Purification and Characterization of Two Plasma Membrane Domains from Ejaculated Bull Spermatozoa

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Abstract. Plasma membranes were detached from ejaculated bull spermatozoa by a brief sonication in a moderately hypotonic medium, and the released plasma membranes were partially purified by differential centrifugation. The resulting fraction was enriched 8- and 15-fold in alkaline phosphatase and 5' nucleotidase activities, respectively, compared with the starting sonicated spermatozoa. This total plasma membrane fraction was separated into two distinct fractions by equilibrium density centrifugation on a continuous linear sucrose gradient. Two peaks of light scattering material were formed at densities of 1.117 and 1.148 g/ml. The denser peak contained most of the protein of the plasma membrane fraction, whereas nearly all the concanavalin A binding activity was found in the lighter peak. The two bands had distinctly different polypeptide compositions when analyzed by SDS PAGE. Polyclonal antibodies were raised in rabbits against a major integral membrane glycoprotein of each fraction (Mr of 92,000 in the light peak and 98,000 in the dense peak). The two antigens were detected on the surface of intact spermatozoa by indirect immunofluorescence microscopy. The 92-kD protein (present in the lighter band) was detected only on the plasma membrane of the acrosomal and anterior postacrosomal regions of the head. The 98-kD antigen, present in the heavier band, was localized to the surface of the postacrosomal region of the head, to the principal piece of the tail, and to the connecting piece between the head and tail. The exclusive localization of the 92-kD polypeptide to the surface of the anterior portion of the head was confirmed by immunoelectron microscopy. These data show that the two fractions isolated on the sucrose gradient originate from different regions of the sperm cell plasma membrane.

The mammalian spermatozoon is a highly polarized cell that has several distinct morphological regions. Within the head, two distinct portions are represented by the acrosomal and postacrosomal regions, whereas the tail is characterized by an anterior portion, which contains mitochondria (the midpiece), and a posterior region, which comprises the principal and end pieces (for a review, see Fawcett, 1975). A corresponding regional diversification of the sperm cell surface has been revealed by a variety of techniques, including freeze-fracture (Friend, 1982), lectin binding (Kinsey and Koehler, 1976; Nicolson et al., 1977; Koehler, 1978; Aguas and da Silva, 1983), lipid probe binding (Bearer and Friend, 1982), and immunolocalization (Myles et al., 1981; Gaunt et al., 1983; Primakoff and Myles, 1983; Naz et al., 1984).

Although the presence of different domains on the surface of the mammalian sperm cell is well established, the degree of compositional overlap between them is not known. To understand how these domains are generated and maintained, it is important to know the composition of the different domains.

Plasma membrane (PM)1-enriched fractions from mammalian spermatozoa have been obtained and characterized (Gillis et al., 1978; Peterson et al., 1980; Noland et al., 1983; Russell et al., 1983). However, the separation and compositional analysis of the different domains has not been reported so far. In this study, we report the separation from ejaculated bull spermatozoa of two membrane fractions that contain vesicles with distinct ultrastructure and polypeptide composition. Major polypeptides of each fraction are localized within different regions of the cell surface of intact sperm, which indicates that the fractions contain membranes that originate from different domains of the spermatozoon surface.

Materials and Methods

Materials

The following reagents were purchased from the following sources: Hanks' solution, Difco Laboratories Inc., Detroit, MI; iodoacetamide, DL-dithiothreitol, benzamidine, β-glycerophosphate, wheat germ agglutinin, concanavalin A (Con A), N-benzoyl-L-arginineethylester-HCl, p-nitrocatechol sulfate, poly-L-lysine, L-cysteine sulfonic acid, 2,6-dichlorophenol-indophenol, phenazine methosulfate, microcopy; HTB, hypotonic Tris buffer; OAM, outer acrosomal membrane; P92, 92-kD polypeptide; P98, 98-kD polypeptide; PM, plasma membrane; TBS, Tris-buffered saline; TS, total sonicate.
pellets (45,000 rpm, 1 h, Beckman 50 Ti rotor), and then resuspended in small volumes of water.

24,000 rpm. 1.3-ml fractions were collected with an Auto Densiflow probe (Buchler Instruments Inc., Fort Lee, N J) connected to a peristaltic pump. 1.3-ml fractions were collected from the column, and 0.1-ml aliquots of the fractions were assayed for acrosin activity by following the hydrolysis of N-benzoyl-L-arginineethylester in 0.5 ml of 50 mM CaCl2, 50 mM Tris-HCl, pH 8.5. The reaction was started by addition of 2.5 ml of a 100 mM solution of the substrate, and ultraviolet absorption at 253 nm was monitored continuously on a double beam spectrophotometer.

