Identification of the Sea Urchin 350-kDa Sperm-binding Protein as a New Sialic Acid-binding Lectin That Belongs to the Heat Shock Protein 110 Family

IMPLICATION OF ITS BINDING TO GANGLIOSIDES IN SPERM LIPID RAFTS IN FERTILIZATION*

Received for publication, July 12, 2003, and in revised form, August 12, 2003
Published, JBC Papers in Press, August 12, 2003, DOI 10.1074/jbc.M307493200

Eri Maehashi‡, Chihiro Sato‡, Kaoru Ohta‡, Yoichiro Harada‡, Tsukasa Matsuda‡, Noritaka Hirohashi¶, William J. Lennarz¶, and Ken Kitajima‡§**‡‡

From the ‡Graduate School of Bioagricultural Sciences, the ¶Biobioscience and Biotechnology Center, and the **Institute for Advanced Research, Nagoya University, Nagoya 464-8601 and the ¶Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, New York 11794-3215

The 350-kDa sperm-binding protein (SBP), a species-specific sperm-binding protein, is localized in the vitelline layer of sea urchin eggs. In this study, we have shown for the first time that sperm gangliosides are ligands for the intact glycosylated SBP. Using recombinant fragments of the SBP, the N-terminal heat shock protein 110-like domain was shown to be responsible for the binding. The intact SBP could bind various gangliosides, and the binding was sialidase-sensitive and inhibited by sialyllactose, thus indicating that it is the sialic acid-binding protein. Calcium and magnesium ions were not required but they did enhance the binding activity of SBP. The observation that bacterially expressed recombinant SBP and the sialidase-treated intact glycosylated SBP lost divalent cation-dependent enhancement of binding activity suggests that the sialylated carbohydrate moieties of the SBP may be involved in this property. Furthermore, the SBP was shown to bind sperm lipid rafts, in which gangliosides are enriched, and this binding was lost upon sialidase treatment of the lipid rafts. Finally, liposomes containing the ganglioside specifically inhibited fertilization. Taken together, these results allow us to identify SBP as a member of a new class of sialic acid-binding lectin belonging to the Hsp110 family, and indicate that SBP may be involved in interaction of sperm with the vitelline layer of the egg.

Glycolipids of the plasma membrane participate in various biological processes such as cell growth, differentiation, adhesion, and signal transduction (1, 2). In gametic cells, the involvement of glycolipids in cell-cell recognition and adhesion during spermatogenesis and fertilization has been described (3–7). In mouse fertilization, the importance of the major glycolipids on the sperm surface has been shown, because a SGG-recognizing protein, sulfolipid immobilizing protein 1 (SLIP1), is present on both egg and sperm surfaces (3, 8). Egg SLIP1 is suggested to be involved in sperm-egg plasma membrane binding through its binding to SGG (9). Recently, a liposome containing SGG has been shown to bind the zona pellucida, the egg extracellular glycoprotein matrix, suggesting the presence of another binding counterpart to SGG in the zona components of the mouse (10). In rainbow trout, possible involvement of the sperm major glycolipid, deaminourenamyl lactosyl ceramide in the sperm-egg interaction has been suggested (11).

In sea urchin sperm, the presence of unique gangliosides as a major component has been well documented (12, 13). Our previous results suggested that sea urchin sperm gangliosides are enriched in a membrane microdomain (or lipid raft) (6). Sperm lipid rafts are also characterized by the presence of both receptor and transducer proteins. Therefore, the lipid rafts may function as sites for binding some egg component to the sperm surface as well as for the subsequent signal transduction into the egg cell (6, 7). It has also been shown that two different anti-sperm ganglioside antibodies specifically inhibit sea urchin fertilization. These results strongly suggest that sperm gangliosides participate in the sperm-egg interaction in sea urchin. However, despite the suggested importance of the gangliosides, a ganglioside-binding protein has not been identified in sea urchin eggs. Therefore, we began searching for an egg component that can bind sperm gangliosides. In our efforts to do so, we have recently made a preliminary observation that the 350-kDa sperm-binding protein (SBP) could bind the major egg ganglioside (5).

SBP was first identified and cloned as the species-specific binding molecule for sperm, and sperm can bind SBP after acrosome reaction (14–17). SBP is a heavily glycosylated glycoprotein and is largely localized in the vitelline layer (18), an egg extracellular matrix corresponding to the mammalian zona pellucida. SBP is known to be involved in interaction with

2 The abbreviations used are: SGG, sulfogalactosylglycerolipid; GPI, glycosylphosphatidylinositol; LacCer, lactosylceramide; LD-DIM, low density, detergent-insoluble membrane; PMV, plasma membrane-vitelline layer; SBP, sperm-binding protein; SBD, sperm binding domain; GST, glutathione S-transferase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ASW, artificial sea water; CFM-ASW, calcium and magnesium-free-ASW; ELISA, enzyme-linked immunosorbent assay.

