT medium (complete culture medium with hypoxanthine and thymidine). Culture supernates were screened by ELISA and indirect immunofluorescence for the presence of antibodies against dried ram spermatozoa immobilized in flat-bottom 96-well microtiter plates or on multi-well printed microscope slides (Carlson Scientific Inc., Peotone, IL). In the ELISA, sperm-associated hybridoma antibody was detected with a secondary antibody that consisted of beta-galactosidase conjugated to $F(ab')_2$ fragments of sheep anti-mouse IgG (light and heavy chain specific, BRL Hybridoma Screening Kit, Cat. No. 9502SA, Bethesda Research Laboratories, Bethesda, MD). In immunofluorescence screening, the secondary antibody was rhodamine-conjugated IgG fraction of rabbit anti-mouse IgG (heavy and light chain specific, Cat. No. 2211-0082, Cappel Laboratories, Cochranville, PA). The slides were mounted using Elvanol (18) and examined for fluorescence under epiillumination using dry 40× objective.

Cloning. One culture that tested positive with ejaculated sperm and negative with testicular sperm was cloned by limiting dilution (42). The clone designated ESA152 was recloned (42) in 0.25% Noble agar.

Antibody Characterization. Commercial immunoglobulin typing kits (Boehringer Mannheim Diagnostics, Inc., Houston, TX [Cat. No. 100 036] and HyClone Laboratories [Cat. No. 55030K]) were used. The ESA152 antibody yielded negative results in all tests for the IgM heavy chain. Strong positives in other tests established that it is an IgG of the G1 subclass. The kappa light chain is present.

Production of ESA152 Ascites Fluid. Pristane-primed BALB/c mice (57) were given intraperitoneal injections of ~10^7 ESA152 hybridoma cells in PBS. Ascites tumors appeared after 5 d. Fluid was withdrawn after 10 d and daily for up to 8 d thereafter until all mice died. The fluid was clarified by centrifugation and stored frozen.

F_{ab} Fragments

The ascites fluid was first tested for its ability to react with protein A by indirect immunofluorescent staining of ejaculated ram sperm using fluorescein-conjugated protein A (Vector Laboratories, Inc., Burlingame, CA). The ascites fluid was then dialyzed against PBS and absorbed to a protein A Sepharose CL-4B column. The IgG fraction was eluted with 0.1 M acetic acid. This fraction was dialyzed against PBS and concentrated to ~0.5 mg/ml by solid dialysis against polyvinylpyrrolidone. Monovalent F_{ab} fragments were produced by digestion in 0.01 M cysteine, 0.002 M EDTA, and 1 mg papain/100 mg protein for 7 h at 37°C with occasional agitation. This digestion mixture was dialyzed at 4°C for 4 h against 500 ml deionized water and for 4 h against each of three 500 ml changes of PBS. The solution was passed through the protein A column to remove F_c fragments, and the nonabsorbed fractions were concentrated by dry dialysis to 1.3 ml which was estimated to contain ~0.2 mg by its absorbance at 280 nm. To prepare fluorescein-conjugated F_{ab}, the solution was diluted with 260 μl of 200 mM carbonate buffer and the pH was adjusted to 9.5. 100 μl of a 1 mg/ml solution of fluorescein isothiocyanate in PBS was then added and the mixture was stirred in the dark for 4 h at room temperature. The sample was dialyzed extensively against PBS.

A similar procedure was used to conjugate a F_{ab} fragment of a goat anti-mouse IgG (Cappel Laboratories) with lissamine rhodamine sulfonyl chloride (Molecular Probes, Eugene, OR).

Labeling of Sperm for FPR Measurements

Washed sperm were diluted to 4 × 10^7 cells/ml in PBS with 0.5% BSA and 0.1% NaN_3. 100 μl of sperm were incubated with either 400 μl of ESA152 or with 10 μl of the fluorescein-F_{ab} fragment for 15 min at room temperature. The suspension was overlayed on PBS with 0.1% NaN_3 and 5% BSA (for the intact ESA152) or 10% BSA (for the fluorescein F_{ab} fragment). This was centrifuged at 480 g_{max} for 10 min at room temperature and the pellet resuspended in PBS with 0.1% NaN_3 and 0.5% BSA. To samples labeled with ESA152, 10 μl of the rhodamine F_{ab} fragment of goat anti-mouse IgG was added, and the solution incubated at room temperature for 15 min. This suspension was overlayed on PBS with 0.1% NaN_3 and 10% BSA and centrifuged and resuspended as before.