For SDS PAGE, samples were solubilized by addition of 2 vol of a solution that contained 95 mM dithiothreitol, 6.7% SDS, 0.005% bromophenol blue, 0.52 M sucrose, 0.3 M Tris-HCl, pH 8.9, boil for 2 min and then alkylated with a 10-fold excess of iodoacetamide. SDS PAGE was done essentially as described by Maizel (1971) on 8-15% gradients or 8% polyacrylamide slabs, 1.5-mm thick. After electrophoresis, gels were stained with silver nitrate and Coomassie Brilliant Blue, and the polypeptides of interest were excised from the gel and alkylated with 20 ml of 0.4 M iodoacetamide. The gel slices were then subjected to digestion with trypsin or trypsin-like proteases.

Preparation of Antibodies

Glycoprotein Identification with 125 I-labeled Con A

Glycoprotein Identification with 125 I-labeled Con A

Electrophoretic transfer of polypeptide to nitrocellulose and radioimmuno-
(1963). Gels were washed for a few days with several changes of incubation buffer. The last washes were without BSA. Gels were then dried and exposed to Kodak-Ormat AR films. The specificity of Con A binding to sugar residues was determined by including L-o-methyl-a-D-glucopyranoside in the Con A incubation mixture. For a quantitative analysis, bands from autoradiograms were cut and treated for a spectrophotometric quantitation, according to Suisa (1983).

Preparation of Protein–Colloidal Gold Conjugates

Colloidal gold particles of ~5-nm diam were prepared according to Faulk and Taylor (1971). Gold–protein A conjugation was done by the method of Slot and Geuze (1981), and Con A was conjugated by the method of Horisberger and Rosset (1977).

Immunolocalization of Sperm Surface Polypeptides

2–4 × 10^9 spermatozoa were pelleted after dilution of fresh ejaculates with cold Hanks’ solution and centrifugation for 5 min at 350 g. Cells were washed twice with Hanks’ and sedimented as above. The final pellet was suspended in freshly prepared 3% paraformaldehyde, 0.2% glutaraldehyde, 0.12 M phosphate buffer, pH 7.4 (for immuno electron microscopy), or in 3% paraformaldehyde alone in the same buffer (for indirect immunofluorescence), and fixed for 1 h at 0°C. The fixed cells were rinsed twice with 0.12 M phosphate buffer.

For indirect immunofluorescence, the fixed and washed cells were resuspended in 0.12 M Tris-HCl, pH 7.4, and drops of the suspension were deposited on polylysine-covered glass slides for 30 min, to allow the spermatozoa to adhere to the substrate. Slides were then rinsed once with Tris-HCl buffer and twice with 0.12 M glycine-NaOH, pH 7.4. After incubation for 30 min in 0.5 M NaCl, 5% BSA, 0.02 M Na+ phosphate buffer, pH 7.4, the slides were covered with the same buffer that contained different dilutions of Ig fractions prepared from immune and preimmune rabbit sera, and incubated for 2 h at room temperature. They were then washed five times with 0.5 M NaCl, 0.02 M phosphate buffer, pH 7.4, and incubated for 90 min with goat anti-rabbit rhodaminated IgG diluted 1:60 in chicken egg albumin buffer. The slides were then washed thoroughly with 0.5 M NaCl, 0.02 M phosphate buffer, pH 7.4, and finally with 5 mM phosphate buffer, pH 7.4. Slides were mounted and observed with a Zeiss Photomicroscope III (Carl Zeiss, Oberkochen, FRG).

For immunolocalization at the electron microscope (EM) level, cells were first embedded in agarose blocks (De Camilli et al., 1983). After two washes with 0.12 M glycine-NaOH, pH 7.4, small agarose pieces were incubated overnight at 4°C in the presence of Ig in 0.5 M NaCl, 5% BSA, 0.02 M Na+ phosphate buffer, pH 7.4. The agarose blocks were then washed in 0.5 M NaCl, 20 mM phosphate buffer, pH 7.4, for 3 h (five changes). After incubation for 2 h with 0.3 ml of gold–protein A properly diluted in PBS that contained 0.5% BSA, blocks were rinsed twice with PBS-0.5% BSA and three times with PBS. The samples were then processed for EM (see below).

Localization of Con A Binding Sites on the Sperm Surface

6 × 10^7 spermatozoa, prepared as described under Cell Preparation, were incubated with 1 ml of gold–Con A complex (0.8 optical density at 520 nm) in TBS for 1 h at 4°C. Control samples were incubated with gold–Con A premixed with 0.1 M L-o-methyl-a-D-glucopyranoside. After the cells were washed three times in TBS, they were fixed and processed for EM (see below).

