Glycoconjugate Journal 17, 205–214, 2000

Co-localization of receptor and transducer proteins in the glycosphingolipid-enriched, low density, detergent-insoluble membrane fraction of sea urchin sperm

Kaoru Ohta[1], Chihiro Sato[1], Tsukasa Matsuda[1], Masaru Toriyama[2], Victor D. Vacquier[3], William J. Lennarz[4] and Ken Kitajima[1]*

[1]*Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan;* [2]*Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Ohya, Shizuoka 422–8529, Japan;* [3]*Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093–0202, U.S.A and* [4]*Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, New York 11794–5215, U.S.A*

The low density, detergent-insoluble membrane fraction (LD-DIM), where gangliosides are likely to be highly enriched, was prepared from sperm of two sea urchin species, *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus*. Immunoblotting showed the presence in the LD-DIM of two receptors for egg ligands, a glycosylphosphatidylinositol (GPI)-anchored protein, and four proteins which may be involved in signal transduction. Co-immunoprecipitation revealed that at least three proteins, the speract receptor, the 63 kDa GPI-anchored protein and the α subunit of a heterotrimeric Gs protein, are localized in the LD-DIM. This suggests that the LD-DIM fraction may be a membrane microdomain for speract–speract receptor interaction, as well as the subsequent signal transduction pathway involved in induction of sperm respiration, motility and possibly the acrosome reaction.

Keywords: **sea urchin, sperm, ganglioside, detergent-insoluble membrane, signal transduction, fertilization, acrosome reaction**

Abbreviations: **ASW, artificial sea water; GPI, glycosylphosphatidylinositol; GSL, glycosphingolipid; HPTLC, high performance thin layer chromatography; LD-DIM, low density detergent-insoluble membrane; PE, phosphatidylethanolamine; PVDF, polyvinylidene difluoride; REJ-1, receptor for egg jelly-1; SBP, sperm binding protein, SLF, sulfatide; $HSO_3(Neu5Ac)_2GlcCer$, HSO_3-8Neu5Acα2 → 8Neu5Acα2 → 6Glcβ1 → Cer; $(Neu5Ac)_2GlcCer$, Neu5Acα2 → 8Neu5Acα2 → 6Glcβ1 → Cer; HSO_3Neu5AcGlcCer, HSO_3-8Neu5Acα2 → 6Glcβ1 → Cer; Neu5AcGlcCer, Neu5Acα2 → 6Glcβ1 → Cer; GD3, Neu5Acα2 → 8Neu5Acα2 → 3Gal β1 → 4Glc**

Introduction

In somatic cells, the glycosphingolipid-enriched membrane domains of the plasma membrane are hot spots for signal transduction [1,2]. The significance of this membrane domain is believed to be that it localizes glycosylphosphatidylinositol (GPI)-anchored and membrane-spanning receptor proteins with transducer proteins [3–6]. This membrane domain can be isolated from various cells as a detergent-insoluble fraction having low buoyant density (the low density detergent-insoluble membrane, or LD-DIM) [7–13]. One interesting feature of this membrane domain is its enrichment in glycolipids in its outer surface [14]. Several lines of evidence show that glycosphingolipids (GSLs) in the LD-DIM are involved in cell adhesion in early development [15], metastasis [16,17] and signal transduction [11,18]. The LD-DIM may function in GSL-associated cell adhesion and recognition, as well as in signal transduction [11,15–18].

In gametic cells, the involvement of GSLs in cell–cell recognition, spermatogenesis, and fertilization has been

*To whom correspondence should be addressed (Fax: + 81-52-789-4128; Phone: + 81-52-789-4130; E-mail: kitajima@agr.nagoya-u.ac.jp)

suggested [19–23]. However, little attention has been paid to the possibility that the LD-DIM is a functional membrane domain for GSL-mediated signaling. Our hypothesis is that the LD-DIM of gametic cells acts as a site of GSL-mediated sperm-egg interaction and signal transduction. To test this hypothesis, we have begun studying the LD-DIM fraction of sea urchin spermatozoon. Recently, we have demonstrated the presence of a LD-DIM domain in sea urchin sperm and established its enrichment in GSLs [24]. In this study, to characterize the sperm LD-DIM as a site of sperm-egg interaction, as well as its possible role in signal transduction, we have determined which glycoproteins are localized in the LD-DIM. We have also carried out immunofluorescence observations of a major ganglioside in sperm using a monoclonal antibody to a sperm ganglioside.

Materials and methods

Materials

Hemicentrotus pulcherrimus were purchased from the local fisheries at Tsushima and Fukushima, Japan, and *Strongylocentrotus purpuratus* were purchased from Marinus (Long Beach, CA, U.S.A.). Sperm was collected by intracoelomic introduction of 0.5 M KCl. Gangliosides from *H. pulcherrimus sperm*, $HSO_3 - 8Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer ($HSO_3(Neu5Ac)_2GlcCer$), $Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer ($(Neu5Ac)_2GlcCer$), $HSO_3 - 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer ($HSO_3Neu5AcGlcCer$) and $Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer (Neu5AcGlcCer), were prepared according to the method of Ijuin *et al*. [22]. A mixture of oligo-*N*-acetylneuraminic acid conjugated with phosphatidylethanolamine, $(8Neu5Ac\alpha2 \rightarrow)_n - PE$, was prepared as described [25]. Authentic samples of $Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 3Gal\beta1 \rightarrow GlcCer$ (GD3) and $HSO_3 \rightarrow 3Gal\beta1 \rightarrow$ Cer (sulfatide, SLF) were purchased from Snow Brand (Japan) and Sigma (St. Louis, MO, U.S.A.). Monoclonal antibody (mAb.3G9), recognizing $HSO_3 \rightarrow 8Neu5Ac$ structure, was prepared as described [24]. Anti-80 kDa speract receptor (rabbit polyclonal IgG), anti-210 kDa receptor for egg jelly (REJ-1) ($J_{4/4}$, mouse monoclonal IgG2a), anti-63 kDa GPI-anchored protein ($J_{17/30}$, mouse monoclonal IgG1), anti-bindin antibody (rabbit polyclonal IgG) and anti-adenylate cyclase (rabbit polyclonal IgG) were prepared as described [26–30]. Anti-guanylate cyclase antibody (rabbit antiserum) that recognizes the *C*-terminal 24 amino acid residues (KPPPQKLTQEAIEIAANRVIPDDV) of the enzyme from *S. purpuratus* sperm [31], was kindly provided by Dr. Norio Suzuki (Hokkaido University). The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (U.S.A.): anti-Gsα/olf (C-18) (rabbit polyclonal IgG), anti-Rho B antibody (119) (rabbit polyclonal antibody), anti-caveolin-1 (N-20) (rabbit polyclonal IgG), anti-protein kinase A catalytic subunit α (C-20) antibody (rabbit polyclonal IgG). FITC-conjugated goat anti-mouse IgM antibody and peroxidase-conjugated goat antibody against rabbit IgG were purchased from ICN/Cappel (Costa Mesa, CA). Peroxidase-conjugated goat antibody against mouse (IgG + IgM) and peroxidase-conjugated rabbit antibody against rat (IgG + IgM) were purchased from American Qualex (San Clemente, CA) and Zymed Laboratories (South San Francisco, CA). A 96-well plastic plate was a product of Nunc (ImmunoPlate MaxiSorp Surface; Denmark).