* This research was supported in part by grants-in-aid for the 21st Century COE Program (to K. K.) and for CREST of Japan Science and Technology Corporation (to K. K.), for Scientific Research (C) (15570096) (to K. K.), and for Young Scientists (B) (14780471) from the Ministry of Education, Science, Sports and Culture (to C. S.), and Mizutani Foundation (to C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Biology, Ochanomizu University, Ohtsu, Kyoto 522-8550, Japan.
¶ To whom correspondence should be addressed. Fax: 81-52-789-4297; E-mail: kitajima@agr.nagoya-u.ac.jp.
The Sialic Acid-binding Lectin from Sea Urchin Egg

Materials—Hemicentrotus pulcherrimus was purchased from local fisheries at Tsushima and Fukushima, Japan and Strongylocentrotus purpuratus was purchased from Marinus Inc. (Venice, CA). Sea urchin sperm and eggs were collected by intracloacal injection of 0.5 mM KCI. Compositions of artificial sea water (ASW) were 444 mM NaCl, 9 mM KCl, 30 mM MgCl₂, 22 mM MgSO₄, 10 mM CaCl₂, 5 mM NaHCO₃ (pH 8.2) and those of calcium- and magnesium-free artificial sea water (CMF-ASW) were 500 mM NaCl, 10 mM KCl, 5 mM NaHCO₃, 5 mM EDTA (pH 8.2). The 350-kDa SBP was purified from the plasma membrane fraction of H. pulcherrimus as described (13). The recombinant fragments of SBP (at indicated amounts in the figure) were prepared as described (18). The antibody recognizes a polypeptide part (amino acids 343–586) of the SBP, as determined using a series of the recombinant fragments as antigens (2). Anti-GST antibody (rabbit polyclonal IgG) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Peroxidase-conjugated goat antibodies against rabbit IgG and mouse (IgG + IgM) were purchased from ICN/Cappel (Costa Mesa, CA) and American Qualex (San Clemente, CA), respectively.

Preparation of the Low Density, Detergent-insoluble Membrane (LD-DIM) Fraction—The LD-DIM fraction was prepared as described (6). Unless otherwise stated, the amount of LD-DIM is expressed as the protein weight that is determined by the BCA assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Preparation of Liposomes—For preparation of liposome with or without the sperm ganglioside, phosphatidylcholine, cholesterol, and (Neu5Ac)₂GlcCer(H) in a ratio of 7:4:3 (w/w/w) or phosphatidylcholine and cholesterol in a ratio of 7:3 (w/w) were dissolved in 1 ml of chloroform/methanol (1:1, v/v). The mixtures were dried by rotary evaporator and subsequently in a desiccator under reduced pressure. Several glass beads and 5 ml of PBS were added and mixed. Vesicles of 100 nm in diameter were prepared by extruding these mixture through a filter (pore size, 100 nm) mounted in the extruder (Lipex Biomembranes, Vancouver, Canada) as described (24). The diameters of liposomes with and without the ganglioside were both 100 nm, as measured by Dynasizer Light Scattering Photometer (HIDLS-7000; Yoko, Kanto, Japan).

(a) Assay for Binding Activity of the SBP Binding of the SBP to Glycolipids—Binding activity of SBP was assayed based on enzyme-linked immunosorbent assay (ELISA). Various glycolipids dissolved in ethanol at varying concentrations (50 μl) were added to the wells, incubated at 37 °C for 2 h and washed three times with PBS. Blocking was carried out by incubating the wells with 100 μl of 1% BSA/PBS at room temperature for 2 h after washing three times with PBS and once with ASW or CMF-ASW, to the wells were added the SBP dissolved in ASW or CMF-ASW (1.8 μg/50 μl/well) and incubated at room temperature for 2 h. After washing three times with ASW or CMF-ASW, the binding was quantitated as described in (24). For washing three times with PBS, the wells were incubated with 50 μl of m35.9 of 1:500 dilution with 1% BSA/PBS at 4 °C for 12 h. After washing three times with PBS, the wells were incubated with 50 μl of the peroxidase-conjugated goat antibody against mouse (IgG + IgM) of 1:500 dilution with 1% BSA/PBS at room temperature for 2 h. After washing five times with PBS, color development was carried out by incubating with 100 μl of 0.05% o-phenylenediamine, 0.1 mM Tris-HCl (pH 7.5) and 0.006% H₂O₂, followed by addition of 100 μl of 1 M H₂SO₄. Absorbance at 492 nm was measured on a Bio-Rad Microplate Reader Model 450.