Electron Microscopy

Cells, subcellular fractions, or agarose blocks were fixed in suspension in ice-cold 2% glutaraldehyde, 0.12 M cacodylate buffer, pH 7.4 for 1–2 h. Samples were then sedimented into pellets, which were washed and postfixed with ice-cold 1.5% osmium tetroxide, 0.12 M cacodylate buffer, pH 7.4, for 1 h. Block staining was in 0.5% uranyl acetate in Veronal buffer, pH 6.0. Dehydration was followed by embedding in Epon 812. Oriented, thin sections were cut on a Reichert Ultracut (C. Reichert AG, Vienna, Austria), stained with uranyl acetate and lead citrate, and examined with a Philips 400 electron microscope (Philips Industries, Eindhoven, The Netherlands).

Results

Preparation and Characterization of a PM-enriched Fraction from Bull Spermatozoa

To optimize conditions of cell disruption that lead to the most efficient and selective detachment of the PM from bull spermatozoa, the distribution of traditional PM marker enzymes between a supernate and a low speed pellet (500 g, 10 min) was determined. The best disruption condition was 3 s of sonication (at a power of 50 W) of the cells suspended in a moderately hypotonic buffered solution (HTB). Under these conditions, ~25% and 40% of the PM marker enzymes alkaline phosphatase and 5′ nucleotidase were recovered in the low speed supernate, respectively, whereas only ~5% of the total protein was released (Table I). Other enzyme activities were measured to test for the release of other cell components. Release of succinate dehydrogenase, a mitochondrial enzyme, was not detected (data not shown), which indicates that mitochondrial fragments had not been released to any great extent. 80% of the arylsulfatase, a marker of acrosomal content, was released to the supernate, which showed that the acrosomal membrane became leaky. Only ~5% of the acrosin activity (measured in one experiment, in which the inhibitor benzamidine was omitted from the buffers) was released (data not shown). This enzyme is known to be difficult to solubilize and is thought to be in part associated with the inner acrosomal membrane (Morton, 1976). The behavior of acid phosphatase, also believed to be a marker for acrosomal content, was intermediate to that of the other two acrosomal enzymes (~19% release).

The disruption procedure seemed to preferentially release the PM, also on the basis of morphological criteria. Fig. 1

![Table I. Distribution of Protein and Enzyme Activities Between Bull Spermatozoon Subcellular Fractions*](http://rupress.org/jcb/article-pdf/102/5/1813/1053225/1813.pdf)

| Fraction from Bull Spermatozoa | Protein (17)* | Alkaline phosphatase (10) | 5′ Nucleotidase (5) | Arylsulfatase (3) | Acid phosphatase (2) |
|-------------------------------|--------------|---------------------------|-------------------|-----------------|-------------------|
| Retained with cells after sonication | 94.7 ± 3.0 | 74.6 ± 2.7 | 62.9 ± 2.5 | 19.0 ± 1.4 | 81.4 ± 10.5 |
| Released by sonication | 6,000 g pellet | 1.3 ± 0.1 | 2.8 ± 0.3 | 7.0 ± 1.4 | 0.1 ± 0.1 | 3.6 ± 1.4 |
| | PM | 1.5 ± 0.1 | 11.0 ± 0.9 | 20.6 ± 1.5 | 0.2 ± 0.2 | 11.3 ± 4.5 |
| | 100,000 g supernate | 2.5 ± 0.3 | 11.3 ± 1.8 | 10.4 ± 2.3 | 80.7 ± 5.4 | 3.7 ± 3.6 |

* Values given are percentage of recovered constituent in subcellular fractions ± SE. Recoveries in the sum of the fractions of TS constituents were: protein, 94.3 ± 3.0%; alkaline phosphatase, 86.3 ± 4.8%; 5′ nucleotidase, 101.5 ± 4.8%; arylsulfatase, 86.7 ± 4.8%; acid phosphatase, 103.4 ± 11.1%. The protein content of TS was 7.6 ± 0.6 mg/10^10 spermatozoa in the unfraccionated ejaculate. Enzyme activities in the TS (in nmol product formed/min per 10^10 spermatozoa in the unfraccionated ejaculate) were: alkaline phosphatase, 47.7 ± 8.6; 5′ nucleotidase, 12.0 ± 3.5; arylsulfatase, 112,150 ± 9,140; acid phosphatase, 22.6 ± 12.1. 35.2 ± 4.9% of the spermatozoa of the fresh ejaculate were recovered in the TS (average of 10 experiments).

