A major flagellum sialoglycoprotein in sea urchin sperm contains a novel polysialic acid, an α2,9-linked poly-N-acetylnuraminic acid chain, capped by an 8-O-sulfated sialic acid residue

Shinji Miyata\(^2\), Chihiro Sato\(^2,3\), Shigeyuki Kitamura\(^2\), Masaru Toriyama\(^4\), and Ken Kitajima\(^1,2,3,5\)

\(^2\)Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan; \(^3\)Bioscience and Biotechnology Center, Nagoya University, Nagoya 464-8601, Japan; \(^4\)Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Ohyoshi, Shizuoka 422-8529, Japan; and \(^5\)Institute for Advanced Research, Nagoya University, Nagoya 464-8601, Japan

Received on April 7, 2004; revised on May 15, 2004; accepted on May 21, 2004

A new type of polysialic acid (polySia) structure was demonstrated to occur in a major unknown sialoglycoprotein with a diverse molecular mass of 40–80 kDa in sea urchin sperm. The polySia-containing glycan structure was determined to be HSO\(_3\)-8Neu5Ac\(_2\)-9(Neu5Ac\(_2\)-9)\(_n\)-2 Neu5Ac\(_2\)-6GalNAc\(_2\)-Ser/Thr (n, on average 15), based on carbohydrate analysis of the sialoglycopeptide obtained by an exhaustive protease digestion of whole sperm, fluorometric anion-exchange high-performance liquid chromatography, and methylation analysis. The sulfate group was predominantly localized to the nonreducing terminus of the polySia chain. This is the first example of an α2,9-linked polySia structure in animal sperm. The polySia-containing sialoglycoprotein was present in sperm flagellum but not in the head. Furthermore, this sialoglycoprotein localized in the sperm lipid raft, which contains an enriched ganglioside (Neu5Ac\(_2\)-8Neu5Ac\(_2\)-6GlcCer), a receptor for sperm-activating peptide (speract), and its associated guanylate cyclase (Ohta \textit{et al.} [2000] \textit{Glycoconj. J.}, 17, 205–214).

Key words: fertilization/polysialic acid/sea urchin/sperm flagellum/sulfated sialic acid

Introduction

Glycan chains on the cell surface have a pivotal role in cell recognition and adhesion in various biologic processes (Varki, 1993). In surface glycan chains, sialic acid (Sia) residues are one of the most important glycan units of the biologic recognition systems, such as host cell–microorganism interactions (Karlsson, 1995), cell–cell and cell–substratum interactions in neuronal development (Ledeen \textit{et al.}, 1998; Rutishauser, 1996), and lymphocyte–endothelial cell interactions (Lasky, 1992). Sia is a family of nine-carbon carboxylated sugars containing nearly 50 members that are derivatives of N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (KDN; 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) (Angata and Varki, 2002).

We recently demonstrated that cell surface Sia residues have an important role in sperm–egg interactions during sea urchin fertilization (Maehashi \textit{et al.}, 2003; Ohta \textit{et al.}, 1999, 2000). Unique Sia-containing glycoconjugates are present in sea urchin sperm and eggs (Hoshi and Nagai, 1975; Ijuin \textit{et al.}, 1996; Kitazume \textit{et al.}, 1994, 1996; Kubo \textit{et al.}, 1990). In the egg jelly and plasma membrane–vitelline layer of Hemicentrotus pulcherrimus and Strongylocentrotus purpuratus, there is a polymeric structure of α2–5Oglycophosphate Neu5Gc in the glycoproteins (Kitazume \textit{et al.}, 1994, 1996). This Neu5Gc polymer is often sulfated (Kitazume \textit{et al.}, 1996) in the egg plasma membrane and vitelline layer. The sulfated and unsulfated Neu5Gc-containing gangliosides, ±HSO\(_3\)-8Neu5Gc\(_2\)-6Glc-Cer, are major egg glycolipid components (Kubo \textit{et al.}, 1990). In sperm, a major ganglioside, Neu5Ac\(_2\)-8Neu5Ac\(_2\)-6Glc-Cer, is sometimes sulfated at the nonreducing terminal Neu5Ac residue (Ijuin \textit{et al.}, 1996). Little is known about the biologic significance of these Sia residues in eggs and sperm. We recently demonstrated, however, that sea urchin fertilization is inhibited by liposomes containing sperm gangliosides (Maehashi \textit{et al.}, 2003) as well as by antibodies against sperm gangliosides (Maehashi \textit{et al.}, unpublished data).

Notably, these sperm gangliosides are enriched in the membrane microdomain (the lipid raft). Lipid rafts are characterized by the colocalization of receptor and transducer proteins (Anderson and Jacobson, 2002; Brown and London, 1998; Simons and Toomre, 2000). These features suggest that the sperm lipid raft functions as the site for ganglioside-mediated interactions and subsequent signal transduction (Ohta \textit{et al.}, 1999; Ohta \textit{et al.}, 2000). The sperm lipid raft is bound by 350-kDa sperm-binding protein (SBP), during which a Sia-recognition domain of SBP is a new sialic acid–binding lectin, designated Hsp-like lectin (Maehashi \textit{et al.}, 2003). SBP is localized mainly in the egg vitelline layer (Hiroshita and Lennarz, 1998), and therefore interactions between SBP and the sperm lipid raft could be important for sperm penetration through the vitelline layer to the egg plasma membrane.

The sperm lipid raft contains Sia residues on glycoproteins as well as on gangliosides. To gain a further insight into biologic significance of Sia residues in the lipid rafts, we thus began to study Sia residues on glycoproteins.