Production of monoclonal antibody against sea urchin sperm gangliosides

mAb.2A11, an IgM monoclonal antibody that recognizes sea urchin sperm ganglioside $HSO_3(Neu5Ac)_2GlcCer$ and $(Neu5Ac)_2GlcCer$, was generated according to described procedures [24]. Briefly, sperm from *H. pulcherrimus* (10 µl undiluted semen) were suspended in 100 µl of 10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), and used as an immunogen. BALB/c mice (8-weeks old female, Nippon SLC, Japan) were immunized by intraperitoneal injection with the suspension of lysed sperm with Freund's complete or incomplete adjuvant three times at two-week intervals. A mouse was injected with the sperm suspension (10 µl undiluted semen) supplemented with 5 µg of $HSO_3(Neu5Ac)_2GlcCer$. Three days after the final injection, splenocytes were isolated and fused with myeloma P3U1 (P3-X63 Ag8.U1) cells. Procedures for preparation of hybridoma, screening, and cloning have been described [32,33]. A hybridoma, which produced antibody that was reactive with $(Neu5Ac)_2GlcCer$ and $HSO_3(Neu5Ac)_2GlcCer$, was cloned, and designated as 2A11. The antibody class was determined by the mouse monoclonal antibody isotyping kit RPN29 (Amersham Life Science, UK). The antibody was partially purified by ammonium precipitation [34].

Immunofluorescence

H. pulcherrimus sperm was suspended in calcium-free sea water (CaFSW, 444 mM NaCl, 9 mM KCl, 40 mM $MgSO_4$, 5 mM $NaHCO_3$ (pH 8.2)) and deposited onto glass slides using a Cytospin. The slides were fixed in 3.7% formaldehyde/CaFSW at room temperature for 10 min, washed with PBS for 10–30 min, and blocked with 3 mg/ml BSA, 0.1 mg/ml normal goat IgG in PBS for 1 h. After washing with PBS, a 1:1000 to 1:10000 dilution of mAb.2A11 was added at 4°C for overnight. The slides were washed three times with PBS for 10 min and incubated with FITC-conjugated goat anti-mouse IgM antibody (a 1:500 dilution) at room temperature for 70 min. After washing three times with PBS and dipping into water, the slides were mounted in 90% glycerol/PBS (pH 8.0) containing 1 mg/ml *p*-phenylenediamine. Cells were observed with a Zeiss Axiovert 135 M microscope equipped with epifluorescence.

Preparation of low density detergent-insoluble membrane (LD-DIM) fractions

The low density, detergent-insoluble membrane (LD-DIM) fraction was prepared as described [24]. Briefly, sperm (150–300 µl undiluted semen) were suspended in 1.0 ml of 10 mM Tris-HCl (pH 7.5) containing 1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, and 75 units/ml aprotinin. The mixture was kept on ice for 20 min and homogenized with 10 strokes of a Dounce homogenizer. After removal of the pellet by centrifugation at $1300 \times g$ for 5 min, the supernatant was mixed with an equal volume of 85% (w/v) sucrose in 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 5 mM EDTA (TNE). The mixture was layered successively with 6 ml of 30% (w/v) sucrose in TNE and with 3.5 ml of 5% (w/v) sucrose in TNE, and centrifuged at $200000 \times g$ for 18 h (4°C). After ultracentrifugation, 1 ml each of 11 fractions were collected from the top of the tube. Fractions were pooled under fraction A for fractions 1–3, B for 4–5, C for 6–7, D for 8–9, and E for 10–11. Each letter designated fraction was subjected to chemical and immunochemical analyses after dialysis against water or PBS. Protein was quantitated by the BCA (bicinchoninic acid) assay (Bio-Rad) using BSA as a standard.

High performance thin layer chromatography (HPTLC)

An acidic lipid fraction was prepared from fractions A-E, and analyzed by HPTLC, as described [24]. After treatment with 7.5 M aqueous ammonia at 25°C for 2 h, the acidic lipid fraction was applied on the HPTLC plate (silica gel 60, Merck, Germany), developed in chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v/v), and sprayed with resorcinol reagent [35], followed by heating at 120°C for 15 min for development. The visible bands were quantitated on ATTO Densitograph using lane and spot analyzer software (ATTO, Japan).

Western blotting

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli [36]. After SDS-PAGE, samples were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, U.S.A.) and immunostained as described [34]. Briefly, the membrane was blocked with 1% BSA/PBST (10 mM sodium phosphate buffer (pH 7.2) − 0.15 M NaCl (PBS), 0.05% Tween 20) at room temperature for 1–2 h. After washing, the membrane was incubated with primary antibodies at 37°C for 2 h, or at 4°C for overnight for IgG antibodies, and at 4°C for overnight for IgM antibodies. The concentration of the primary antibodies was: 1:2000 dilution for anti-speract receptor; 1 µg/ml for anti-REJ-1; 0.2 µg/ml for anti-63 kDa protein; 1:1000 dilution for anti-bindin; 0.4 µg/ml for anti-Gsα; 0.2 µg/ml for anti-Rho B; 0.4 µg/ml for anti-caveolin-1; 1:1000 dilution for anti-adenylate cyclase; 1:5000 dilution for anti-guanylate cyclase; 0.4 µg/ml for anti-protein kinase A. After washing twice with PBST, incubation of the membrane with secondary antibodies at 1:2000 dilution with 1% BSA/PBST were carried out at 37 °C for 45 min. After 3 washes in PBST, the blot was developed using enhanced chemiluminescence (ECL) reagent and Hyperfilm™ MP (Amersham, England). Stained bands were densitometrically analyzed on ATTO Densitograph.