| Numbers in parentheses indicate the number of experiments. |
illustrates the ultrastructure of spermatozoa at various stages of the disruption procedure. Cells fixed in isotonic solution showed closely apposed PM and outer acrosomal membrane (OAM) (Fig. 1 a). Exposure of the cells to hypotonic medium resulted in a swelling of the space between PM and OAM, but most of the cells retained their PM (Fig. 1 b). A 3-s sonication resulted in detachment of PM from many of the cells, swelling and clearing of the acrosome, but retention of the acrosomal membrane (Fig. 1, c and d). 120 profiles for each sperm region were counted. The PM was absent from the acrosomal region of 74% of the profiles, from the postacrosomal region of 54% of the profiles, from 64% of the observed profiles of the principal piece of the tail, and from 25% of the tail midpiece profiles. The inner acrosomal membrane remained attached to the cell in 100% of the profiles after sonication, and 95% of the observed head profiles also had the OAM. Unfortunately, the low release of OAM could not be confirmed biochemically, because of the lack of well-
established marker enzymes for the acrosomal membranes. A fraction enriched in PM was obtained by pelleting membranes from the low speed supernate obtained from the sonicated cells. Table I shows how various marker enzymes were distributed during the fractionation procedure. The relative specific activities of marker enzymes in the PM fraction can be derived from the data of the table, by dividing the enzyme recovery values by the protein recovery value (1.5%). Thus, the PM fraction contained ~10 and 20% of the total alkaline phosphatase and 5’ nucleotidase, respectively, with relative specific activities of ~7 and 14. The higher recovery of 5’ nucleotidase than of alkaline phosphatase in the PM fraction can be explained by the localization of alkaline phosphatase to other membranes besides the PM (Gordon, 1973). Alternatively, the two enzymes could be localized on different regions of the PM, which were released with different efficiencies by the sonication procedure. Arylsulfatase, a marker of acrosomal contents, was recovered largely in the high speed supernatant. Unexpectedly, 11% of acid phosphatase was found in the PM fraction and had a relative specific activity of 7.5.

Examination of the fractions by EM showed that the low

Figure 2. Electron micrographs of the PM fraction and subfractions thereof. (a) PM fraction. A mixed population of round-shaped and flattened vesicles is present. After equilibration on a continuous sucrose gradient, the PM fraction gives rise to two distinct peaks (b–d). (b) Peak II, which equilibrates at $\rho = 1.148$, is composed primarily of round-shaped vesicles. (c) Peak I, which equilibrates at $\rho = 1.117$, is enriched in flattened structures. (d) Enlargement of the area framed in c: flattened single-walled (arrowheads) or double-walled (arrows) vesicles are seen. Double-walled spherical vesicles (asterisks) are also characteristic of this membrane fraction. Bars: (a, b, and c) 2 µm; (d) 0.4 µm.
speed pellet contained the head and the midpiece of the sperm cells, the 6,000 g pellet was enriched in fragments from the principal piece of the tail, whereas the 100,000 g supernate contained small membrane fragments (micrographs not shown). These membrane fragments could explain the presence of PM marker enzymes in the 100,000 g supernate (Table I). The PM fraction (Fig. 2 a) was composed of a heterogeneous population of round-shaped vesicles and flattened membranous structures.

The PM fraction was enriched in polypeptides that were barely visible or not detectable at all in the TS (Fig. 3A). To determine which of these polypeptides might be integral membrane proteins, the PM fraction was extracted with 0.1 M Na₂CO₃, pH 11, and the polypeptide compositions of the membrane pellet and the alkaline extract were analyzed by SDS PAGE (Fig. 4). Only low molecular weight polypeptides (<21 kD) and some material not entering the gel were extracted by the alkaline treatment. The major polypeptides, with \( M_r > 21,000 \) in the PM fraction, are therefore candidates for integral membrane proteins. These polypeptides also partitioned into the detergent phase of Triton X-114 after phase separation (not shown), which also indicates that they are integral membrane proteins (Bordier, 1981).

**Subfractionation of PM Vesicles**

After equilibrium density centrifugation of the PM fraction on a linear sucrose gradient, two bands of light-scattering material could be seen (Fig. 5). Stars and asterisks show the positions of the two peaks in Fig. 6. A minor peak (I) equilibrated at 1.117 g/ml and a major peak (II) equilibrated at a density of 1.148 g/ml. Most of the protein of the gradient fractions (Fig. 6a) was contained in the major peak (II).