\(^1\)To whom correspondence should be addressed; e-mail: kitajima@agr.nagoya-u.ac.jp
Interestingly, gangliosides and glycoproteins in sperm share common glycan epitopes, such as the 8-O-sulfated Neu5Ac (Neu5Ac8S) structure (Miyata et al., unpublished data). We identified several known glycoproteins and an unknown sialoglycoprotein as Neu5Ac8S-containing glycoproteins. This unknown sialoglycoprotein is unique because it contains a Neu5Ac8S epitope common to the sperm ganglioside and because it has a diverse molecular mass ranging from 40 to 80 kDa. Furthermore, it is a major sialoglycoprotein in sea urchin sperm and appears to contain a poly saccharide (polySia) structure (a polymeric structure of sialic acid) with different properties from α2,8-linked polySia, which is commonly found in bacteria and animals (Mühlenhoff et al., 1998; Troy, 1996).

The aim of the present study was to determine the precise structure of the novel polySia on the unknown glycoprotein and to elucidate the sub-localization of this novel glycoprotein in sperm. We demonstrated that the novel polySia chain is made up of a polymerized form of α2,9-linked Neu5Ac residues and is capped by a Neu5Ac8S residue. It is attached to the major sialoglycoprotein in sea urchin sperm. This is the first report of polySia-containing glycoprotein in animal sperm. We also demonstrated that this glycoprotein is localized in the flagellum of sperm. Notably, it is present in the sperm lipid raft, which suggests that the polySia chain has a regulatory function in some lipid raft-mediated interactions in sperm activation.

Results

Preparation of a major Sia-containing glycopeptide fraction (SGP) from sea urchin sperm

Dry sperm (150 ml) was delipidated and digested with Actinase E. The digested product contained approximately 17 mg Sia. It was then chromatographed on a Sephadex G-50 column to give two Sia-positive peaks (Figure 1A). The flow-through fraction was then subjected to DEAE-Toyopearl 650 M anion-exchange chromatography. The Sia-positive fractions eluted at 0.25–0.38 M NaCl were pooled (Figure 1B). The pooled fractions were further purified by successive chromatography on Sephacryl S-300 (Figure 1C) and Sephacryl S-100 columns (data not shown). Fractions under a single peak containing Sia were pooled and designated SGP. About 85% of total Sia in delipidated sperm was recovered in SGP.

Carbohydrate composition of SGP

The carbohydrate composition of SGP is shown in Table I. Only two monosaccharide components, GalNAc and Sia, were detected in 1.0:16.0 (mol/mol) proportion. The Sia species was identified as Neu5Ac (see later discussion). This composition suggests the presence of O-linked glycan chains. After alkaline borohydride treatment, all GalNAc residues were converted to GalNAccol (Table I). These data indicate that all the GalNAc residues in SGP are attached to Ser/Thr residues of the core peptide.

As already described, some sperm glycoproteins are recognized by monoclonal antibody (mAb) 3G9, which binds to the Neu5Ac8S epitope (see Figures 11 and 12 later). Therefore, 1,2-diamino-4,5-methyleneoxybenzene (DHB) derivatization/fluorometric high-performance liquid chromatography (HPLC) analysis was performed to chemically demonstrate the presence of the sulfated Sia residues in SGP. The HPLC profile of the authentic DMB

Fig. 1. Preparation of SGP from the Actinase E digest of sea urchin sperm. (A) Sephadex G-50 chromatography of the Actinase E digest of delipidated H. pulcherrimus sperm. The column (1.5 x 57.5 cm) was equilibrated and eluted with 0.1 M NaCl. V0, void volume. Vt, total volume. Major Sia-containing fractions (fractions 28–38) indicated by the bar were pooled. (B) DEAE-Toyopearl 650 M chromatography of the major Sia-containing fraction in (A). The fraction was applied to a DEAE-Toyopearl 650 M column (Cl− form; 2.2 x 15 cm), and eluted with a linear gradient of NaCl (0–0.6 M) in 10 mM Tris–HCl (pH 8.0). The NaCl concentration is shown by the straight line. The Sia-containing fractions (fractions 52–75 at 0.25–0.38 M NaCl) were pooled as indicated by the bar. (C) Sephacryl S-300 chromatography of the pooled fraction in (B). The column (0.8 x 95 cm) was eluted with 0.1 M NaCl. V0, void volume. Vt, total volume. The major Sia-containing fractions (fractions 60–67) were pooled as indicated by the bar and designated SGP. Elution profiles were monitored by absorbance (open circles) at 230 nm (A230) in A and B or 210 nm (A210) in C, and by the sialic acid analysis by the thiobarbituric acid method (closed circles; A549 in A and B) or the resorcinol method (closed circles; A580 in C).
derivatives of sulfated and unsulfated Sia is shown in Figure 2A. All six derivatives could be separated from each other. The HPLC profile for SGP gave two peaks that corresponded to the DMB derivatives of Neu5Ac8S and Neu5Ac (Figure 2B). Neu5Gc was not detected. The molar ratio of Neu5Ac8S to Neu5Ac was 1.0:5.0.

Structural elucidation of the PolySia chains in SGP
Anion-exchange HPLC of sialoglycan alditols released from SGP by the alkaline borohydride treatment. The high Neu5Ac/GalNAc molar ratio in SGP (Table I) suggests the presence of a polySia structure. The anion-exchange HPLC profile of the sialoglycan alditols released from SGP by the alkaline borohydride treatment is shown in Figure 3. The released glycans gave a series of peaks with degrees of polymerization (DP) ranging from 2 to ~20 (similar to the DPs for α2,8-linked oligo/polyNeu5Ac). These results suggest that there are a series of polySia structures in SGP. The average DP was estimated to be 15, based on the molar ratio of Neu5Ac/GalNAcol (Table I).