(b) Binding of SBP to the Sialidase-treated Sperm Gangliosides—The wells were coated with the ganglioside (Neu5Ac)₂GlcCer at 3.5 μg of sialic acid/well, and blocked with 1% BSA/PBS. The wells were incubated with or without a mixture of 2 milliunits/well each of A. ureafaciens and C. perfringens sialidases in 50 μl of 50 mM sodium acetate (pH 5.5) at room temperature for 3 h. After washing twice with ASW containing 0.1 mM Neu5Ac2en and twice with ASW, SBP was added and the binding was assayed as described in (a), except that m35.9 was used at 1:100 dilution.

(c) Effect of Divalent Cations—After the glycolipid-coating and blocking, the wells were washed three times with PBS containing 5 mM EDTA, and the SBP that had been dissolved in 10 mM Tris-HCl (pH 8.0), 0.5 mM NaCl containing CaCl₂ (1, 5, or 10 μM), MgSO₄ (1, 10, or 50 μM), or both 10 mM CaCl₂ and 50 mM MgSO₄ was added. The SBP dissolved in 10 mM Tris-HCl (pH 8.0), 0.5 mM CaCl₂ and 1 mM EDTA was also added to the wells, and the binding was assayed as described in (a).

(d) Binding of the Recombinant Fragments of SBP to Glycolipids—The recombinant fragments of SBP (at indicated amounts in the figure) was used instead of SBP. Incubation with a GST fusion protein (GST-hexonophorin) that was kindly provided by our laboratory (Nagoya University) was also run as a negative control. The bound recombinant SBP was then quantitated as described in (a) except that rabbit anti-GST antibody (1:1500 dilution) and the peroxidase-conjugated goat anti-rabbit IgG (1:2000 dilution) were used instead of m35.9 and the peroxidase-conjugated goat anti-mouse IgG + IgM, respectively.

(e) Binding of the Sialidase-treated SBP to the Sperm Ganglioside—SBP (270 μg) was incubated with a mixture of 0.125 unit each of A. ureafaciens and C. perfringens sialidases in 640 μl of 50 mM sodium acetate (pH 5.5) at 25 °C for 3 h. The digest was ultrafiltered through
Microcon YM-10. To the concentrated SBP, 50 μl of 50 mM sodium acetate (pH 5.5) was added, followed by the ultracentrifugation again. The resultant SBP was used for the ELISA-based binding assay after diluting with ASW or CMF-ASW. The combined filtrates were analyzed for the presence of released sialic acid by the fluorometric analysis (25, 26). For quantitating content of sialic acid, the SBP (1.9 μg) was hydrolyzed in 0.1 N trifluoroacetic acid at 50 °C for 2 h, and the released sialic acid was fluorometrically quantitated. The sialidase activity was assayed as described (27). For binding assay, coating, and blocking of the wells were described in (a). To the coated wells, 5.5 μl of 1 mM Neu5Ac2en or water and 50 μl of the SBP or the sialidase-treated SBP in ASW or CMF-ASW (1.5 μg/50 μl/well) were added and incubated at room temperature for 2 h. The bound SBP or sialidase-treated SBP was quantitated as described in (a).

**Inhibition of the SBP Binding to (Neu5Ac)₂GlcCer by Sialyllactose**—Inhibition experiment of SBP binding to the ganglioside by sialyllactose was based on inhibition ELISA procedures as follows. The wells were coated with (Neu5Ac)₂GlcCer (1.8 μg/50 μl/well) in the presence (●) or absence (○) of divalent cations at the same concentrations as in sea water (10 mM Ca²⁺ and 52 mM Mg²⁺) at room temperature for 2 h. The bound SBP was quantified as described under “Experimental Procedures.” The wells coated with (Neu5Ac)₂GlcCer at 250 ng of sialic acid/well were treated (+) or untreated (−) with sialidases, and incubated with SBP (1.8 μg/50 μl/well) in the presence of divalent cations as above. Binding is expressed as a relative value compared with the extent of binding to the sialidase-untreated ganglioside set at 100%. D, the inhibition of sialyllactose to SBP binding to ganglioside. The wells were coated with (Neu5Ac)₂GlcCer (250 ng of sialic acid/well) and incubated with sialyllactose-pretreated SBP (1.8 μg/50 μl/well). The bound SBP was quantified as described under “Experimental Procedures.” Binding is expressed as a relative value compared with the extent of binding to the sialyllactose-non-treated SBP set at 100%. All the experiments were carried out at least in duplicate, and deviations are expressed by the error bars.