~20 μl of sperm were then placed between an acid-washed slide and coverslip for viewing and/or FPR measurements.

Labeling of Sperm for Photomicroscopy

Printed microscope slides with 6-mm wells were treated twice with MICRO Laboratory Cleaner (International Products Corp., Trenton, NJ) at 90°C and

rinsed with deionized water. They were dried in an oven and stored in a dessicator over activated charcoal. 5 μl of a suspension of poly-D-lysine (0.01 mg/ml) were added to each well and allowed to dry. Slides were washed with water followed by PBS. Sperm were pelleted and diluted 50-fold in PBS. 20 μl of sperm were placed in each well and allowed to adsorb for 5–15 min at 4°C. Slides were rinsed with cold PBS; each well was aspirated and 20 μl of ESA152 was immediately added to it. After incubation for 45 min at 4°C, 20 μl of a 1:10 dilution of second antibody, either rhodamine-labeled rabbit anti-mouse IgG (Cappel Laboratories) or the rhodamine-labeled F_{ab} fragment of goat anti-mouse, was added and incubation was continued for 45 min at 4°C. The slide was then washed with PBS. The final wash was done with PBS that contained 25 μg/ml gentamicin. Excess PBS was shaken off and a 22 × 50-mm coverslip applied. Excess buffer was aspirated away and the edges sealed with mineral oil.

Photomicroscopy

Photomicroscopy was done on an Olympus BHS phase contrast fluorescence microscope with an Olympus Photomicroscopy System using a Zeiss 63 × 1.4 numerical aperture Apochromat objective and standard rhodamine filters. Photomicrographs were made on Kodak Tri-X and developed in D76.

FPR Measurements

The technique of FPR has been described in detail elsewhere (1, 27, 51). FPR provides two measures of lateral diffusion in the plane of the membrane: (*a*) the fraction of the component that is free to diffuse (%R), and (*b*) the diffusion coefficient (D) of that fraction. Our instrument is similar to published designs (27, 51) and its particulars have been described elsewhere (58, 59). The beam exp (−2) radius was determined (40) to be (0.9 ± 0.2) μm. For rhodamine bleaching, times were ~5 ms at ~10 mW and monitoring intensities were ~1 μW. For fluorescein bleaching, times were ~20 ms at 0.2 mW and monitoring intensities were ~0.02 μW (fluorescein). Rhodamine was excited at 514.5 nm and fluorescein at 488 nm. The counting interval was 30 ms. Data were fitted by nonlinear least squares programs after Bevington (5), according to algorithms described by Barisas and Leuther (3) and Wolf and Edidin (51). Data were corrected for sperm geometry. The one- and two-dimensional solutions appropriate to the tail and head, respectively, can be found in the literature (1, 26). A solution appropriate for the midpiece was developed using standard procedures and assuming a Gaussian beam and diffusion on a cylinder. Identical results are obtained when either a spot or a line is used for bleaching. Details of this solution will be published elsewhere (Wolf, D. E., manuscript in preparation).

Partial Characterization of the ESA152 Antigen

ESA152 monoclonal antibodies were used with indirect immunofluorescence, ELISA, and dot blot assays of extracts to examine ejaculated and testicular spermatozoa for the presence of the corresponding antigen. All assays demonstrated the presence of the ESA152 antigen on mature spermatozoa and none yielded evidence of antigen in immature spermatozoa. In immunofluorescence studies ring staining of intact spermatozoa was seen (Fig. 1). When unfixed sperm were damaged on the slide, they often lost cell-associated fluorescence and released fluorescent debris. Spermatozoa extracted with 0.9% Triton X-100 were negative in immunofluorescence, and the presence of the antigen in Nonidet P-40 and SDS extracts was readily demonstrated by dot blot assays. These observations define ESA152 as a maturation-dependent surface antigen (Fairbanks, G., R. G. Lewis, and J. K. Voglmayr, manuscript in preparation).