Mild acid hydrolysis/thin-layer chromatography (TLC) analysis of SGP. Mild acid hydrolysis/TLC analysis was performed to characterize the polySia structure in SGP. When treated at pH 4.8 at 50°C for 4 h, SGP gave no oligomers of Sia, whereas the α2,8-linked polyNeu5Ac chain gave a series of oligoNeu5Ac (data not shown). SGP was then partially hydrolyzed under stronger conditions with 0.01 N trifluoroacetic acid (TFA) at 50°C for 2 h. A ladderlike profile of bands was obtained on TLC of the hydrolysate (Figure 4A). The fastest migrating band contained both Neu5Ac and Neu5Ac8S. None of the other bands in Figure 4A (lane 2), however, corresponded to the bands for a series of α2,8-linked oligo/polyNeu5Ac (Figure 4A, lane 1). These results suggest that the ketosidic linkages in polyNeu5Ac of SGP are not α2,8-linkages.

Endo-N-acylneuraminidase (Endo-N) digestion of SGP. Endo-N catalyzes the hydrolysis of α2,8-linked oligo/polySia with DPs of at least five and more (Hallenbeck et al., 1987). SGP was digested with Endo-N, and the digest was analyzed by TLC. SGP was resistant to depolymerization by Endo-N (Figure 4B, lane 4). In contrast, authentic α2,8-linked polyNeu5Ac was depolymerized (Figure 4B, lane 2). These results are consistent with the conclusion that Neu5Ac residues in SGP are joined by a different type of ketosidic linkage than the usual α2,8-ketosidic linkage.

Periodate fluorometric C7/C9 analysis of SGP. α2,8-linked polyNeu5Ac is resistant to depolymerization by periodate oxidation, and all internal Neu5Ac residues remained intact [C9(Neu5Ac)] on fluorometric C7/C9 analysis. The nonreducing terminal residue was oxidized to give the C7 compound [C7(Neu5Ac)] (Rohr and Troy, 1980). In contrast, all the Neu5Ac residues in α2,9-linked polyNeu5Ac were sensitive to periodate oxidation and produced C7(Neu5Ac) (Bhattacharge et al., 1975; Egan et al., 1977). Consistent with these descriptions, authentic α2,8-linked polyNeu5Ac and α2,9-linked polyNeu5Ac gave exclusive peaks of DMB derivatives of C9(Neu5Ac) and C7(Neu5Ac), respectively, on fluorometric C7/C9 analysis (Figures 5A and 5B). Therefore, SGP was subjected to fluorometric C7/C9 analysis to determine the ketosidic linkage of polyNeu5Ac chains in SGP. The majority (85%) of Neu5Ac residues in SGP were converted to C7(Neu5Ac), and the remaining DMB derivative of Sia (i.e., 15%) in SGP was Neu5Ac8S (Figure 5C). There were trace amounts of C9(Neu5Ac). The presence of

---

**Table I. Carbohydrate composition of SGP before and after alkaline-borohydride treatment**

<table>
<thead>
<tr>
<th></th>
<th>GalNAc</th>
<th>GalNAcol</th>
<th>Neu5Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>1.0</td>
<td>NDb</td>
<td>16</td>
</tr>
<tr>
<td>After treatment</td>
<td>NDb</td>
<td>1.0</td>
<td>15</td>
</tr>
</tbody>
</table>

aValues are molar ratios relative to GalNAc or GalNAcol taken as 1.0.
bNot detected.
Neu5Ac8S is consistent with the result in Figure 2. Together, these results indicate that the ketosidic linkages of the polyNeu5Ac chains in SGP were exclusively α2,9-linked.

Mild acid hydrolysis/fluorometric anion-exchange HPLC analysis. To further characterize the polySia chains of SGP, mild acid hydrolysis/fluorometric anion-exchange HPLC analysis was performed. The HPLC profile of...
DMB derivatives of sialyl oligo/polymers released from SGP by mild acid hydrolysis is shown in Figure 6A. SGP contained only Neu5Ac and Neu5Ac8S (Figure 2), and therefore polySia chains of SGP consisted of these two Sia species. A semi-logarithmic plot of the eluted NaCl concentration of the peak for the DMB derivative of each oligo/polymer against the tentative DP is shown in Figure 6C. This plot is considered a structure-HPLC parameter correlation, and the plots for a series of Sia homo-oligomers should be on a straight line (Kitazume et al., 1992). The two plots depicted are linear; one is a series of peak numbers 3, 4, 5 . . . and the other is for S2, S3, S4, and S5 fractions. These results suggest that two different series of homo-oligomers are present in the oligo/polymers of Sia from SGP.

Because sulfated Sia residues are resistant to exosialidase digestion (Ijuin et al., 1996; Kitazume et al., 1996; Kubo et al., 1990), DMB derivatives of Sia that remain after exosialidase digestion are considered to be sulfated oligo/polySia chains. Peaks S1, S2, S3, S4, and S5 in Figure 6A were resistant to exosialidase (Figure 6B). S1 eluted at the same position as the DMB derivative of the authentic Neu5Ac8S monomer. The DMB/HPLC analysis of the pooled S1, S2, S3, S4, and S5 fractions revealed the presence of Neu5Ac8S in these peak fractions (data not shown). These peaks are on the same straight line (Figure 6C), suggesting that S2, S3, S4, and S5 are 8-O-sulfated Neu5Ac linked to monomers, dimers, trimers, and tetraters of Neu5Ac, respectively. The intensity proportion of these peaks remained unchanged after exosialidase digestion, except for peaks S4 and S5, which comigrated with peaks 8 and 9, respectively, before exosialidase digestion. This result suggests that the nonreducing terminus of each oligosialyl chain is capped by a Neu5Ac8S residue in S2–S5 and that no additional Neu5Ac or Neu5Ac8S residue is present on the terminal Neu5Ac8S residue.