Alkaline and acid phosphatase activities (Fig. 6, b and c) were enriched in the major peak 1.5-fold with respect to the starting PM fraction. The distribution of 5' nucleotidase could not be determined, because the activity was partially lost after the
sucrose gradient centrifugation. The morphologically heterogeneous structures in the total PM fraction were separated into two distinct sets of vesicles on the sucrose gradient (Fig. 2). The higher density peak (II) contained mostly spherical vesicles (Fig. 2b), and the lower density peak (I) was enriched in flattened membranous structures (Fig. 2c).

Peaks I and II displayed very different polypeptide compositions (Fig. 3c). Most of the major polypeptides of the PM fraction were present in either one or the other of the two peaks of the gradient. In particular, the 92-, 73-, and 48-kD bands as well as the carbonate extractable 17-kD polypeptide were exclusive to peak I, whereas peak II contained the 98-, 66-, 42-, and 21-kD polypeptides.

Immunocytochemical Localization of Polypeptides of the Two Membrane Subfractions

To determine the origin of the two membrane fractions, polyclonal antibodies against major polypeptides exclusive to each of the fractions were raised in rabbits and used for immunolocalization studies. Antibodies were raised either against the 92-kD polypeptide or the 98-kD polypeptide (P92 and P98), characteristic of peaks I and II, respectively (see Fig. 3). The specificities of the antibodies for the immunogen were demonstrated by immunoblotting. When tested against the total M fraction, each antiserum reacted only with the polypeptide that had been used as immunogen (Fig. 7). Additional antigens were not revealed when the antisera were tested against TS (data not shown). The antibodies were then used to immunolocalize the two membrane antigens on intact ejaculated bull spermatozoa. Fig. 8 shows that the 92-kD antigen was localized on the anterior portion of the head region, which includes the acrosomal and part of the postacrosomal region. Immunolocalization at the EM level con-
figure 7. Radioimmunostaining of electroblotted PM proteins with anti-P92 and anti-P98 antibodies. The total PM fraction (50 μg of protein/lane) was run on a 8% gel and then transferred to a nitrocellulose filter. Strips were incubated with the following Ig preparations: lane A, anti-P92 (0.02 mg/ml); lane B, preimmune Ig from the same rabbit as in A (0.02 mg/ml); lane C, anti-P98 (0.05 mg/ml); lane D, preimmune Ig from same rabbit as in C (0.05 mg/ml). Bound antibodies were revealed with 125I-protein A. The molecular weight (× 10^3) of a standard is indicated on the left.

firmed the immunofluorescence results (Fig. 8, e–i). P92 was found on the PM, delimiting the acrosomal region and the anterior part of the postacrosomal region, whereas labeling was low or completely absent on the rest of the cell surface. During the preparation for immunolocalization, cells occasionally lost their PM, exposing a ruptured OAM. However, no labeling of the OAM was ever observed (micrographs not shown). Thus, P92 was a component of the PM. Its localization to the acrosomal region indicated that the membranes in peak I came from the PM of the acrosomal region.

The distribution of P98, enriched in peak II, was more complex (Fig. 9). Surface immunofluorescence localized this polypeptide to the surface of the postacrosomal region and on the principal piece of the tail (Fig. 9). A bright fluorescent spot was also present on the connecting piece, between the neck region of the head and the midpiece of the tail. This region was also weakly positive with preimmune Igbs (Fig. 9 d). Postacrosomal region of the head, connecting piece, and principal piece of the tail also gave a positive signal with an antiserum raised against the 42-kD polypeptide of peak II (results not shown). Thus, these three noncontiguous regions may have these two polypeptides in common.

Discussion

The mammalian spermatozoon is one of the most striking examples of a polarized cell. In this study, we have separated and characterized two PM subfractions from bull spermatozoa, and presented evidence that they derive from different domains of the cell surface.

A very light sonication under moderately hypotonic conditions was found to be the most efficient and selective means to release the PM from the sperm. 38% of the activity of the traditional PM marker enzyme, 5' nucleotidase, was released into the low speed supernate. 8-fold and 15-fold enrichments with respect to the TS were obtained, respectively, for alkaline phosphatase and 5' nucleotidase activities in the final PM fraction, with a 10–20% recovery of their respective total activities. About 95% of the cells retained their OAM after this treatment. Thus, the membranes of the PM fraction were probably derived from the cell surface, with little contamination by OAM and mitochondria.

Nearly all the major polypeptides of the PM fraction were resistant to alkali extraction and therefore are likely to be integral membrane proteins. Only a group of low molecular weight components (M_r < 21,000) were extracted by the pH 11 treatment. Russell et al. (1983) have described a group of polypeptides in that molecular weight range in the boar, which they believe to be peripheral components of the membrane contributed by the seminal plasma. Other more loosely bound peripheral components may have been lost during our fractionation procedure.