The ESA152 antigen is robust, in that it survives in SDS and chloroform–methanol extracts and its immunofluorescence is not attenuated by aldehyde fixation. However, pretreatment of spermatozoa for 15 min at 36°C with trypsin, chymotrypsin, or pronase (0.05 mg/ml) did not remove surface antigen demonstrable by immunofluorescence; attempts to immunoprecipitate an ^{125}I-labeled protein from Nonidet P-40 extracts of radioiodinated spermatozoa have so far yielded negative results. Although it is very efficiently extracted by chloroform–methanol, the antigen did not migrate in thin-layer chromatographic systems used for glycolipid characterization. After phase separation by addition of chloroform and water to chloroform–methanol extracts in the

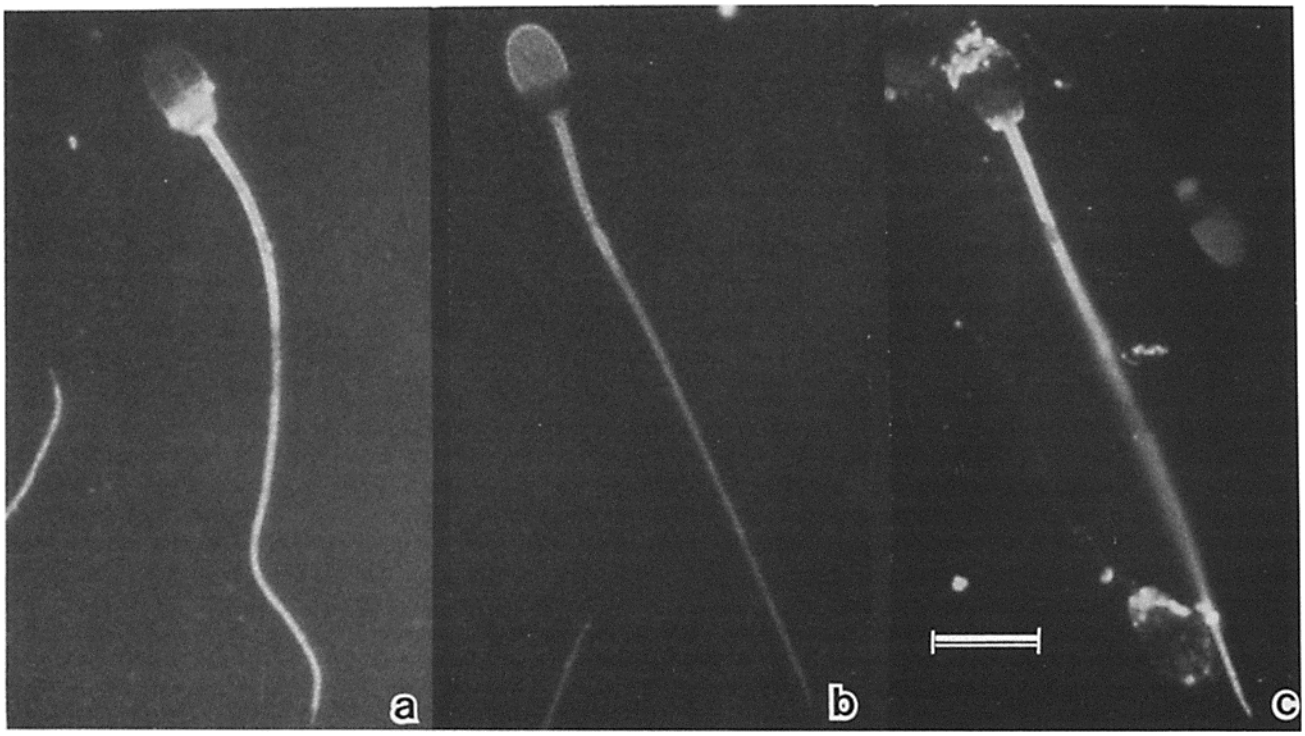

Figure 1. Fluorescence photomicrographs showing ESA152 staining patterns. Specific details of staining are given in the text (*a*). Sperm fixed in 5% glutaraldehyde and then labeled with intact ESA152 antibody followed by a rhodaminated rabbit anti-mouse IgG. Similar staining was observed in unfixed cells labeled directly with a fluorescein-labeled F_{ab} fragment of ESA152. ESA152 is seen on all regions of the sperm surface, being most intense on the posterior region of the head (*b*). Unfixed sperm labeled with intact ESA152 followed by a rhodamine-labeled F_{ab} fragment of a goat anti-mouse IgG. Cross-linking by first antibody results in a redistribution of antigen, excluding it from the posterior region of the head (*c*). Unfixed sperm labeled with intact ESA152 followed by a rhodamine-labeled rabbit anti-mouse IgG. In addition to the redistribution observed in *b*, intact second antibody leads to patching of the antigen. The particular samples in *a* and *c* were plated on poly-D-lysine as described, whereas *b* was labeled as described for FPR measurements. Bar, 10 μm.

method of Bligh and Dyer (7), the antigen was recovered at the interface. When the interface material was solubilized in SDS and fractionated electrophoretically in 10–20% polyacrylamide gradient gels (28), the antigen was detected by immunoblotting (21) at a position that corresponded to apparent M_r ~30,000. This zone of reactivity was eliminated by treatment with pronase or proteinase K in SDS before electrophoresis. These observations imply that, although the ESA152 antigen has amphipathic properties akin to lipids, it is a protein or glycoprotein. Further characterization of this interesting plasma membrane antigen is in progress, and these results will be presented separately in greater detail.