The peaks designated as 2, 3, 4, for DMB derivatives of oligo/polyNeu5Ac with the DPs of 2, 3, 4 . . . in SGP were all exosialidase-sensitive (Figures 6A and 6B). To determine the ketosidic linkages in these oligo/polyNeu5Ac, authentic DMB derivatives of a series of oligo/polyNeu5Ac with the α2,8- and α2,9-ketosidic linkages were coinjected into the HPLC column with the DMB derivatives of SGP-derived oligo/polyNeu5Ac chains. DMB derivatives of α2,8-linked polyNeu5Ac and those of α2,9-linked polyNeu5Ac were separately eluted for dimers up to hexamers and o-eluted for heptamers up to at least octadecamers (Figure 7A). The exosialidase-sensitive peaks derived from SGP exhibited a simple profile when cochromatographed with the DMB derivatives of authentic α2,9-linked polyNeu5Ac (Figure 7B), whereas there was a series of doublet peaks when cochromatographed with the DMB derivatives of authentic α2,8-linked polyNeu5Ac (Figure 7C). These results demonstrate that the ketosidic linkages of the exosialidase-sensitive oligo/polyNeu5Ac in SGP were α2,9-linkages.

Methylation analysis/GLC-MS of polySia in SGP. Per-methylated SGP was methanolyzed, acetylated, and analyzed by gas-liquid chromatography (GLC) mass spectrometry (MS). A total ion chromatogram and a mass chromatogram (mass: m/z 129, a characteristic fragment ion of permethylated derivatives of Neu5Ac, as shown in Figure 8D) for permethylated Neu5Ac derivatives obtained
The mass spectra confirmed that peaks a and b (retention time 12.5 and 14.0 min, respectively) were identical to those of the 2,4,7,8-tetramethyl Neu5Ac methyl ester obtained from authentic 2,9-linked polyNeu5Ac. The presence of 9-O-acetyl-2,4,7,8-tetramethyl Neu5Ac methyl ester confirmed that the ketosidic linkages of Neu5Ac residues in SGP were α2,9-linkages.

Elucidation of the core structure of polySia-containing O-glycan chains

As described, all GalNAc residues were O-glycosidically attached to Ser/Thr of SGP and were linked by the α2,9-linked polyNeu5Ac residue. To determine the linkage between GalNAc and Neu5Ac, methylation analysis/GLC-MS of glycans obtained by alkaline borohydride treatment of SGP was performed. All GalNAc were converted to GalNAcO in the glycans (Table 1). A mass spectrum of the peak corresponding to authentic 6-O-substituted GalNAcO on a gas chromatogram (data not shown) is shown in Figure 9. The mass spectrum shows a typical profile for 6-O-acetyl-1,3,4,5-tetramethyl-2-deoxy-2-(N-acetylamino)-galactitol, indicating the presence of 6-O-substituted GalNAcO. GLC-MS revealed no terminal, 3-O-substituted, or 3,6-O-substituted GalNAcO. These results confirm that the reducing terminal structure was Neu5Acα2→6GalNAc1→Ser/Thr. Supporting evidence for this terminal structure was obtained by periodate oxidation/GLC analysis of SGP. Periodate oxidation destroys 6-O-substituted GalNAc residues but not 3- or 4-O-substituted GalNAc residues. Composition analysis of SGP after periodate oxidation shows no GalNAc, indicating that all the GalNAc residues in SGP were sensitive to periodate oxidation (data not shown). Together our results indicate that all GalNAc residues were located at the reducing termini of novel 8-O-sulfated α2,9-linked polyNeu5Ac-containing O-glycan chains of SGP and were joined with Neu5Ac through an 2,6-linkage (Figure 10).

Localization of the sulfated polySia-containing glycoprotein in sea urchin sperm

Sperm are highly compartmentalized, terminally differentiated cells, composed of a head, containing an acrosomal vesicle and a nucleus, and a long flagellum. Thus localization of the novel 8-O-sulfated α2,9-linked polyNeu5Ac-containing glycoprotein in sperm was examined. The head and flagellum fractions were prepared from fresh sea urchin sperm and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)/immunostaining using mAb 3G9, which recognizes Neu5Ac8S residues. As shown in Figure 11A, the 3G9 epitope was exclusively localized to the flagellum fraction. The head and flagellum fractions were also subjected to mild acid hydrolysis/fluorometric anion-exchange HPLC analysis. A typical profile for
DMB derivatives of the oligo/polyNeu5Ac released from SGP was obtained for the flagellum fraction as well as whole sperm, whereas there was no such profile for the head fraction (Figure 11B). These results indicate that the unique sulfated oligo/polyNeu5Ac-containing glycoprotein was confined to the flagellum. A dimer and a trimer of α2,8-linked Neu5Ac as well as Neu5Ac8S were also detected in the head and flagellum fractions and whole sperm (Figure 11B). Because the head and flagellum fractions were not delipidated, these components derive from known gangliosides (Ijuin et al., 1996) or unknown glycoproteins.