Immunoprecipitation

Unless stated otherwise, all procedures were carried out at 4 °C. The LD-DIM fraction was dialyzed against PBS and concentrated to 1 ml using a Microcon YM10 (Millipore, U.S.A.). This was applied for 1.5 h to 30–100 µl of Protein G-Sepharose (Pharmacia Biotech, Sweden), which had been pretreated with 1% BSA/PBS for 1 h. After centrifugation at $200 \times g$ for 2 min, the supernatant was applied to 30–100 µl of 50% (v/v) suspension of Protein G-Sepharose that had been preincubated with 200–500 µl of 1% BSA/PBS containing anti-80 kDa speract receptor antibody or anti-63 kDa protein ($J_{17/30}$) for 1.5 h, followed by washing three times with 1% BSA/PBS. After overnight incubation and the subsequent centrifugation, the pellet was washed four times with PBS, and subjected to SDS-PAGE/Western-blot analysis.

Results

Localization of a major ganglioside, $(Neu5Ac)_2GlcCer$, of sea urchin sperm

A monoclonal antibody (IgM) which recognized $(Neu5Ac)_2GlcCer$ and $HSO_3(Neu5Ac)_2GlcCer$ was obtained and designated mAb.2A11. As shown in Figure 1, using an ELISA assay, mAb.2A11 reacted with $HSO_3(Neu5Ac)_2$ GlcCer, $HSO_3Neu5AcGlcCer$ and $(Neu5Ac)_2GlcCer$, but not with Neu5AcGlcCer. Also, $(8Neu5Ac\alpha2 \rightarrow)n - PE$, GD3, and sulfatide were not recognized by this antibody (data not shown). mAb.2A11 is thus specific to the (Neu5Acα2 or HSO_3) → 8Neu5Acα2 → 6Glc structure.

H. pulcherrimus sperm was observed by immunofluorescence after exposure to mAb.2A11. As shown in Figure 2a, the entire cell surface was reactive in most sperm. In some sperm, staining was observed at the tip of the sperm heads and the midpiece (Figure 2d). However, the staining pattern of the tail region was uniform in all sperm. The secondary antibody alone (negative control) showed no fluorescence (data not shown).

Distribution of the major gangliosides of *H. pulcherrimus* and *S. purpuratus* sperm in fractions A–E.

After sucrose density gradient centrifugation of the Triton X-100 sperm lysate, fractions A-E were collected (Scheme 1). Fraction B consisted of a light scattering band that corresponded to the LD-DIM, as we have reported previously [24]. Gangliosides in each fraction were quantitated by densitometric analysis of an HPTLC plate as developed by the resorcinol reagent. As shown in Figure 3a,

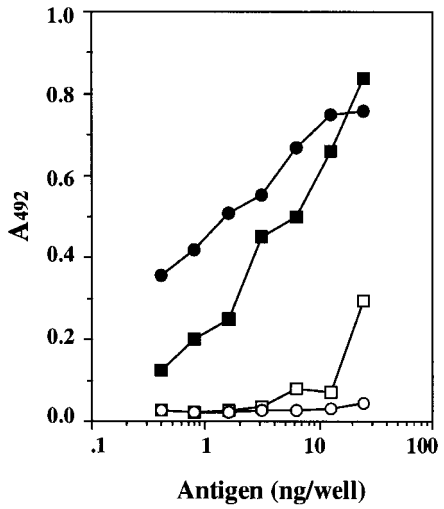


Figure 1. Determination of antigenic specificity of mAb.2A11. The plastic wells were coated with various amounts of glycolipids (0.4–25 ng as Neu5Ac), and incubated with 30 μg/ml of mAb.2A11 at 4°C for 12 h. After washing with PBS-T, the wells were incubated with 2 μg/ml of peroxidase-conjugated goat anti-mouse (IgG + IgM) at 37°C for 1 h. After washing five times with PBS-T, 100 μl of 0.05% *o*-phenylenediamine in 0.1 M Tris-HCl (pH 7.5) containing 0.006% H_2O_2 was added and incubated at room temperature for 10–20 min, followed by measurement of absorbance at 492 nm after adding of 100 μl of 2 N H_2SO_4. $HSO_3(Neu5Ac)_2GlcCer$ (■), $(Neu5Ac)_2GlcCer$ (□), $HSO_3Neu5AcGlcCer$ (●), and Neu5AcGlcCer (○).

$(Neu5Ac)_2GlcCer$ and $HSO_3(Neu5Ac)_2GlcCer$ migrated at R_f values of 0.55 and 0.45. Most of these components were distributed in the LD-DIM in *H. pulcherrimus* sperm, and 54% and 62% of the total ganglioside was present in this fraction (Figure 3c). In *S. purpuratus* sperm, a major ganglioside with almost the same R_f value (0.58) as $(Neu5Ac)_2GlcCer$ of *H. pulcherrimus* was also found in the LD-DIM (Figures 3b and d). Thus, the LD-DIM contains a large proportion of the ganglioside components.

Western blot analysis of fractions A–E

To identify which proteins were present in the LD-DIM of *S. purpuratus* sperm, fractions A-E were subjected to Western blotting using various specific antibodies against *S. purpuratus* sperm proteins and to other proteins involved in signal transduction (Figure 4). The results are summarized in Table 1. In the whole sperm lysate, an 80 kDa protein (the speract receptor), a 210 kDa protein (REJ-1), a 63 kDa protein (63 kDa GPI-anchored protein), a 31 kDa protein (bindin), a 48 kDa protein (α subunit of a heterotrimeric G protein, Gsα), a 190 kDa protein (adenylate cyclase), a 133 kDa protein (guanylate cyclase), a 47 kDa protein (protein kinase A catalytic subunit α), a 25 kDa protein (a small G protein, Rho B) and a 21 kDa protein (caveolin-1) were visualized by each specific antibody. Of these, seven proteins, the 80 kDa (speract receptor), 210 kDa (REJ-1), 63 kDa (the 63 kDa GPI-anchored protein), 48 kDa (Gsα), 190 kDa (adenylate cyclase), 133 kDa (guanylate cyclase) and 47 kDa (protein kinase A) were detected in the LD-DIM fractions. Bindin, caveolin-1 and Rho B were only detected in the detergent-soluble fractions, but not in the LD-DIM fraction. The antibodies against *S. purpuratus* sperm proteins recognized the corresponding proteins of *H. pulcherrimus* sperm, and the distribution of the proteins in the A–E fractions was the same as in *S. purpuratus* sperm, except for REJ-1 and Gsα epitopes. In *H. pulcherrimus* sperm, the REJ-1 protein was detected at 115 kDa, but not 210 kDa, which could be due to proteolysis of the 210 kDa form (Table 1). Three different Gsα isoforms at 52 kDa, 49 kDa and 42 kDa were observed, although signals of the 49 kDa and 42 kDa isoforms were faint in the whole sperm lysate (Figure 4b). Interestingly, the 52 kDa and 42 kDa isoforms were highly enriched in the LD-DIM fraction (Figure 4b), while the 49 kDa isoform was more abundant in the detergent-soluble fraction than in the LD-DIM. In *S. purpuratus* sperm, anti-Gsα antibody also reacted with three bands at molecular masses of 50 kDa, 48 kDa and 47 kDa; the 48 kDa isoform was only detected in the LD-DIM (Figure 4a). It was reported that the 48 kDa and 45 kDa isoforms are also immunochemically detectable in *S. purpuratus* sperm, and the 48 kDa isoform was considered to be functional based on the sensitivity to ADP-ribosylation by cholera toxin [37].