When the PM fraction was subjected to density equilibrium centrifugation on a continuous linear sucrose gradient, it separated into two peaks: the lower density peak contained a small fraction of the total protein, but nearly all the Con A binding activity of the starting PM fraction, and the higher density peak contained most of the protein and phosphatase
Figure 8. Immunolocalization of the 92-kD polypeptide of peak 1. (a and c) Immunofluorescence images obtained with anti-P92 and preimmune Ig (both at 0.2 mg/ml), respectively. (b and d) Phase contrast images of the same fields shown in a and c, respectively. (e–i) Protein A–gold ultrastructural immunolocalization of P92. Cells were incubated with anti-P92 at a concentration of 1 mg/ml. Immunoreactivity is restricted to the PM of the acrosomal (e) and of the anterior part of the postacrosomal region (f). Immunoreactivity is almost undetectable on the plasma membrane of the posterior part of the postacrosomal region (g) and on the midpiece (h) and principal piece (i) of the tail. Bars: (a–d) 25 μm; (e–i) 0.2 μm.
Figure 9. Immunolocalization of the 98-kD polypeptide of peak II. (a and c) Immunofluorescence images obtained with anti-p98 at a concentration of 0.7 mg/ml. Immunofluorescence is restricted to the posterior part of the head, and to the connecting (arrows) and principal pieces of the tail. It is absent from the midpiece. At higher magnification (c), it can be seen that immunofluorescence on the principal piece of the tail is more intense on the borders, as expected for a PM antigen. Differences in the intensity of immunofluorescence on the positive segment are due to out-of-focus effects. (d) Immunofluorescence image obtained with preimmune Ig (0.7 mg/ml). (b and e) Phase contrast images of the same fields shown in a and d, respectively. Arrows indicate the connecting piece. Bars, 20 μm.

activities of the PM fraction. Ultrastructural analysis revealed that the two peaks were composed of different kinds of vesicles. Flattened and/or double-walled vesicles were enriched in peak I, whereas spherical vesicles bounded by single bilayers were concentrated in peak II. Striking differences between the polypeptide compositions of the two peaks were also observed. Each band contained specific polypeptides that were either absent or very poorly represented in the other one.

Having obtained the separation of two different membrane populations, the problem was to determine whether both of them were derived from the PM, and if so, from which regions. Monospecific, polyclonal antibodies raised to specific polypeptides of each fraction demonstrated that peak I contains membranes that derive from the PM of the anterior portion of the head of the spermatozoon. This conclusion was confirmed by the observation that Con A receptors, which were shown by EM localization studies to be concentrated at the surface of the anterior portion of the head, were highly enriched in peak I. In the case of peak II, antibodies against two of its major polypeptides stained the surface of the sperm cell, which demonstrates that this fraction also was derived from the cell surface. However, because of the triple localization of the two antigens, to the posterior portion of the head, the principal piece of the tail, and the connecting piece, we cannot decide whether the vesicles in peak II derive from all these three regions, or only from one or two of them. Because these three regions appear to share two antigens, one wonders whether they have identical polypeptide composition and derive from the same PM during biogenesis. However, in other species, these regions show differences in antigen composition (Myles et al., 1981). The relationship between these three surface regions in the bull remains to be defined by further studies.

Previous studies have been published on mammalian sperm cell PM isolation and characterization by other authors (Gillis et al., 1978; Peterson et al., 1980; Noland et al., 1983; Russell et al., 1983). However, until the present, the separation of vesicle populations that derive from different domains of the cell surface had not been reported. Noland et al. (1983) reported the separation of two membrane peaks from bovine epididymal sperm disrupted by nitrogen cavitation, but con-
cluded that one of the peaks derived from cytoplasmic droplets. The ejaculated spermatozoa we used, purified on a Percoll gradient, were devoid of cytoplasmic droplets, as assessed by phase-contrast microscopy. The most extensive biochemical characterization of mammalian sperm cell PM has been done on the boar (Russell et al., 1983). 20 major polypeptides were detected on one-dimensional gels with Coomassie Blue staining, in good agreement with our SDS PAGE pattern of the PM fraction. Two-dimensional gels, however, revealed at least 250 spots. We do not know whether our PM fraction would also reveal such a complex pattern with a more refined analysis.