Results

Distribution of ESA152 on Ram Sperm Surfaces

Fig. 1 *a* shows the fluorescence staining pattern of ejaculated ram spermatozoa fixed in 5% glutaraldehyde and indirectly labeled with bivalent antibody. Fixed sperm were similarly stained when intact ESA152 followed by rhodamine-conjugated F_{ab} fragments of an anti-mouse IgG were used. Unfixed sperm also yielded this same pattern when labeled directly with fluorescein-conjugated F_{ab} fragments of ESA152 IgG. The labeling is detectable over the entire surface of ejaculated sperm, but staining is most intense on the posterior region of the head. The staining observed is specific. In the case of indirect labeling, staining does not occur when the ESA152 is replaced by normal mouse serum or omitted altogether. In the case of the direct labeling, staining with F_{ab} fragment does not occur when incubation is done in the presence of excess ascites fluid that contains intact ESA152.

Fig. 1 *b* shows the staining pattern of unfixed ejaculated sperm labeled indirectly with intact ESA152 followed by the rhodamine-conjugated F_{ab} fragment of anti-mouse IgG. Similar staining occurs on sperm fixed after the addition of ESA152. The presence of bivalent ESA152 results in a redistribution of antigen such that it is much more prominent in the equatorial region of the head and excluded from the posterior region of the head where staining is otherwise strongest.

Fig. 1 *c* shows the staining pattern of unfixed ejaculated sperm labeled with intact ESA152 followed by rhodamine-conjugated intact rabbit anti-mouse IgG. Under these conditions, the distribution of stain is similar to that observed in Fig. 1 *b*, except that when both antibodies are bivalent there is considerable patching of the antigen–antibody complexes.

Neither the intensity nor the distribution of ESA152 staining was measurably reduced by pretreatment of sperm with proteolytic enzymes, as described above. Pre-labeling of sperm with a variety of fluorescent lectins—concanavalin A (1 mg/ml), wheat germ agglutinin (0.8 mg/ml), soybean (1 mg/ml), *Dolichos biflorus* (1 mg/ml), *Ulex europius* Agglutin 1 (1 mg/ml), peanut agglutinin (1 mg/ml), *Ricinus communis* Agglutin 1 (1 mg/ml) (Vector Laboratories)—also had no effect on ESA152 staining intensity or distribution.