Co-immunoprecipitation analysis of the LD-DIM

The LD-DIM fraction of *H. pulcherrimus* sperm was immunoprecipitated with antibody to the 80 kDa speract receptor, and the precipitate was analyzed by Western blotting using various antibodies described above. As shown in Figure 5a, not only the 78 kDa protein (recognized by anti-80 kDa speract receptor antibody), but also the 62 kDa protein (recognized by anti-63 kDa GPI-anchored protein antibody) and 52, 49 and 42 kDa components (positive for anti-Gsα antibody recognition) were precipitated. REJ-1, adenylate cyclase, guanylate cyclase and protein kinase A were not detected in the immunoprecipitate (data not shown). Anti-63 kDa protein antibody precipitated anti-80 kDa receptor and anti-Gsα antibody epitopes, in addition to the 63 kDa protein (Figure 5b). REJ-1, adenylate cyclase, guanylate cyclase and protein kinase A were not co-precipitated with anti-63 kDa protein antibody (data not shown). TLC-immunostaining of the precipitates using anti-gangliosides antibody mAb.2A11 showed that gangliosides were also co-localized in the LD-DIM (data not shown). These results showed that the speract receptor, the GPI-anchored protein and the Gsα protein were co-localized in the LD-DIM containing mAb.2A11-reactive gangliosides. This co-localization suggests a possible functional association of these signaling proteins.

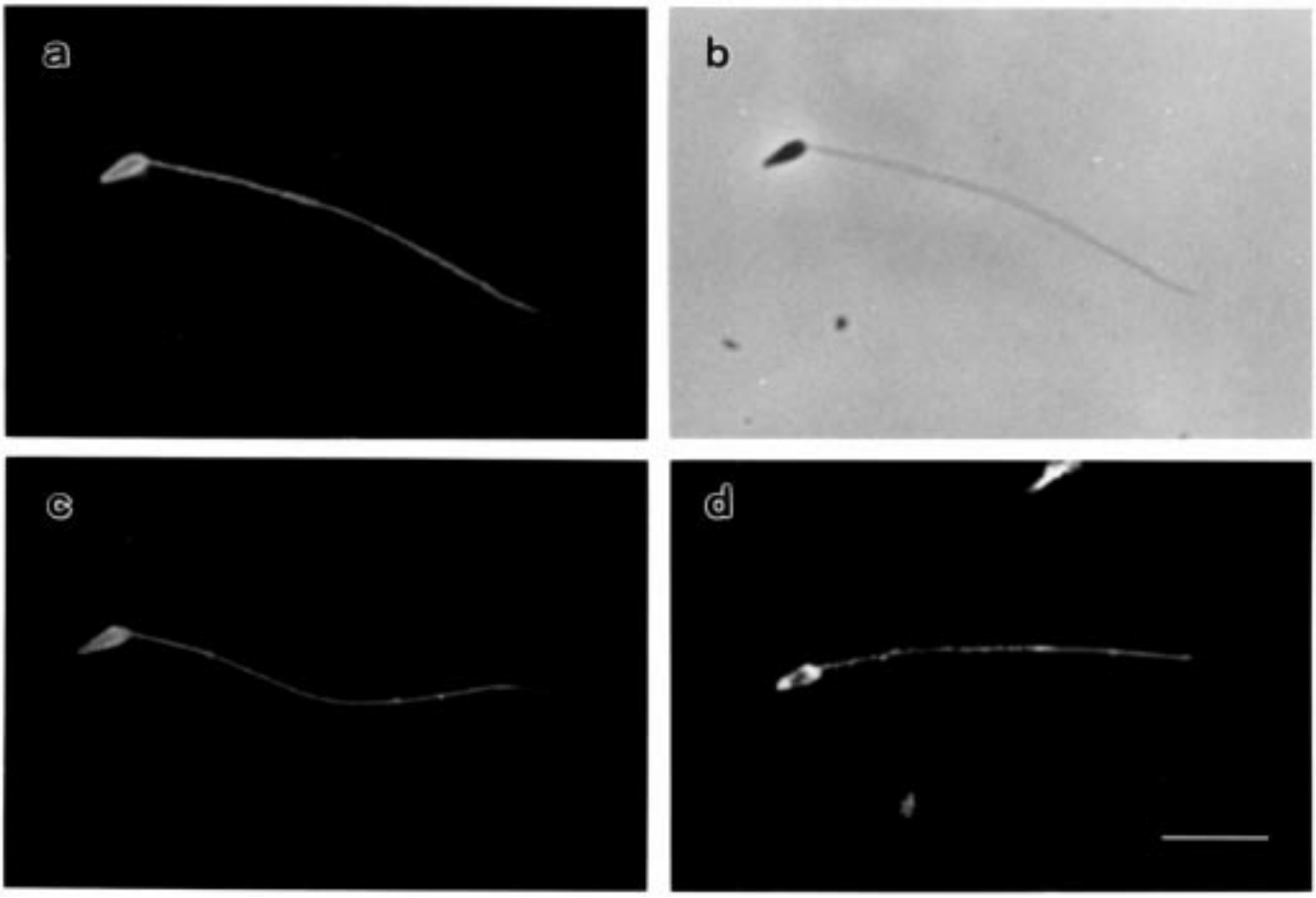


Figure 2. Immunolocalization of the 2A11 epitope in sea urchin sperm. Fixed *H. pulcherrimus* sperm was incubated with mAb.2A11 as primary antibody and then with FITC-conjugated goat anti-mouse IgM as secondary antibody. (a) Epifluorescence microscopy showed the entire surface of the sperm was stained. (b) A phase-contrast image of the same sperm as in (a). (c) Another sperm was shown, in which the 2A11 epitope was distributed over the entire surface. (d) In this sperm the tip of sperm head and the vicinity of the midpiece were preferentially stained. (a), (c), (d) epifluorescence, (b) phase contrast. Bar in (d), 10 μm.