The most striking finding of this study is the distinctly different polypeptide composition of two membrane fractions derived from the surface of the same cell. This different composition could be so clearly demonstrated because of the excellent separation between the two fractions, thanks to the homogeneous physical properties of each vesicle population. How domains with such different molecular components are generated and maintained remains a mystery. The problem of the generation of membrane domains in polarized cells has been investigated most intensely in epithelial cells, where it has been found that newly synthesized surface molecules characteristic of the basolateral or apical regions are delivered directly to their respective domains (Simons and Fuller, 1985). In epithelial cells, tight junctions have also been implicated in the maintenance of distinct membrane domains (Gumbiner and Louvard, 1985). Sperm cells, of course, do not have any intercellular junctions, and the definition of the different domains may thus be more complex. For example, it has generally been thought that acrosomal and postacrosomal regions represent two domains, and that some barrier to
protein diffusion might exist between these two regions. However, in the present study it was found that P92 extended over the entire acrosomal region and to part of the postacrosomal region of the head, which suggests that an additional barrier between P92 and antigens characteristic of the postacrosomal region remains to be defined by double-labeling at the EM level.

A recent study (Virtanen et al., 1984) on the cytoskeletal structure of human sperm cells has shown the presence of distinct cytoskeletal domains, which seem to correspond well to the surface regions observed in this and other studies (Koehler. 1978; Myles et al., 1981; Bearer and Friend. 1982; Eddy and Koehler, 1982, Gaunt et al., 1983; Wolf and Voglmayr. 1984). This suggests a role for the cytoskeleton in the organization of different membrane domains. The possibility of purifying and characterizing different PM fractions should represent a starting point for the study of the biogenesis of these domains as well as of the functions that their molecular constituents play in the biology of sperm maturation and sperm–egg interaction.

We are grateful to Pietro De Camilli, Barry Gumbiner, and Kai Simons for helpful suggestions, to Paolo Tinelli and Franco Crippa for photographic assistance, and to Joyce de Bruyn and Annie Steiner for typing this manuscript. We are also grateful to Dr. Marina Camatini of the Department of Biology of the University of Milan for her suggestions, which were very helpful especially during the initial part of this work, and in whose laboratory a part of the electron microscopy work was done.

Received for publication 4 November 1985, and in revised form 17 January 1986.

References

Agius, A. P., and P. Pinto da Silva. 1983. Regionalization of transmembrane glycoproteins in the plasma membrane of boar sperm head is revealed by fracture-label. J. Cell Biol. 97:1356–1364.

Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphates. Methods Enzymol. 8:115–118.

Appelmann, F., and C. de Duve. 1955. Tissue fractionation studies. III. Further observations on the binding of acid phosphatase by rat liver particles. Biochem. J. 59:426–433.

Arrighi, O., and T. P. Singer. 1962. Limitations of the phenazine methosulfate assay for succinic and related dehydrogenases. Nature (Lond). 193:1256–1258.

Bearer, E. L., and D. S. Friend. 1982. Modifications of anionic lipid domains preceding membrane fusion in guinea pig sperm. J. Cell Biol. 92:604–615.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114. J. BioL Chem. 256:1604–1607.

Borgese, N., D. Macconi, L. Parola, and G. Pietrini. 1982. Rat erythrocyte Na+K+-ATPase. Characterization and comparison between the membrane-bound and soluble forms using an antibody against the rat liver enzyme. J. Biol. Chem. 257:13854–13861.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

De Camilli, P., S. M. Harris, Jr., W. B. Hutton, and P. Greenberg. 1983. Synapsin I (protein 1), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agogose-embedded synaptosomes. J. Cell Biol. 96:1355–1373.

Eddy, E. M., and J. K. Koehler. 1982. Restricted domains of the sperm surface. Scanning Electron Microsoc. 3:1313–1322.

Fawcett, D. W. 1975. The mammalian spermatozoon. Dev. Biol. 44:394–436.

Faulk, W. P., and G. M. Taylor. 1971. An immunocollodiod method for the electron microscope. Immunochemistry. 8:1081–1083.

Friend, D. S. 1982. Plasma membrane diversity in a highly polarized cell. J. Cell Biol. 93:243–249.

Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. 93:97–102.

Gaunt, S. J., C. R. Brown, and R. Jones. 1983. Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. Exp. Cell Res. 144:275–284.

Gillis, G. R., Peterson, L. Russel, L. Hook, and M. Freund. 1978. Isolation and characterization of membrane vesicles from human and boar spermatozoa: methods using nitrogen cavitation and ionophore induced vesiculation. Prep. Biochem. 8:363–378.

Gordon, M. 1973. Localization of phosphate activity on the membranes of the mammalian sperm head. J. Exp. Zool. 185:111–120.

Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of hog kidney by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. 93:97–102.

Gaunt, S. J., C. R. Brown, and R. Jones. 1983. Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. Exp. Cell Res. 144:275–284.