**RESULTS**

SBP binds sperm gangliosides. It was previously shown that acrosome-reacted sperm binds to SBP of sea urchin eggs (15, 16, 28). Since SBP is mainly localized in the vitelline layer (18), sperm surface gangliosides could interact with it during sperm penetration of the vitelline layer. In a search for molecules that could bind to sperm gangliosides, we found that SBP from *S. purpuratus* eggs could bind the sperm ganglioside, (Neu5Ac)₂GlcCer (5). Following this finding, we have now further characterized binding activity of the SBP to sperm gangliosides. As shown in Fig. 1, A and B, SBP bound not only (Neu5Ac)₂GlcCer but also Neu5AcGlcCer, in a concentration-dependent manner. (Neu5Ac)₂GlcCer is a major ganglioside and Neu5AcGlcCer is a minor ganglioside (13). These results indicate that SBP has the ability to bind sea urchin sperm gangliosides. When (Neu5Ac)₂GlcCer was pretreated with sialidase, no binding was observed (Fig. 1C). Moreover, the binding of SBP to (Neu5Ac)₂GlcCer was inhibited by co-incubation with sialyllactose in a dose-dependent manner (Fig. 1D). At 1 mM of sialyllactose, the binding was inhibited by 21%. These results clearly indicate that the sialic acid residue is necessary for SBP binding. Binding of SBP to sperm gangliosides was observed in the absence of Ca²⁺ and Mg²⁺, showing that the binding does not require divalent cations. However, greater binding was observed in the presence of divalent cations than in their absence (Fig. 1, A and B), indicating that divalent cations enhance the binding activity of SBP. SBP was also shown to be present in *H. pulcherrimus* eggs, and similar binding ability to the sperm gangliosides was observed (data not shown).

**Binding Properties of SBP to Various Gangliosides**—To gain further insight into the binding properties of SBP, various
Fig. 2. SBP binds various gangliosides. A, wells of ELISA plate were coated with GM3 (0–500 ng of sialic acid/well) or LacCer (0–500 ng/well), and incubated with SBP (1.8 μg/well) in the presence or absence of divalent cations (10 mM Ca2+ and 52 mM Mg2+) at room temperature for 2 h. The bound SBP was quantified as described in “Experimental Procedures.” GM3 in the presence of divalent cations (closed circle); GM3 in the absence of divalent cations (open circle); LacCer in the presence of divalent cations (open diamond). B, wells were coated with GM1, GD3, or GD1a (0–500 ng of sialic acid/well), sulfatide, or GlcCer (0–500 ng/well), and incubated with SBP (1.8 μg/well) in the presence of the divalent cations at room temperature for 2 h. The bound SBP was quantified. GM1 (open triangle); GD3 (closed square); GD1a (open square), sulfatide (closed square), or GlcCer (closed diamond). All the experiments in A and B were carried out at least in duplicate on the same ELISA plate, and deviations are expressed by error bars. The figures show the representative data, and the results are highly reproducible.

glycolipids were tested as ligands. As shown in Fig. 2, A and B, SBP had the ability to bind gangliosides GM3, GM1, GD3, and GD1a, which do not exist in sea urchin sperm. No binding to GlcCer or LacCer was observed. These results indicate that SBP recognizes sialic acid residues of the gangliosides. GD1a was a poor ligand for the SBP, compared with other gangliosides. This result suggests that not only sialic acid residues themselves, but also the underlying structures appear to affect SBP binding. The SBP also bound sulfatide, but this binding was not strong relative to other gangliosides. This result suggests that SBP recognizes sialic acid rather than sulfate residues in these acidic glycolipids. The results confirming that GD1a and sulfatide were poorer ligands than GM3, GM1, or GD3 were highly reproducible. However, quantitative evaluation of binding strength of SBP to gangliosides by static and kinetic analyses are necessary for better understanding of the binding specificity of SBP.

Binding of SBP to GM3 was also observed in the absence of Ca2+ and Mg2+ and enhanced by the presence of these cations (Fig. 2A), as was observed for the sperm gangliosides (Fig. 1, A and B).

Effects of Divalent Cations on Binding of Gangliosides by SBP—Effects of divalent cations on binding of gangliosides to SBP were examined using the sperm ganglioside, (Neu5Ac)2GlcCer (Fig. 3). In these experiments, ranges of Ca2+ and Mg2+ concentrations were 0–10 mM and 0–50 mM, respectively, similar to concentration ranges occurring in sea water where fertilization occurs. SBP was shown to bind ganglioside in the absence of Mg2+ and Ca2+. When either Ca2+ or Mg2+ alone was added to the incubation solution, binding activity of the SBP to the ganglioside was dose-dependently enhanced. Ca2+ was more effective than Mg2+. These results indicate that the binding activity of SBP to ganglioside does not require divalent cations, although it is effectively enhanced by Ca2+.