Localization of the sulfated polySia-containing glycoprotein in the sperm lipid raft
To examine which glycoprotein in sperm contains the sulfated polySia chains, we performed immunochemical and chemical analyses of glycoprotein components that were separated by SDS-PAGE followed by electronic transfer.
to a polyvinylidene difluoride (PVDF) membrane. The Neu5Ac8S-containing glycoproteins were detected as a smear from 40 to 80 kDa in addition to discrete bands at 220, 137, and 80 kDa (Figure 12A, IB:3G9). Consistent with these results, fluorometric HPLC analysis of the pieces cut from the PVDF membrane revealed that Neu5Ac8S was detected in all the pieces, and 83% of the Neu5Ac8S was present in piece numbers 6–10 (Figure 12A, Neu5Ac8S). These results also indicate that 83% of total Neu5Ac8S residues in sperm glycoproteins were present in the 40–80 kDa molecular mass range. The α2,9-linked Neu5Ac residues were exclusively detected in piece numbers 6–10 or in the 40–80 kDa range (Figure 12A). The quantity of the 2,9-linked Neu5Ac residues in each piece was calculated based on the summation of peak areas of a series of oligo/polySia components with DPs of at least 3 on the fluorometric anion-exchange HPLC. The α2,9-linked Neu5Ac residues are present exclusively in the 40–80 kDa range, and the residue amount accounts for ~80% of the total Neu5Ac in sperm glycoproteins. These results indicate that almost all Neu5Ac8S-capped α2,9-linked polySia structures were glycoproteins in the 40–80 kDa range. Because 85% of Sia residues in total sperm glycoproteins were present in SGP as described, we concluded that SGP originated from the 40–80-kDa glycoproteins. As shown in Figure 3, the O-linked polysia-containing glycans released from SGP by alkaline borohydride treatment shows an extensive heterogeneity in the DP as well as in negative charge, and this heterogeneity might have caused the diffuse SDS–PAGE profiles in the 40–80 kDa range. This finding suggests that the 40–80-kDa glycoprotein is a carrier protein of the sulfated α2,9-linked polysia glycans.

Previously, we reported that the 3G9-positive sulfated ganglioside, HSO3⁻8NeuAcα2→9( NeuAcα2→9)ₙ-2NeuAca2→6GalNac1α→(Xaa-Ser/Thr-Xaa-N), was concentrated in sperm lipid rafts. As described here, there is evidence that the Neu5Ac8S-capped α2,9-linked polySia is present in the 40–80-kDa glycoprotein and is recognized by mAb 3G9. Therefore, to determine
whether the 40–80-kDa glycoprotein was also localized in the sperm lipid raft, we performed western blotting of the sperm lipid raft. About 10% of the 40–80-kDa glycoprotein was present in the lipid raft fraction, and the rest was found in the non–lipid raft fraction (Figure 12B). The localization of this glycoprotein to the lipid raft indicates that the 3G9 epitope common to glycolipids and glycoproteins was colocalized in the lipid raft.

We do not know the biologic significance of the common 3G9 epitope, but a sulfate group in the 3G9 epitope might protect the Neu5Ac residue from degradation by sialidase digestion and could provide clustered epitopes recognized by an unknown carbohydrate-binding molecule.

Discussion

The present study demonstrated that a new type of polySia, 8-O-sulfated Neu5Ac-capped α2,9-linked polyNeu5Ac, occurs in SGP (a major sialoglycopeptide fraction) prepared from sea urchin sperm and that it is attached to the 6-position of a GalNAc residue that is α-O-glycosidically linked to Ser/Thr residues of the peptide. (On proton nuclear magnetic resonance spectra of SGP, signals for α-anomeric protons of GalNAc were observed; Miyata et al., unpublished data.) The proposed carbohydrate structure is shown in Figure 10. Based on the molecular mass of SGP and an average molecular mass of a sialoglycan, a molecule of SGP is estimated to contain four to five sialoglycan chains. The α2,9-linkage of the oligo/polySia is strongly suggested by the reactivity toward periodate oxidation as estimated on fluorometric C7/C9 analysis, the comigration of mild acid hydrolysate of SGP with authentic oligo/polySia on anion-exchange HPLC, and the sensitivity toward exosialidase specific to the α-configuration of Sia residue. These analyses, however, cannot exclude the possibility of an α2,4-linked oligo/polyNeu5Ac structure. Therefore methylation analysis was performed to firmly establish an exclusive α2,9-linkage. The DP of the α2,9-linked oligo/polyNeu5Ac chain is estimated to be 15 from the Neu5Ac/GalNAc ratio and ranges from 2 to 20 on the anion-exchange chromatography. 8-O-sulfation often occurs in the oligo/polyNeu5Ac chain. About 17% of the total Neu5Ac in SGP was modified by sulfate. Considering that the average DP of the oligo/polyNeu5Ac was 15, it is likely that both the terminal and internal residues were sulfated. (If sulfation were to occur exclusively at the nonreducing termini, then 7% of the total Neu5Ac would be sulfated Neu5Ac residues.) The fluorometric anion-exchange HPLC of mild acid hydrolysate of SGP suggested that sulfation occurred predominantly at nonreducing termini of oligo/polyNeu5Ac structures. We do not know the reason for this, but acid instability of the sulfate group in the oligo/polyNeu5Ac structure could have been responsible, and this possibility will be examined in the future.

Naturally occurring polySia structures have a large diversity, primarily arising from the diversity in the Sia components (Neu5Ac, Neu5Gc, and KDN), and in the intersialyl linkages (α2,5, α2,8, α2,9, and α2,8/9) (Troy, 1996). The α2,8-linked polySia structure is the most common in...
S. Miyata et al.