Gillis, G. R., Peterson, L. Russel, L. Hook, and M. Freund. 1978. Isolation and characterization of membrane vesicles from human and boar spermatozoa: methods using nitrogen cavitation and ionophore induced vesiculation. Prep. Biochem. 8:363–378.

Gordon, M. 1973. Localization of phosphate activity on the membranes of the mammalian sperm head. J. Exp. Zool. 185:111–120.

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Gaunt, S. J., C. R. Brown, and R. Jones. 1983. Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. Exp. Cell Res. 144:275–284.

Gillis, G. R., Peterson, L. Russel, L. Hook, and M. Freund. 1978. Isolation and characterization of membrane vesicles from human and boar spermatozoa: methods using nitrogen cavitation and ionophore induced vesiculation. Prep. Biochem. 8:363–378.

Gordon, M. 1973. Localization of phosphate activity on the membranes of the mammalian sperm head. J. Exp. Zool. 185:111–120.

Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of hog kidney by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. 93:97–102.

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Gillis, G. R., Peterson, L. Russel, L. Hook, and M. Freund. 1978. Isolation and characterization of membrane vesicles from human and boar spermatozoa: methods using nitrogen cavitation and ionophore induced vesiculation. Prep. Biochem. 8:363–378.

Gordon, M. 1973. Localization of phosphate activity on the membranes of the mammalian sperm head. J. Exp. Zool. 185:111–120.

Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of hog kidney by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. 93:97–102.

Gaunt, S. J., C. R. Brown, and R. Jones. 1983. Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. Exp. Cell Res. 144:275–284.

Gillis, G. R., Peterson, L. Russel, L. Hook, and M. Freund. 1978. Isolation and characterization of membrane vesicles from human and boar spermatozoa: methods using nitrogen cavitation and ionophore induced vesiculation. Prep. Biochem. 8:363–378.

Gordon, M. 1973. Localization of phosphate activity on the membranes of the mammalian sperm head. J. Exp. Zool. 185:111–120.
Monoclonal antibody to a human germ cell membrane glycoprotein that inhibits fertilization. Science (Wash. DC). 225:342–344.

Nicolson, G. L., N. Usui, R. Yanagimachi, H. Yanagimachi, and J. H. Smith. 1977. Lectin binding sites on the plasma membrane of rabbit spermatozoa. Changes in surface receptors during epididymal maturation and after ejaculation. J. Cell Biol. 74:950–962.

Noland, T. D., G. E. Olson, and D. L. Garbers. 1983. Purification and partial characterization of plasma membranes from bovine spermatozoa. Biol. Reprod. 29:987–998.

Peterson, R., L. Russell, D. Bundman, and M. Freund. 1980. Evaluation of the purity of boar sperm plasma membranes prepared by nitrogen cavitation. Biol. Reprod. 23:637–645.

Primakoff, P., and D. G. Myles. 1983. A map of the guinea pig sperm surface constructed with monoclonal antibodies. Dev. Biol. 98:417–428.

Russell, L. D., R. N. Peterson, T. A. Russell, and W. Hunt. 1983. Electrophoretic map of boar sperm plasma membrane polypeptides and localization and fractionation of specific polypeptides subclasses. Biol. Reprod. 28:393–413.

Simons, K., and S. Fuller. 1985. Cell surface polarity in epithelia. Ann. Rev. Cell Biol. 1:295–340.

Sloot, J. W., and H. J. Geuze. 1981. Sizing of protein A-colloidal gold probes for immunoelectronmicroscopy. J. Cell Biol. 90:533–536.

Stanley, K. K., M. R. Edwards, and J. P. Luzio. 1980. Subcellular distribution and movement of 5′-nucleotidase in rat cells. Biochem. J. 186:59–69.

Suissa, M. 1983. Spectrophotometric quantitation of silver grains eluted from autoradiograms. Anal. Biochem. 133:511–514.

Switzer, R. C., III, C. R. Mervis, and S. Shifrin. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal. Biochem. 98:231–237.

Virtanen, J., R. A. Badley, R. Pasivuo, and V.-P. Lehto. 1984. Distinct cytoskeletal domains revealed in sperm cells. J. Cell Biol. 99:1083–1091.

Wolf, D. E., and J. K. Voglmayr. 1984. Diffusion and regionalization in membranes of maturing ram spermatozoa. J. Cell Biol. 98:1678–1684.

Yang, C. H., and P. N. Srivastava. 1974. Purification and properties of aryl sulfatases from rabbit sperm acrosomes. Proc. Soc. Exp. Biol. Med. 145:721–725.