Discussion

Here, the LD-DIM was obtained by sucrose-density gradient centrifugation after detergent treatment of sperm of two species of sea urchin. Gangliosides were enriched in the LD-DIM in sperm of both species (Figure 3). In *H. pulcherrimus* sperm, $(Neu5Ac)_2GlcCer$ was shown to be a major ganglioside [22,38]. A disialylated GlcCer that migrated near $(Neu5Ac)_2GlcCer$ of *H. pulcherrimus* sperm (Figure 3b) was the most abundant ganglioside in *S. purpuratus* sperm. A large percentage of the total sperm gangliosides in *H. pulcherrimus* ($> 54\%$) and *S. purpuratus* ($> 44\%$) were recovered in the LD-DIM.

Proteins that were detected in the LD-DIM by Western blots were classified into two groups. Group I proteins are those which orient their functional domain to the outer surface of sperm plasma membrane, including two receptor proteins for egg components, 80 kDa speract receptor and 210 kDa REJ-1 and a GPI-anchored protein of molecular mass of 63 kDa, whose function remains unknown. Bindin, an acrosomal protein which is known to reside on the outer surface of the inner acrosomal membrane, was not detected in the LD-DIM. Bindin may dissociate from the acrosomal membrane during the Triton X-100 treatment, because it has no anchoring structure such as GPI-conjugation, fatty acylation nor membrane spanning domain. Group II proteins are expected to be linked to the inner surface of the membrane. These include proteins involved in signal transduction, such as Gsα, adenylate cyclase, guanylate cyclase and protein kinase A. (In sea urchin sperm the guanylate cyclase is a transmembrane

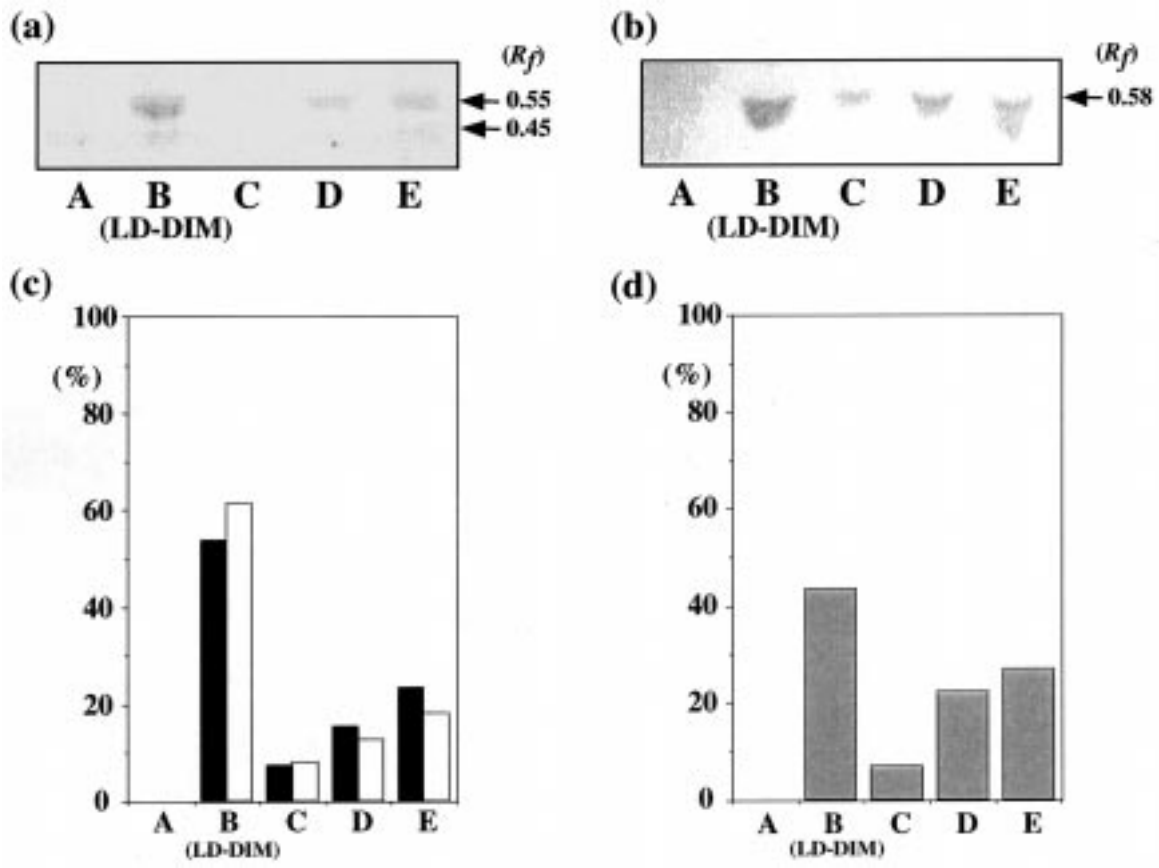
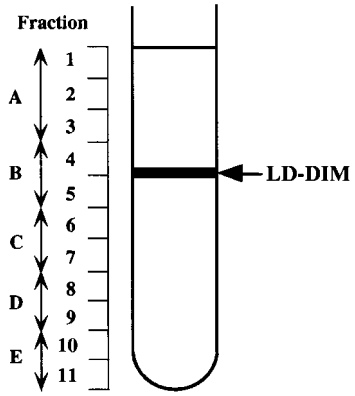


Figure 3. Major sperm gangliosides are enriched in the LD-DIM fraction. (a, b) HPTLC of the acidic lipid fraction prepared from fractions A–E obtained by the sucrose-density gradient centrifugation of sperm extracted with Triton X-100 treatment. Gangliosides were visualized by spraying with the resorcinol reagent followed by heating at 120°C for 15 min. (c, d) Distribution of major gangliosides in the A–E fractions, as densitometrically quantitated. The proportion (w/w%) of the amount of gangliosides in each fraction to the total amount of these gangliosides are shown. (a, c) *H. pulcherrimus* and (b, d) *S. purpuratus*. Closed and open bars represent % recovery for $(Neu5Ac)_2GlcCer$ and HSO_3 $(Neu5Ac)_2GlcCer$. Gray bars represent % recovery for disialyl GlcCer. See Scheme 1 for reference with fractions A–E.



Scheme 1. Preparation of fractions A–E upon the sucrose-density gradient centrifugation of the lysed sperm.

protein with about 50% of the sequence on the outside of the plasma membrane and 50% on the inside.) All these results indicate that the sperm-derived LD-DIM can be also characterized by the presence of both receptor proteins and transducer molecules, as is the case with those from somatic cells [1,2]. Caveolin-1 is a cytosol-oriented membrane protein and a specific marker of caveolae, a flask-shaped invagination of the plasma membrane [1,39]. Caveolae are known to give rise to a detergent-insoluble membrane fraction during treatment with 1% Triton X-100 [40]. Caveolin-1 was not detected in the sperm LD-DIM, although a weak signal was observed in the whole sperm lysate. This indicates that most of the LD-DIM in sea urchin sperm may be of a non-caveolae-type detergent-insoluble membrane, as observed for mouse melanoma B16 cells [17].