Fig. 12. Characterization of the Neu5Ac8S-capped oligo/polyNeu5Ac-containing glycoprotein. (A) Quantification of Neu5Ac8S and α2,9-Neu5Ac residues in glycoprotein components of sea urchin sperm. Whole sperm lysate was subjected to the SDS–PAGE, followed by electronic blotting on the PVDF membrane. The PVDF membrane was cut into 12 equal pieces according to molecular masses. Each piece was analyzed for the quantity of total Neu5Ac (Total Neu5Ac) and Neu5Ac8S (Neu5Ac8S) by the fluorometric HPLC method and α2,9-linked Neu5Ac residues by fluorometric anion-exchange HPLC method (α2,9-Neu5Ac) as described under Materials and methods. Western blotting of the whole sperm lysate using mAb 3G9 (IB: 3G9) is also shown. (B) Western blotting of the detergent-insoluble membrane fraction (the raft fraction), 1 μg/lane (lane 1), and the detergent-soluble membrane fraction (the non-raft fraction), 1 μg/lane (lane 2), using mAb 3G9. Standard molecular masses are indicated by the bars with numbers.

different glycoproteins. This structure has a DP ranging from 8 to 200 sialyl residues and occurs in capsular components of neuroinvasive bacteria (Bhattacharjee et al., 1975; Rohr and Troy, 1980), such as Escherichia coli K1 and Neisseria meningitidis group B, and glycoproteins of fish eggs (Sato et al., 1993), mammalian brain (Finne, 1982), and breast milk (Yabe et al., 2003). In mammals, only three polySia-containing glycoproteins have been identified: neural cell adhesion molecules, the α-subunit of the voltage-sensitive sodium channel (Zuber et al., 1992), and CD36 from human and mouse milk (Yabe et al., 2003). In contrast, an α2,9-linked polySia structure exists in capsular polysaccharides of N. meningitidis group C (Bhattacharjee et al., 1975), but it is found much less frequently in glycoproteins. The presence of the α2,9-linked disialic acid structure (Neu5Acα2→9Neu5Acα2→9) in glycoproteins was first established in human teratocarcinoma cells (Fukuda et al., 1985). Recently, the existence of an α2,9-linked polySia structure has been suggested in an unidentified protein in mouse neuroblastoma cells, based on its sensitivity toward periodate oxidation and the HPLC elution profile (Inoue et al., 2002). Our study unequivocally demonstrates the presence of α2,9-linked polyNeu5Ac structure in sea urchin sperm. Importantly, this finding confirms the ubiquitous occurrence of the α2,9-linked polyNeu5Ac structure in animal glycoproteins and also extends the diversity of polySia structures.

Sulfation of polySia structure is exemplified in the glycopeptides of the plasma membrane–vitelline layer of sea urchin eggs, where a sulfate group caps the terminus of α2,5Oglycolyl-linked polyNeu5Gc structure (Kitazume et al., 1996). Although the 8-O-sulfated Neu5Ac residues have been shown to occur in gangliosides of sea urchin sperm, this is the first example of the presence of 8-O-sulfated Neu5Ac residues in polySia structures. Sulfation of Sia residues might serve as a stop signal for the elongation of polySia chain as well as a protection mechanism for the polySia structure, because the sulfated Sia residues are resistant to bacterial sialidases. Considering its presence at the sperm surface, sulfated Sia might be important for recognition processes during sperm–egg interaction at fertilization.

Escherichia coli K1 expresses α2,8-linked polySia as its capsular polysaccharide, whereas E. coli K92 expresses alternating α2,8/2,9-linked polySia residues. Genes for these polysialyltransferases have been cloned, and Steenbergen and Vimr (2003) recently demonstrated that the 30 amino acids in the N-terminal region of these polysialyltransferases are important for their linkage specificity. In mammals, six α2,8-sialyltransferases (ST8Sia I to VI) are involved in the formation of α2,8-linkages (Harduin-Lepers et al., 2001). Among them, ST8Sia II and ST8Sia IV are considered to be responsible for α2,8-linked polySia structures (Angata and Fukuda, 2003). To date, no α2,9-sialyltransferase gene has been reported in any animal. Two completely different types of polySia structures have now been demonstrated in sea urchin: α2,5Oglycolyl-linked polySia in egg jelly (Kitazume et al., 1994) and vitelline layer glycoproteins (Kitazume et al., 1996) and α2,9-linked polySia in sperm glycoprotein. In addition, α2,8-linked dimeric to tetrameric
structures are present in sperm glycolipids (Hoshi and Nagai, 1975; Ijui et al., 1996). Thus three different types of ketosidic linkage are biosynthesized in sea urchin. Experiments are currently under way in our laboratory to clone the genes for these sialyltransferases and to elucidate the structure-specificity relation of the enzymes to better understand the origin of the structural diversity of polySia.

The present study demonstrated that 100% of the sulfated Neu5Ac-capped α,2,9-linked oligo/polyNeu5Ac present in whole sperm glycoproteins resides in the 40–80-kDa glycoprotein, and SGP is obtained from this glycoprotein by Actinase E digestion. The dispersed nature of this glycoprotein on SDS–PAGE appears to be due to heterogeneity in the DP of the α,2,9-linked oligo/polyNeu5Ac in a number of O-glycans on the polypeptide backbone. In addition, this glycoprotein is difficult to stain with Coomassie brilliant blue. These unique properties have prevented it from being identified before. Thus the 40–80-kDa protein is considered a novel glycoprotein, which we are now trying to identify. Biologic function of the 40–80-kDa glycoprotein containing the sulfated polySia structure in sperm is exclusively localized in the sperm flagellum. The function of this new polySia glycoepitope is not known and is currently under study in our laboratory.

Materials and methods

Materials

H. pulcherrimus were purchased from the local fisheries at Tsushima and Fukushima, Japan. Sperm was collected by intracellular introduction of 0.5 M KCl, and stored at –30°C until use. The sperm lipid raft was prepared as described previously (Ohta et al., 1999, 2000). Arthrobacter ureafaciens sialidase was purchased from Nacalai (Kyoto, Japan). Clostridium perfringens sialidase was purchased from Sigma (St. Louis, MO). Actinase E was purchased from Kaken (Tokyo). Endo-N was prepared from bacterium on SDS–PAGE appears to be due to heterogeneity in the DP of the α,2,9-linked oligo/polyNeu5Ac in a number of O-glycans on the polypeptide backbone. In addition, this glycoprotein is difficult to stain with Coomassie brilliant blue. These unique properties have prevented it from being identified before. Thus the 40–80-kDa protein is considered a novel glycoprotein, which we are now trying to identify. Biologic function of the 40–80-kDa glycoprotein containing the sulfated polySia structure in sperm is exclusively localized in the sperm flagellum. The function of this new polySia glycoepitope is not known and is currently under study in our laboratory.