Binding Properties of Recombinant Fragments of SBP to Various Glycolipids—It was previously shown that SBP contains two sperm binding domains (SBD), the genus-nonspecific SBD (amino acids 96–342), and the genus-specific SBD (amino acids 380–411), respectively (20). Therefore, we tested which SBD is responsible for binding to the sperm ganglioside. The recombinant fragments, G-(96–586) containing both the genus-specific and genus-nonspecific SBDs, G-(96–342) corresponding to the genus-nonspecific SBD, and G-(343–586) showing properties of the genus-specific SBD (20), were prepared and tested. As shown in Fig. 4, G-(96–586) and G-(96–342) bound the major sperm ganglioside, (Neu5Ac)2GlcCer, while only a very weak binding was observed for G-(343–586). No significant binding with hyosophorin-GST (negative control) was observed, and no color was developed in the absence of GST fusion proteins, either, indicating that GST is not involved in the binding (Fig. 4). As recombinant fragment G-(96–342) retained binding activity, it appears that the genus-nonspecific SBD is important for binding to sperm gangliosides.

We further tested if the recombinant SBP had the same binding specificity as intact SBP using the recombinant G-(96–
As shown in Fig. 5, A–D, a GST fusion protein with the recombinant SBP also bound GM3 in addition to (Neu5Ac)_2GlcCer and Neu5AcGlcCer, but not GlcCer. These results suggest that the protein moiety of SBP had the binding activity to sialic acid residues in gangliosides, although the extent of binding was different depending on the gangliosides tested. The recombinant SBP appears to preferentially bind Neu5AcGlcCer rather than (Neu5Ac)_2GlcCer, or GM3. It should be noted that binding of the recombinant SBP to gangliosides was not affected by the presence or absence of Ca^{2+} and Mg^{2+} (Fig. 5, A–C). Considering that binding of the intact SBP is enhanced by divalent cations (Figs. 1–3), these results suggest that the carbohydrate moieties of SBP may be involved in divalent cation-dependent enhancement of binding, or that SBP contains bound cations.

**Ganglioside Binding Properties of Sialidase-treated SBP—** As suggested above, the carbohydrate moieties of SBP may be involved in divalent cation-dependent enhancement of the binding activity. Sialic acids are known to have high affinity for Ca^{2+} (29), and SBP is a heavily sialylated glycoprotein (about 30% by weight) (30). Therefore, we examined effects of sialidase treatment of SBP on its binding activity using (Neu5Ac)_2GlcCer as a ligand.

The sialidase treatment released 58% of the total Neu5Gc in the SBP. Thus, 42% of the total Neu5Gc residues were retained in the sialidase-treated SBP. No further release of Neu5Gc was observed after additional incubation with the sialidases. This result may be partly due to the presence of 9-O-sulfated Neu5Gc residues (31), which are sialidase-resistant. Actually, SBP used here had Neu5Gc and 9-O-sulfated Neu5Gc residues in a molar proportion of 5:1, based on fluorometric analysis of sialic acid (data not shown).

Fig. 6 shows that: (a) the sialidase-treated SBP retained binding to sperm ganglioside irrespective of the presence or absence of divalent cations, (b) in the presence of divalent cations, the sialidase-treated SBP bound the sperm ganglioside more weakly than the intact SBP, and (c) in the absence of divalent cations, the sialidase-treated SBP bound the ganglioside as well as the intact SBP. These results indicate that a 58% reduction of sialic acid residues in SBP results in a loss of
divalent cation-dependent enhancement of the binding activity. In other words, the sialic acid residues on SBP may be involved in the divalent cation dependence.

**SBP Binds the Sperm LD-DIM**—SBP is exclusively localized in the vitelline layer of the egg (18). Sperm gangliosides could become associated with SBP when sperm are passing through the vitelline layer. Because sperm gangliosides are highly enriched in LD-DIM, or lipid rafts (5), it is hypothesized that SBP interacts with the gangliosides-enriched LD-DIM. To provide evidence supporting this linkage, we measured binding of SBP to sperm LD-DIM. As shown in Fig. 7A, SBP bound sperm LD-DIM in a dose-dependent manner. Divalent cation-dependent enhancement was also observed for binding of SBP to sperm LD-DIM. These LD-DIM binding properties are similar to those observed for ganglioside binding, thus suggesting that SBP may be binding to gangliosides on the sperm LD-DIM. To estimate how sialic acid residues of gangliosides on the sperm LD-DIM contribute to SBP binding, LD-DIM was treated with sialidases and subsequently analyzed for SBP binding. As shown in Fig. 7B, binding was greatly reduced, depending on the amount of sialidase added. This result indicates that sialic acid residues in the sperm LD-DIM are necessary for binding between SBP and sperm LD-DIM.

**Liposomes Containing Sperm Ganglioside Inhibit Sea Urchin Fertilization**—To assess the biological importance of sperm gangliosides at fertilization, the effects of co-incubation of eggs with liposomes containing the major sperm ganglioside, H. pulcherrimus eggs were co-incubated with liposomes containing the sperm ganglioside (Neu5Ac,GlcCer)H or control liposomes followed by the fertilization procedures as described under "Experimental Procedures." Two independent experiments were carried out on different days, and the deviation from the average is shown by error bars.