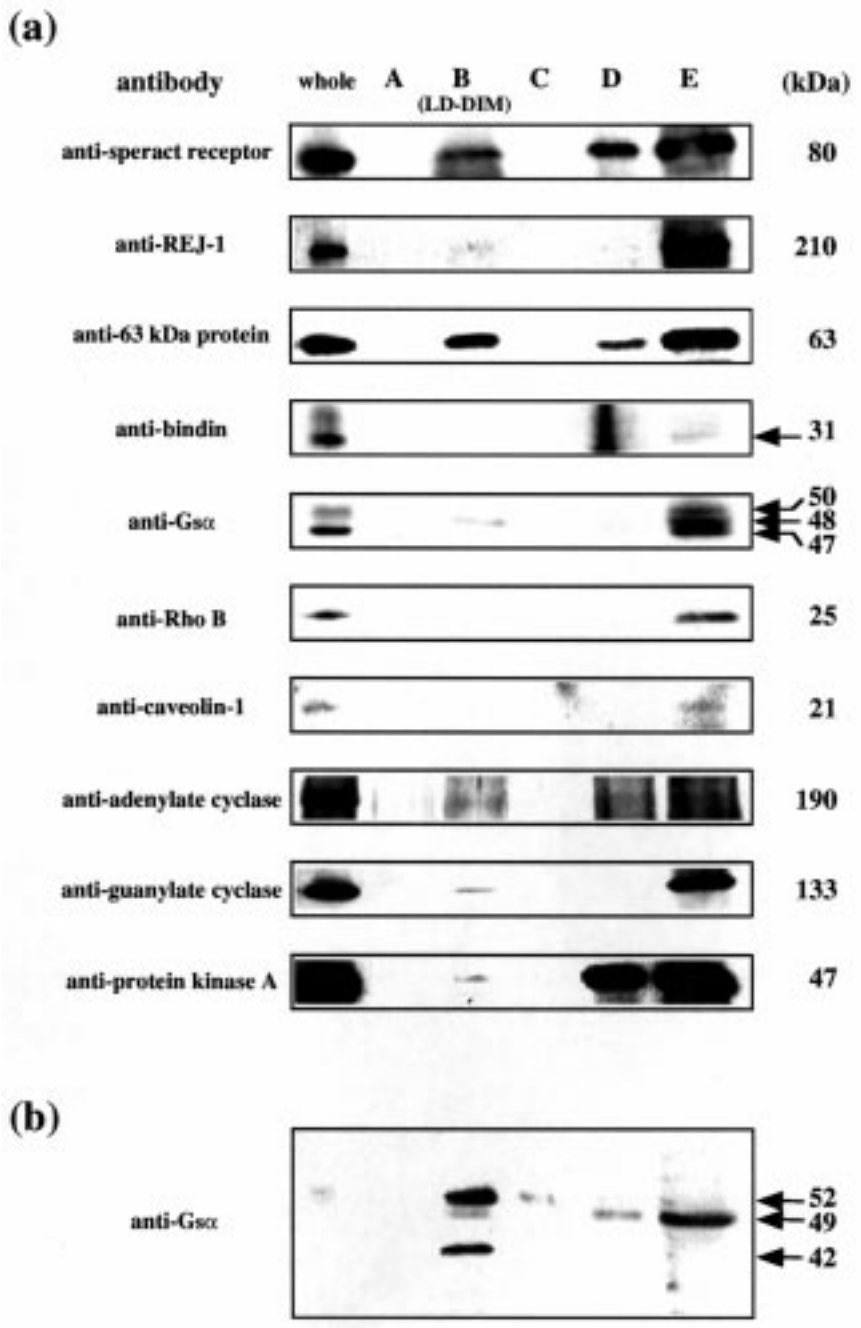


Figure 4. Western blots of sperm proteins in the fractions A–E obtained by sucrose-density gradient centrifugation of the detergent extract of sperm of (a) *S. purpuratus* and (b) *H. pulcherrimus*. Each of the fractions A–E was subjected to SDS-PAGE (7.5% PAG for 210 kDa REJ-1, adenylate cyclase and guanylate cyclase; 9% PAG for protein kinase A; 10% PAG for 80 kDa speract receptor, 63 kDa GPI-anchored protein and Gsα; 15% PAG for bindin, Rho B and caveolin-1), transferred to PVDF membrane. Sperm proteins present in each fraction were visualized by immunostaining using the antibodies indicated at the left side of each panel. In (b), the results of immunostaining by anti-Gsα antibody are only shown. An estimated molecular mass for each protein given at the right side of each panel. Whole, lysate of whole sperm. See Scheme 1 for reference to fractions A–E.

The ganglioside species and the proteins that were detected in the LD-DIM were also present in the detergent-soluble E fraction. This is partly because the membrane microdomain (often called a lipid raft), that is Triton X-100-insoluble, may be unstable in Triton X-100 depending on subtle differences in conditions (temperature, pH, the detergent-lipid ratio). Alternatively, it may be because recruitment of proteins into the LD-DIM is known to undergo dynamic changes, depending on

Table 1. Immunolocalization of various sperm proteins in the LD-DIM (B) and the detergent-soluble (E) fraction

	Localization					
	S. purpuratus			*H. pulcherrimus*		
Proteins	*Molecular mass*	*LD-DIM*	*Detergent-soluble*	*Molecular mass*	*LD-DIM*	*Detergent-soluble*
Speract receptor	80 kDa	+	+	78 kDa	+	+
REJ-1	210 kDa	+	+	115 kDa	+	+
63 kDa protein	63 kDa	+	+	62 kDa	+	+
Bindin	31 kDa	n.d.[a]	+	31 kDa	n.d.	+
Gsα	50, 48, 47 kDa[b]	+	+	52, 49, 42 kDa[b]	+	+
Rho B	25 kDa	n.d.	+	26 kDa	n.d.	+
Caveolin-1	21 kDa	n.d.	+	20 kDa	n.d.	+
Adenyate cyclase	190 kDa	+	+	192 kDa	+	+
Guanylate cyclase	133 kDa	+	+	135 kDa	+	+
Protein kinase A	47 kDa	+	+	46 kDa	+	+

[a]n.d., not detected.
[b]See also Figure 4.

the structure of the lipid components, the state of the cells (resting or activated), or the association with particular ligands [14,41]. Thus, some portion of the resident proteins in the LD-DIM can always be solubilized with Triton X-100. However, the validity of the Triton X-100-insoluble feature as the criterion for association of the proteins with a membrane microdomain raft is substantiated by the fact they can be isolated by ultracentrifugation [4,40].