Materials and methods

H. pulcherrimus were purchased from the local fisheries at Tsushima and Fukushima, Japan. Sperm was collected by intracellular introduction of 0.5 M KCl, and stored at –30°C until use. The sperm lipid raft was prepared as described previously (Ohta et al., 1999, 2000). Arthrobacter ureafaciens sialidase was purchased from Nacalai (Kyoto, Japan). Clostridium perfringens sialidase was purchased from Sigma (St. Louis, MO). Actinase E was purchased from Kaken (Tokyo). Endo-N was prepared from bacteriophage K1F as previously reported (Hallenbeck et al., 1987). DMB was purchased from Dojindo (Kumamoto, Japan). 4-Methylumbelliferyl (4MU) derivatives of the following sulfated and unsulfated Sia were kindly provided by Dr. K. Furuhata (Kitasato University): Neu5Ac, Neu5Ac8S, Neu5Ac9S, Neu5Gc, Neu5Gc8S, and Neu5Gc9S. Colominic acid (α2,8-linked Neu5Ac polymer) was purchased from Wako (Osaka, Japan). α2,9-Linked Neu5Ac polymer isolated from capsular polysaccharide of N. meningitidis group C was a generous gift from Dr. H. J. Jennings (National Research Council of Canada) and Dr. Y. Inoue (Academia Sinica, Taiwan). DEAE-Toyopearl was purchased from Tosoh (Tokyo). Sephacryl S-300 and S-100 and Sephadex G-25 and G-50 were obtained from Amersham (Tokyo).

Purification of a novel Sia-containing glycopeptide fraction from sea urchin sperm

Acetone powder was prepared from 150 ml sea urchin sperm, and extracted with 300 ml chloroform/methanol (2:1 by volume) and then with 300 ml chloroform/methanol (2:1) at room temperature for 2 h (Yu et al., 1991). The delipidated residue was washed with 300 ml ethanol, suspended in 100 ml 0.1 M Tris–HCl buffer (pH 8.0) containing 10 mM CaCl2, and incubated with 4.5 mg Actinase E at room temperature with moderate shaking. After 24 h, 4.5 mg Actinase E was added and incubated for additional 24 h. The Actinase E digest was centrifuged at 10,000 × g for 20 min. The supernatant was applied to a Sephadex G-50 column (1.8 × 57.5 cm), and the column was eluted with 0.1 M NaCl. Fractions were assayed for Sia as will be described. The major Sia-containing fraction was desalted by passage through a Sephadex G-25 column (1.2 × 75 cm) with 10% ethanol and applied to a DEAE-Toyopearl 650 M column (2.2 × 15 cm) equilibrated with 10 mM Tris–HCl (pH 8.0). The column was eluted with a linear gradient of NaCl (0–0.6 M) in 10 mM Tris–HCl (pH 8.0). The fractions containing Sia were pooled and applied to a Sephacryl S-300 column (0.8 × 95 cm) that was eluted with 0.1 M NaCl. Sia-positive fractions were pooled and applied to a Sephacryl S-100 column (0.8 × 106 cm). The column was eluted with 0.1 M NaCl. The obtained Sia-containing fraction was designated SGP and desalted by passage through a Sephadex G-25 column (1.2 × 75 cm). Before analyses described later, SGP was treated with 0.5 N NaOH at room temperature for 1 h to saponify possible lactones and O-acetyl groups and desalted by passage through a Sephadex G-25 column (1.2 × 75 cm) after neutralization with 1 N HCl.

Carbohydrate analysis

The monosaccharide composition of SGP was determined by GLC as described previously (Nomoto et al., 1982). Sia residues were quantitated by the thiobarbituric acid method (Aminoff, 1961; Uchida et al., 1977) or the resorcinol method (Svennerholm, 1957). A species of Sia was determined by a DMB derivatization/fluorometric HPLC method as described previously (Hara et al., 1989; Kitzum-Kawaguchi et al., 1997; Sato et al., 1998). Briefly, SGP (1 μg as Sia) or 4MU derivatives of sulfated and unsulfated Sia (100 pmol each) were hydrolyzed in 200 μl 0.1 N TFA at 80°C for 2 h. The hydrolysates were subjected to derivatization with DMB as previously described (Hara et al., 1989). For HPLC, a Capcellpak C18 type MG column (250 × 4.6 mm) (Shiseido, Tokyo) and CH3OH/CH3CN/0.05 % (v/v) TFA (9:7:84, v/v) as the solvent system were used.

Analysis of sialoglycan alditols obtained by alkaline borohydride treatment of SGP

The SGP (0.4 mg as Sia) was treated with 0.5 ml of 0.1 N NaOH containing 1 M NaBH4 at 37°C. After 48 h, the reaction mixture was neutralized with 1 N HCl and desalted by passage through a Sephadex G-25 column (1.2 × 75 cm). The sialoglycan alditols thus obtained were subjected to carbohydrate analyses (see previous description) and to anion-exchange HPLC. For HPLC, the sample was applied to a Mono Q column (1 ml, 5 × 50 mm) and eluted with a linear gradient from 0 to 0.4 M NaCl in 20 mM Tris–HCl (pH 8.0). An elution profile was monitored by measuring the absorbance at 210 nm.
Chemical detection of PolySia

For analyses of polySia structure, a mild acid hydrolysis/TLC analysis (Kitajima et al., 1988), a mild acid hydrolysis/fluorometric anion-exchange HPLC analysis (Sato et al., 1999), and a fluorometric C$_2$/C$_3$ analysis (Sato et al., 1998) were carried out. For the mild acid hydrolysis of SGP for the TLC and HPLC analyses, SGP (10 µg as Sia) was incubated in 10 µl 50 mM sodium acetate buffer (pH 4.8) at 50°C for 4 h or in 0.01 N TFA at 50°C for 2 h. For the mild acid hydrolysis/HPLC analysis of the solubilized proteins from intact sperm and the head and flagellum fractions (see later description), the samples were treated with 0.01 N TFA at 50°C for 1 h, followed by DMB derivatization and fluorometric HPLC.