**DISCUSSION**

SBP is the species-specific sperm binding molecule that was first identified in sea urchin eggs (14–17). Sperm can bind to this glycoprotein after the acrosome reaction to initiate sperm-egg binding at fertilization. SBP has two SBD, a genus-specific and a genus-nonspecific SBD (20), corresponding to amino acids 96–342 and 380–411, respectively. The binding counterpart of the genus-specific SBD is shown to be bindin (21). Bindin is a major acrosomal protein that is associated with the sperm plasma membrane after acrosome reaction, and mediates adhesion and fusion between sperm and egg (32, 33). However, the binding counterpart of the genus-nonspecific SBD had not previously been identified. In this study we have clearly demonstrated that the binding counterpart for the genus-nonspecific SBD is sialic acid residues of sperm gangliosides. Thus, in search for the binding counterpart for the sea urchin sperm gangliosides, we first found that SBP has the ability to bind the major sperm ganglioside, (Neu5Ac,GlcCer). This binding was not observed when sialic acid residues were removed from the sperm ganglioside by sialidase treatment. Binding experiments using various glycolipids as ligands revealed that SBP could bind the sialic acid-containing glycolipids (gangliosides) GM3, GM1, GD1a, Neu5AcGlcCer and (Neu5Ac),GlcCer, but not LacCer, GM3, GM1, GD3, and GD1a are not sea urchin sperm components. These results indicate that SBP recognizes sialic acid residues. Bacterially expressed recombinant proteins consisting of the N-terminal half of SBP (amino acids 96–586 and 96–342) retained binding activity for sialic acid residues in various gangliosides. Thus, the polypeptide chain of the molecule has the activity to bind sialic acids, although SBP is heavily glycosylated (70% by weight) and sialylated (30% by weight). Based on these results, SBP can be regarded as a sialic acid-binding lectin. As has been pointed out, the sequence identity in the N-terminal 500 amino acid residues of SBP is 63% as compared with the N-terminal half of the hamster Hsp110 (17). Therefore, the N-terminal Hsp110-like domain of SBP is responsible for the binding activity.

3-O-Sulfated Gal-containing glycolipid (sulfatide) could also be a ligand for SBP, but it was a poor ligand compared with α,2,3-sialylated Gal-containing glycolipids, GM3. This is also the case with the recombinant form of SBP consisting of amino acid residues 96–342 (20), corresponding to amino acids 96–342 and 380–411, respectively. The binding counterpart of the genus-nonspecific SBD had not previously been identified. In this study we have clearly demonstrated that the binding counterpart for the genus-nonspecific SBD is sialic acid residues of sperm gangliosides. Thus, in search for the binding counterpart for the sea urchin sperm gangliosides, we first found that SBP has the ability to bind the major sperm ganglioside, (Neu5Ac,GlcCer). This binding was not observed when sialic acid residues were removed from the sperm ganglioside by sialidase treatment. Binding experiments using various glycolipids as ligands revealed that SBP could bind the sialic acid-containing glycolipids (gangliosides) GM3, GM1, GD1a, Neu5AcGlcCer and (Neu5Ac),GlcCer, but not LacCer, GM3, GM1, GD3, and GD1a are not sea urchin sperm components. These results indicate that SBP recognizes sialic acid residues. Bacterially expressed recombinant proteins consisting of the N-terminal half of SBP (amino acids 96–586 and 96–342) retained binding activity for sialic acid residues in various gangliosides. Thus, the polypeptide chain of the molecule has the activity to bind sialic acids, although SBP is heavily glycosylated (70% by weight) and sialylated (30% by weight). Based on these results, SBP can be regarded as a sialic acid-binding lectin. As has been pointed out, the sequence identity in the N-terminal 500 amino acid residues of SBP is 63% as compared with the N-terminal half of the hamster Hsp110 (17). Therefore, the N-terminal Hsp110-like domain of SBP is responsible for the binding activity.
The Sialic Acid-binding Lectin from Sea Urchin Egg

acids 96–586 (data not shown). Recently, Mamelak et al. (22) have reported that recombinant SBP (amino acids 96–586) can bind sulfatide, but not the ganglioside GM1, as determined by thin-layer chromatography overlay assay. However, we unambiguously demonstrated that SBP bound gangliosides. As they did, we also tested with the recombinant protein to show that it bound GM1 as well as other gangliosides, although the binding was relatively weak (data not shown, but similar to GM3 in Fig. 5) when compared with Neu5AcGlcCer and (Neu5Ac)2GlcCer. We also observed that sulfatide was a poor ligand, even when we used the sulfatide species that was reported to be most strongly bound by the recombinant protein (22). Examination of the results of Mamelak et al. (22) suggests that binding of the recombinant protein to sulfatide is weak as compared with that of the recombinant mouse testis-specific Hsp70. Therefore, it can be concluded that SBP is a sialic acid-binding protein rather than a sulfated Gal-binding protein.