The 78 kDa protein in *H. pulcherrimus* sperm and the 80 kDa protein in *S. purpuratus* sperm can be referred to as the speract receptor, although the molecular mass of the protein was reported to be 77 kDa and 71 kDa in *H. pulcherrimus* and *S. purpuratus* sperm [42,43]. As shown in Figure 5, anti-80 kDa speract receptor antibody precipitated the 63 kDa GPI-anchored protein and Gsα together with the speract receptor, while anti-63 kDa protein antibody precipitated 80 kDa speract receptor and Gsα together. These results indicate that these three proteins are localized in the same LD-DIM microdomain. Since the 80 kDa speract receptor is known to play a role in activation of membrane-bound guanylate cyclase and the subsequent stimulation of the respiration and motility of sperm when it is bound by the 10 amino acid peptide, speract, found in the jelly layer of eggs, it is suggested that 63 kDa GPI-anchored protein and Gsα also are involved in these processes. It is noted that the GPI-anchored protein in *H. pulcherrimus* sperm is suggested to be near the speract receptor in the plasma membrane [44]. However, the 135 kDa guanylate cyclase was not detected in the same LD-DIM membrane that contained the speract receptor and the 63 kDa GPI-anchored protein. One explanation of this may be because the LD-DIM contains only a small amount of guanylate cyclase as detected in these immunoprecipitation experiments. An alternative explanation may be that the association of guanylate cyclase with the LD-DIM is dynamic, depending on the state of phosphorylation of the enzyme. In this regard, it is interesting to note that the IgεRI receptor is dynamically recruited into the DIM fraction only after it forms an oligomeric structure in the presence of IgE [14]. No functional linkage between the speract receptor and the trimeric Gs protein is known. It would be interesting to elucidate the dynamic structural changes and functions of this speract receptor-containing LD-DIM during its interaction of sperm with speract at fertilization.

Immunofluorescence microscopy of *H. pulcherrimus* sperm using mAb.2A11, which recognizes a major ganglioside of the sea urchin sperm, showed that this ganglioside was distributed on the entire surface of sperm (Figure 2), although it was reported that some topographical differences were observed in the localization of gangliosides [45]. The 80 kDa speract receptor, 63 kDa GPI-anchored protein and Gsα are localized mainly in the sperm flagellum [28,37]. Some population of the major ganglioside species of sperm, which is localized in the flagellum, is suggested to participate in the formation of the speract receptor-containing LD-DIM. Recently, we have shown that a population of the LD-DIM fraction has the ability to bind to the egg surface 350 kDa sperm binding protein (SBP) [46]. However, it remains to be elucidated if the speract receptor-containing LD-DIM can bind to the SBP. Current efforts are focussed on the identification of sperm LD-DIM molecules interacting with the 350 kDa SBP.

Acknowledgments

This research was supported in part by Grants-in-Aid for International Scientific Research, Joint Research 10044265, and for Scientific Research on Priority Areas 10134216 (to KK) from the Ministry of Education, Science, Sports and

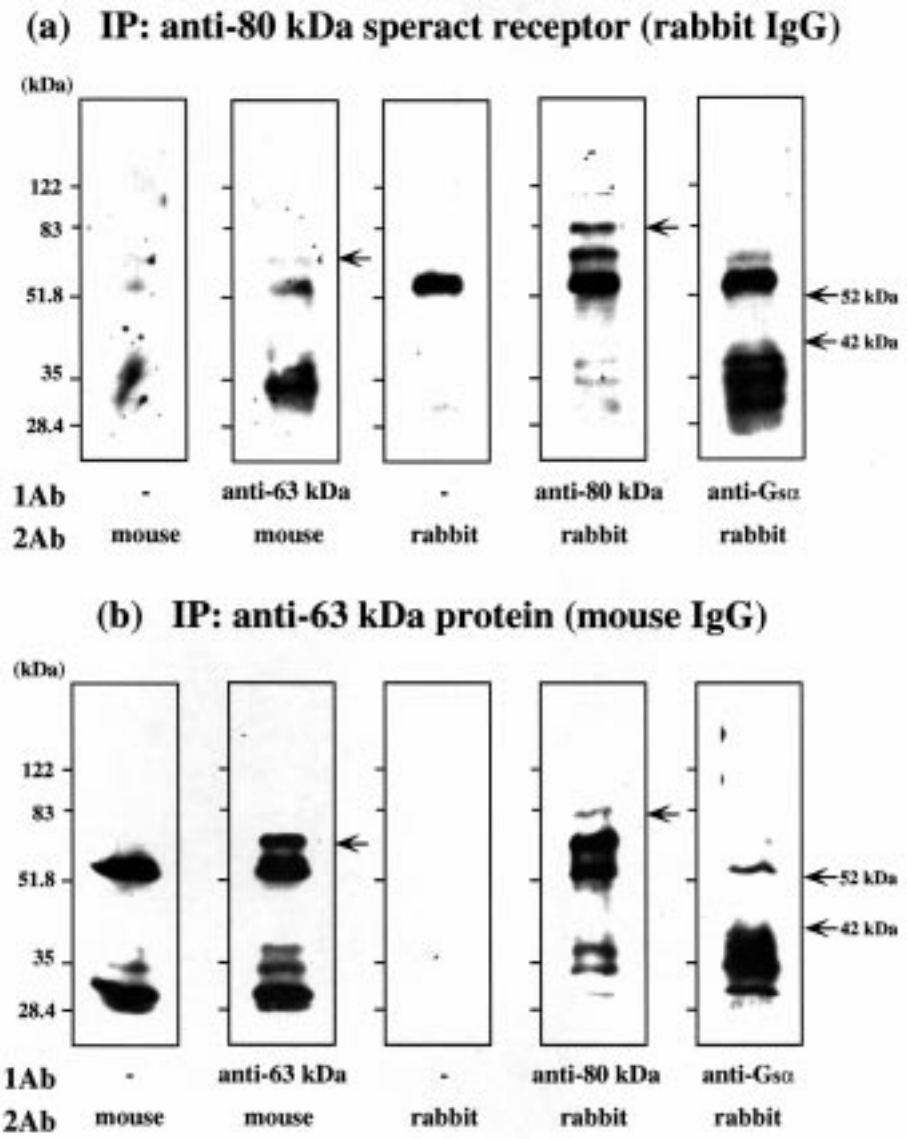


Figure 5. Co-immunoprecipitation of 80 kDa speract receptor, 63 kDa GPI-anchored protein and Gsα in the DIM fraction with anti-80 kDa or anti-63 kDa protein. The LD-DIM fraction of *H. pulcherrimus* was incubated with Protein G-Sepharose resin conjugated with anti-80 kDa or anti-63 kDa protein antibody. The resins were subjected to SDS-PAGE/Western blot analysis using anti-63 kDa, anti-80 kDa or anti-Gsα antibody as a primary antibody (1Ab). Panel (a), immunoprecipitation (IP) with anti-80 kDa speract receptor protein; Panel (b), IP with anti-63 kDa GPI-anchored protein. The blotted membranes were probed with the antibodies indicated at the bottom of each panel. Locations of 80 kDa speract receptor, 63 kDa GPI-anchored protein and Gsα are indicated by arrows on the right of each panel. 2Ab mouse, peroxidase-conjugated anti-mouse IgG + IgM; 2Ab rabbit, peroxidase-conjugated anti-rabbit IgG as secondary antibodies.