Table I. Diffusion of Fluorescein-conjugated F_{ab} Fragments of ESA152 on Ejaculated Ram Spermatozoa

	D × 10^9 s/cm^2	%R	*n*
ah	1.31 ± 0.21	50 ± 3	34
ph	1.03 ± 0.19	51 ± 3	28
m*	6.98 ± 1.63	28 ± 3	16
t*	2.57 ± 0.21	54 ± 3	24

Values are means ± standard error of the mean. ah, anterior region of the head; ph, posterior region of the head; m, midpiece; t, tail. *n*, number of measurements.

* Results were similar for the midpiece and tails using either spot or line bleaching. The particular values reported here are from line bleaching.

Table II. Diffusion of Intact ESA152 on Ejaculated Ram Spermatozoa

	D × 10^9 s/cm^2	%R	*n*
ah	4.1 ± 0.6	61 ± 2	29
ph	NP	NP	NP
m	2.0 ± 0.2	50 ± 3	27
t	2.7 ± 0.3	66 ± 3	29

Values are means ± standard error of the mean. ah, anterior region of the head; ph, posterior region of the head; m, midpiece; t, tail. NP, not present. *n*, number of measurements.

FPR Measurements

Tables I and II show the results of FPR measurements of the lateral diffusion of ESA152 on the different regions of ejaculated ram sperm labeled either directly, using the fluorescein-conjugated F_{ab} fragment of ESA152, or indirectly, using intact antibody followed by rhodamine-conjugated F_{ab} fragments of a goat anti-mouse IgG. Table III shows by Student's *t* test the interregional comparisons as well as comparison between monovalent and intact ESA152 measurements. Where the monovalent label is used ~50% of the antigen is free to diffuse on all regions, except for the midpiece where only 28 ± 3% is free to diffuse. The diffusion rate is the same ~1 × 10^{-9} cm^2/s on the two regions of the head, but it is faster on the midpiece (~7.0 × 10^{-9} cm^2/s) and tail (~2.6 × 10^{-9} cm^2/s) (see Table I). Thus, diffusion of ESA152 differs on the morphologically distinct regions of the sperm surface. Use of intact ESA152 antibody alters the diffusion as well as the distribution of the antigen. Most significant are: an increase in both the extent (to 61 ± 2%) and rate (to ~4 × 10^{-9} cm^2/s) of diffusion on the anterior region of the head, and a striking increase in the extent of diffusion (to 50 ± 3%)

Table III. Student's t Test Comparisons for ESA152

	D				%R			
	ah	ph	m	t	ah	ph	m	t
ah	<0.0005	NS	≤0.0005	≤0.0005	<0.0005	NS	≤0.0005	NS
ph	NP	NP	≤0.0005	≤0.0005	NP	NP	≤0.0005	NS
m	<0.0005	NP	≤0.005	≤0.0005	<0.005	NP	<0.0005	≤0.0005
t	<0.025	NP	<0.05	NS	NS	NP	<0.0005	<0.005

P values above the diagonal test interregional comparisons with the fluorescein F_{ab} direct stain (Table I). *P* values below the diagonal test interregional comparisons with the intact antibody (Table II). Diagonal values compare the same region with the two staining procedures. NP, not present. NS, not significant (*P* value ≥0.01). ah, anterior region of the head; ph, posterior region of the head; m, midpiece; t, tail.

coupled with a decrease in the rate (to ~2.0×10^{-9} cm^2/s) on the midpiece (see Table II).

Diffusion is completely arrested when sperm are treated with 5% glutaraldehyde at pH 7.4 for 1 h on ice before labeling. Furthermore, the fluorescein-labeled F_{ab}s are not measurably competed off by incubation with excess intact antibody (ascites fluid) after labeling. These two controls demonstrate that the results evaluate lateral diffusion of the membrane antigen rather than hopping of the F_{ab} (51) by rapid association–dissociation at the surface.