Lingwood and co-workers (22, 34) demonstrated that the Hsp70 family possesses a unique binding specificity for 3-O-sulfated Gal-containing lipids. As the N-terminal half of SBP is homologous to the ATP-binding domain of the Hsp70 family, the difference in binding specificity must be attributed to the particular differences in the N-terminal half of the sequence. In this regard, it is interesting to note that SBP is a member of the Hsp110 family, a distant subfamily of molecular chaperones among the large Hsp70 families (35). Therefore, our results on the binding specificity of SBP suggest that members of the Hsp110 subfamily have different specificities than other members of the Hsp70 family. It would be interesting to determine structural elements required for recognition of sialic acids in the Hsp110-like domain in SBP, and this line of experiments is underway in our laboratory.

The sialic acid-binding lectins play important roles in various biological phenomena, such as cell-cell interactions (36, 37). Siglecs and selectins are two large families of sialic acid-binding lectins (36, 38). This study shows that SBP is a sialic acid-binding lectin that belongs to the Hsp110 family (17). Therefore, the Hsp110-like lectins could represent a new family of the sialic acid-binding lectins, although the binding specificity of other members of the Hsp110 family will have to be determined.

SBP required no Ca$^{2+}$ or Mg$^{2+}$ in sea water for binding activity. However, in the presence of these cations, binding activity was enhanced by 40–60%. Ca$^{2+}$ is effective at lower concentrations than Mg$^{2+}$ in enhancing the binding activity of SBP. Interestingly, this divalent cation-dependent enhancement of binding activity was lost when examined with the recombinant protein consisting of amino acids 96–586. As this recombinant protein was devoid of carbohydrate moieties, enhanced binding activity is possibly dependent on the presence of carbohydrates on SBP. To confirm this possibility, effects of sialidase treatment on the binding properties of SBP were examined, because sialic acids are known to have a relatively high affinity for Ca$^{2+}$ (29). SBP contains oligomers of Neu5Gc capped by the 9-O-sulfated Neu5Gc residues (30). Based on fluorometric analysis of the sialic acid, the SBP used here is composed of Neu5Gc and 9-O-sulfated Neu5Gc residues in a molar proportion of 5:1, and 58% of the total Neu5Gc were released from the SBP by exhaustive sialidase treatment. Therefore, the sialidase treatment was extensive but not complete. This result may be due to the presence of 9-O-sulfated Neu5Gc residues, which are sialidase-resistant. Since the sialidase-treated SBP was composed of Neu5Gc and 9-O-sulfated Neu5Gc (1.5:1, mol/mol), sulfate group-capped oligomers with on average two or three Neu5Gc residues are estimated to remain undigested in the molecule. This estimation is consistent with the previously reported average chain length of the oligoNeu5Gc moieties (31). Binding experiments using sialidase-treated SBP showed that desialylation of 58% resulted in loss of divalent cation-dependent enhancement of binding. This result indicates that the sialylated carbohydrate moieties in particular sites of SBP molecule may be involved in the divalent cation-dependent enhancement of binding activity. In this regard, it should be noted that oligo- or polymeric forms of sialic acid are known to bind Ca$^{2+}$ with relatively high affinity (29). Thus, complex formation of the particular sialyl carbohydrate moieties with divalent cations may stabilize the functional structure of SBP. Alternatively, the divalent cation-carbohydrate complex may possibly be involved in direct binding to the gangliosides through carbohydrate-carbohydrate interaction. This interaction is possible because of the fact that acrosome-reacted sperm can bind carbohydrate chains that are released from SBP and conjugated with bovine serum albumin (19). Furthermore, we cannot exclude the possibility that alterations of higher order structure of SBP by removal of sialic acid residues affect the binding ability to the gangliosides as well as its divalent cation-dependent property. It is possible that the sialidase-treated SBP forms such a complex that reduces the binding ability either via the underlying carbohydrates or via other ionic or hydrophobic interactions, since the sialic acid content of SBP is high enough to prevent aberrant complex formation. However, an underlying mechanism for these divalent cation- and/or carbohydrate-dependent binding properties should be elucidated by further experiments, e.g. binding experiments between the carbohydrate moieties of SBP and the sperm gangliosides in the presence and absence of Ca$^{2+}$.