Culture. Support was also provided to VDV from NIH (12896) and to WJL from the NIH (HD 18590).

References

1 Parton RG, Simon K, *Science* **269**, 1398–9 (1995).
2 Simon K, Ikonen E, *Nature* **387**, 569–72 (1997).
3 Brown DA, Rose JK, *Cell* **68**, 533–44 (1992).
4 Verkade P, Simon K, *Histochem Cell Biol* **108**, 211–20 (1997).
5 Brawn DA, London E, *Biochem Biophys Res Commun* **240**, 1–7 (1997).
6 Kenworthy AK, Edidin M, *J Cell Biol* **142**, 69–84 (1998).
7 Fiedler K, Kobayashi T, Kurzchalia TV, Simon K, *Biochemistry* **32**, 6365–73 (1993).
8 Lisanti MP, Scherer PE, Vidugiriene J, Tang Z, Hermanowski-Vosatka A, Tu YH, Cook RF, Sargiacomo M, *J Cell Biol* **126**, 111–26 (1994).
9 Rogers W, Rose JK, *J Cell Biol* **135**, 1515–23 (1996).
10 Yamamura S, Handa K, Hakomori S, *Biochem Biophys Res Commun* **236**, 218–22 (1997).
11 Kasahara K, Watanabe Y, Yamamoto T, Sanai Y, *J Biol Chem* **272**, 29947–53 (1997).

12 Maekawa S, Sato C, Kitajima K, Funatsu N, Kumanogoh H, Sokawa Y, *J Biol Chem* **274**, 21369–74 (1999).
13 Manes S, Mira E, Gometz-Mouton C, Lacalle RA, Keller P, Labrador JP, Martinez-A C, *EMBO J* **18**, 6211–20 (1999).
14 Harder T, Simon K, *Curr Opin Cell Biol* **9**, 534–42 (1997).
15 Yu S, Withers DA, Hakomori S, *J Biol Chem* **273**, 2517–25 (1998).
16 Iwabuchi K, Yamamura S, Prinetti A, Handa K, Hakomori S, *J Biol Chem* **273**, 9130–8 (1998).
17 Iwabuchi K, Handa K, Hakomori S, *J Biol Chem* **273**, 33766–73 (1998).
18 Prinetti A, Iwabuchi K, Hakomori S, *J Biol Chem* **274**, 20916–24 (1999).
19 Hoshi M, Nagai Y, *Biochim Biophys Acta* **388**, 152–62 (1975).
20 Kotchetokov NK, Smirnova GP, Chekareva NV, *Biochim Biophys Acta* **424**, 274–83 (1976).
21 Yu S, Kitajima K, Inoue S, Khoo KH, Morris HR, Dell A, Inoue Y, *Glycobiology* **5**, 207–18 (1995).
22 Ijuin T, Kitajima K, Yu S, Kitazume S, Inoue S, Haslam SM, Morris HR, Dell A, Inoue Y, *Glycoconjugates J* **13**, 401–13 (1996).
23 Tanphaichitr N, Smith J, Mongkolsirikieart S, Gradil C, Lingwood C, *Dev Biol* **156**, 165–75 (1993).
24 Ohta K, Sato C, Matsuda T, Toriyama M, Lennarz WJ, Kitajima K, *Biochem Biophys Res Commun* **258**, 616–23 (1999).
25 Sato C, Kitajima K, Inoue S, Seki T, Troy FA II, Inoue Y, *J Biol Chem* **270**, 18923–8 (1995).
26 Podell SB, Vacquier VD, *Exp Cell Res* **155**, 467–76 (1984).
27 Trimmer JS, Trowbridge IS, Vacquier VD, *Cell* **40**, 697–703 (1985).
28 Nishioka D, Trimmer JS, Poccia D, Vacquier VD, *Exp Cell Res* **173**, 606–16 (1987).
29 Vacquier VD, *Exp Cell Res* **153**, 281–6 (1984).
30 Bookbinder LH, Moy GW, Vacquier VD, *J Cell Biol* **111**, 1859–66 (1990).
31 Shimizu T, Takeda K, Furuya H, Hoshino K, Nomura K, Suzuki N, *Zool Sci* **13**, 285–94 (1996).
32 Kohler G, Milstein C, *Nature* **256**, 495–7 (1975).
33 Kohler G, Milstein C, *Eur J Immunol* **6**, 511–9 (1976).
34 Sato C, Kitajima K, Inoue S, Inoue Y, *J Biol Chem* **273**, 2575–82 (1998).
35 Svennerholm L, *Biochim Biophys Acta* **24**, 604–11 (1957).
36 Laemmli UK, *Nature* **227**, 680–5 (1970).
37 Cuéllar-Mata P, Martínez-Cadena G, Castellano LE, Aldana-Veloz G, Novoa-Martínez G, Vargas I, Darszon A, García-Soto J, *Develop Growth Differ* **37**, 173–81 (1995).
38 Nagai Y, Hoshi M, *Biochim Biophys Acta* **388**, 146–51 (1975).
39 Parton RG, *Curr Opin Cell Biol* **8**, 542–8 (1996).
40 Hooper NM, *Mol Membr Biol* **16**, 145–56 (1999).
41 Keller P, Simons K, *J Cell Biol* **140**, 1357–67 (1998).
42 Dangott LJ, Garbers DL, *J Biol Chem* **259**, 13712–6 (1984).
43 Shimizu T, Yoshino K, Suzuki N, *Develop Growth Differ* **36**, 209–21 (1994).
44 Suzuki N, in *The male gamete: from basic science to clinical applications* (Gagnon C ed), Cache River Press (1999), pp 258–65.
45 Osawa T, Nagai Y, *Biochim Biophys Acta* **389**, 69–83 (1975).
46 Ohta K, Sato C, Matsuda T, Hirohashi N, Lennarz WJ, Kitajima, K, *Glycoconjugate J* **16**, S63 (1999).