Discussion

The observation that ESA152 is present over the entire surface of ejaculated ram sperm, puts us in a position to extend the studies of Wolf and Voglmayr (59) to a membrane protein, and invites comparison with the work of Myles et al. (31) on a highly regionalized guinea pig sperm membrane protein, PT-1. Our diffusion measurements show that, as was the case for the lipid analogue, C_{16diI} (59), ESA152 exhibits different diffusibility (both D and %R) between the morphologically distinct surface regions. The diffusion rates for ESA152 are in the range 10^{-8}–10^{-9} cm^2/s. Significant nondiffusing fractions were observed on all surface regions. On other cell types (for review see reference 35) membrane proteins show diffusion rates $\leq 10^{-10}$ cm^2/s with significant immobile fractions, while lipids show diffusion rates of 10^{-8}–10^{-9} cm^2/s with complete diffusion. Fluid dynamic calculations of membrane protein diffusion do not predict immobile fractions and predict lipid-like diffusion rates (36, 49, 50). Thus, in most cell plasma membranes, membrane protein diffusion is not lipid fluidity limited. In some cases, disruption of membrane cell contact by blebbing causes membrane proteins to diffuse at this limit and to diffuse completely (44, 60). Thus other factors, possibly interactions with cyto and/or exoskeletons, control membrane protein diffusion. ESA152 is similar to PT-1 in that its diffusion rate is at or near this fluid dynamic limit. It is dissimilar in that it exhibits large nondiffusing fractions. Further characterization of this antigen and the nature of the epitope recognized may enable us to biochemically distinguish the diffusible and nondiffusible fractions.

The redistributions and alterations in diffusibility induced by intact ESA152 are interesting for a number of reasons. Redistribution of antigen from the posterior head to the anterior head and/or midpiece is reminiscent of ligand-induced capping (41, 45). Two differences however, must be considered. First, this redistribution occurs in the presence of azide, while capping, in general, does not. Second, when the second antibody is monovalent the fluorescence redistribution occurs without first patching. Such is uncommon in capping phenomena. A notable exception to this is capping of the artificial lipopolysaccharide stearoyl dextran on T lymphocytes (57). One can imagine several mechanisms that would lead to such redistribution in the absence of metabolic energy and patching, such as: a breakdown of an interregional barrier, lateral phase segregations induced by cross-linking of a glycolipid or glycoprotein component, and cytoskeletal rearrangements induced by cross-linking a surface receptor. This induced redistribution is probably most significant phenomenologically, in that it demonstrates that a stage-specific surface component, potentially with a receptor role, can be redistributed upon interaction with a ligand.

It is also interesting to note that unlike previously studied capping systems, where capping results in nearly complete immobilization of receptor (10, 54, 56), ESA152 after redistribution from the posterior head diffuses more rapidly and completely on both the anterior region of the head and the midpiece. Evidently, in this case, redistribution does not require immobilization.

These studies raise a number of questions about the ESA152 antigen, its diffusion, and regionalization which we are currently pursuing: (*a*) Since ESA152 is present on the surface of mature spermatozoa, but wholly absent from testicular spermatozoa, it is reasonable to ask whether it is secreted by the epithelia of the epididymis, and inserted from the luminal fluid of the epididymis (9, 47, 48). (*b*) Does the mechanism of its appearance account for its relatively high hydrophobicity and lipid-like diffusion rates? (*c*) Do the diffusible and nondiffusible fractions represent structurally distinct populations of ESA152? (*d*) Are there natural conditions that result in the redistribution of ESA152, such as hyperactivation, the acrosome reaction, or capacitation? (*e*) Do arrays of intramembranous particles by virtue of particle proximity induce anisotropies in diffusion within a region or restrictions to diffusion between regions? (*f*) Does antibody-induced cross-linking or redistribution of ESA152 affect physical properties of the membrane such as the diffusion of lipid analogues or intramembranous particle distribution or physiological properties of the sperm such as flagellar wave pattern or acrosomal fusion capacity?

Underlying these questions about ESA152 distribution and diffusion are two questions common to all membranes and membrane components: what is the physical basis for immobilization of a membrane component?; and, what is the physical relationship between mobile and immobile fractions?

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