It is also noted that native and recombinant SBPs show different binding specificities against the two sperm gangliosides, Neu5AcGlcCer and (Neu5Ac)2GlcCer. Thus, SBP binds these gangliosides with comparable affinity, while the recombinant SBP, (96–586), binds Neu5AcGlcCer better than (Neu5Ac)2GlcCer. We currently do not know the reason for the difference, but the large C-terminal and the heavy carbohydrate parts of native SBP, both of which are deficient in the recombinant SBP, may affect the binding specificity. For example, as described above for unique binding property of the sialidase-treated SBP, a particular conformation of the sialic acid-binding domain of the recombinant SBP due to carbohydrate deficiency, unmasking effects of the sialic acid-binding domain occupied by the sialic acid residues on the same SBP molecule, or formation of such aberrant complex with low binding ability to the gangliosides may cause the change in substrate specificity. These issues should be addressed in future studies.

Previously, we showed that sperm gangliosides are highly enriched in the lipid rafts or the LD-DIM (6). The sperm lipid rafts of sea urchin are suggested to be the sites of sperm-egg interaction as well as subsequent signal transduction, because the receptor and transducer proteins are co-localized in the lipid rafts (7). We have tested if SBP binds sperm LD-DIM in order to determine whether binding of SBP to sperm ganglioside is involved in interaction and signaling on the LD-DIM. The results showed that SBP bound sperm LD-DIM and that this binding was mainly dependent on sialic acid residues on the LD-DIM. We previously showed that sulfatide is also enriched in the LD-DIM, and amounts to two-thirds of the gangliosides (6). However, because binding of SBP to sialidase-treated LD-DIM is very low, sulfated Gal residues in the LD-DIM are not critical for interaction with the SBP. It is thus concluded that sperm is associated with the genus-nonspecific SBD of SBP through the gangliosides present on sperm lipid rafts. In order to elucidate the significance of this interaction in
the context of fertilization processes, effects of co-incubation of sea urchin sperm and eggs with phosphatidylcholine/cholesterol liposomes containing the sperm ganglioside (Neu5Ac)2GlcCer were examined. The sperm ganglioside-containing liposomes inhibited fertilization, while the liposomes without ganglioside had no effects. Furthermore, we have recently shown that fertilization is inhibited by the two anti-sperm ganglioside antibodies mAb.3G9 and mAb.2A11, and the Fab fragment of mAb.3G9.2 This result clearly indicates that gangliosides on the sperm surface are important at fertilization.

It has been shown that SBP is largely localized in the vitelline layer and plays a role in species-specific binding to the vitelline layer by acrosome reacted sperm (18, 39, 40). During passage through the vitelline layer, SBP may interact with at least two kinds of molecules on the sperm surface, bindin and gangliosides. Previously, we reported that bindin was not detected on sperm lipid rafts (6). However, we have most recently found that bindin is associated with sperm lipid rafts when they are prepared in a way that more closely approximates physiological conditions.4 Therefore, bindin and gangliosides appear to be co-localized in sperm lipid rafts, enabling SBP to be associated with the lipid rafts. Thus, SBP mediates protein-protein interaction with bindin as well as the carbohydrate interaction with gangliosides on lipid rafts. It would be interesting to reveal how these two different interactions are coordinated during the initial binding of sperm to the vitelline layer and guidance of bound sperm to the plasma membrane at fertilization.

Finally, the importance of this study is 2-fold. First, SBP is a new type of sialic acid-binding lectin that belongs to the Hsp110 family, a distinct subfamily of the Hsp70 families of molecular chaperones. The Hsp110-like domain of the molecule is responsible for recognition of sialic acids. This is the first report that the Hsp-related proteins bind sialic acids. Second, this sialic acid-binding lectin functions in an important biological context, i.e. the initial binding to and passage through the vitelline layer (an extracellular matrix protein layer of egg), to allow the sperm to reach the egg plasma membrane. The binding of this lectin to lipid rafts through both protein-protein and protein-carbohydrate interactions may occur during the process of sperm-egg membrane interaction and fusion.

Acknowledgment—We thank Dr. Kazunari Akiyoshi (Kyoto University) for his help in preparation of the liposomes containing the sperm gangliosides.


REFERENCES

Identification of the Sea Urchin 350-kDa Sperm-binding Protein as a New Sialic Acid-binding Lectin That Belongs to the Heat Shock Protein 110 Family: IMPLICATION OF ITS BINDING TO GANGLIOSIDES IN SPERM LIPID RAFTS IN FERTILIZATION
Eri Maehashi, Chihiro Sato, Kaoru Ohta, Yoichiro Harada, Tsukasa Matsuda, Noritaka Hirohashi, William J. Lennarz and Ken Kitajima

doi: 10.1074/jbc.M307493200 originally published online August 12, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307493200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 38 references, 12 of which can be accessed free at http://www.jbc.org/content/278/43/42050.full.html#ref-list-1