Endo- and exosialidase digestion

SGP and colominic acid (10 µg each as Sia) were digested with 90 microunits of Endo-N at 37°C for 5 h in 50 mM Tris–HCl (pH 7.5). The digests were analyzed by TLC. For exosialidase digestion of DMB derivatives of oligo/polySia liberated from SGP by the mild acid conditions described, a digestion with 10 milliunits of A. ureafaciens sialidase and 10 milliunits of C. perfringens sialidase was performed in 20 µl 50 mM sodium acetate buffer (pH 5.5) at 37°C for 2 h. The digest was analyzed by fluorometric anion-exchange HPLC.

Methylation analysis

Methylation analysis of sialyl glycans obtained by the alkaline borohydride treatment of SGP was carried out as reported previously (Anumula and Taylor, 1992; Ijuin et al., 1996). Partially methylated, partially acetylated sugars were determined by GLC-MS. GLC was performed on a GC system G1530A gas chromatograph equipped with a 30 m x 0.25 mm DB-1 capillary column, 0.25 µm film (Hewlett-Packard, Palo Alto, CA). The column was coupled to a Mstation JMS-700 mass spectrometer (Jeol, Tokyo). The analyses were performed in the electron ionization mode (ionization energy, 70 eV).

Periodate oxidation

Periodate oxidation and reduction procedure was carried out as described previously (Yoshima et al., 1980). Briefly, SGP (10 µg as Sia) was dissolved in 30 µl 0.05 M sodium acetate buffer (pH 5.5) containing 0.08 M NaIO$_4$ for 96 h at 25°C. Excess oxidant was destroyed by adding 5 µl 3% ethylene glycol. After 30 min, 32 µl 0.1 M NaBH$_4$, 0.1 M sodium borate buffer (pH 8.0) were added and the mixture was left at 4°C overnight. After neutralization with acetic acid, the reaction mixture was desalted by passing through a Dowex 50w-X2 column. The intact and oxidized SGP were analyzed for carbohydrate composition by GLC as described.

Isolation of the head and flagellum fractions

The head and flagellum fractions of sea urchin sperm were obtained as reported previously (Gary and Drummond, 1976). Briefly, 1 ml dry sperm was diluted in 7 ml cold solution A (475 mM NaCl, 25 mM KCl, 10 mM Tris–HCl [pH 8.0], 1 mM CaCl$_2$). Cellular debris and impurities were removed by centrifugation at 250 x g for 5 min. Sperm in the supernatant were centrifuged at 500 x g for 7 min at 4°C, and the pellet was resuspended in 7 ml cold solution A. Flagella were detached from sperm by passing the sperm suspension 16 times through a 22.5-gauge hypodermic needle. Thereafter the broken flagella were separated by placing the suspension over 5 ml solution B (25% sucrose, 10 mM Tris–HCl [pH 8.0], 1 mM CaCl$_2$), followed by centrifugation at 650 x g for 15 min at 4°C. The top 6 ml (flagellum fraction) and the pellet (head fraction) were separately stored on ice. The isolated flagella were pelleted by centrifugation at 3000 x g for 30 min at 4°C.

SDS-PAGE and immunostaining

To solubilize membrane glycoproteins, whole sperm and the head and flagellum fractions were incubated with 1 ml 10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 5 mM ethylenediamine tetra-acetic acid, protease inhibitors (1 µg leupeptin, 2 µg antipain, 10 µg benzamidine, 1 µg pepstatin, 0.9 µg aprotinin) on ice for 20 min. After removal of the pellet by centrifugation at 1300 x g for 5 min, solubilized proteins were quantitated by BCA assay kit (Bio-Rad, Hercules, CA). For SDS-PAGE and immunostaining, the solubilized proteins were incubated with Laemmli buffer (Laemmli, 1970) containing 5% mercaptoethanol at 65°C for 15 min. The samples were then electrophoresed on 7.5% polyacrylamide gels and visualized by Coomassie brilliant blue staining or electroblotted on PVDF membrane using a semidry blotting apparatus. The PVDF membrane was blocked with 10 mM sodium phosphate buffer (pH 7.2), 0.15 M NaCl, containing 0.05% Tween 20, and 1% bovine serum albumin at 4°C overnight. The membranes were incubated with primary antibody 3G9 (1.6 µg/ml) at 4°C overnight. As the secondary antibody, peroxidase-conjugated anti-mouse IgM (1:5000 dilution) were used at 37°C for 1 h, and the color development was carried out as described previously (Sato et al., 2000).

Acknowledgments

This research was supported in part by Grants-in-Aid for Scientific Research (C) (15570096) (to K.K.); CREST of Japan Science and Technology Corporation (to K.K.); the 21st Century COE Program (to K.K.); Young Scientists (B) (14780471) (to C.S.) from the Ministry of Education, Science, Sports and Culture; and Mizutani Foundation (to C.S.). We thank Drs. N. Hirohashi (Ochanomizu University, Japan) and V. D. Vacquier (Scripps Institution of Oceanography, University of California, San Diego) for their help with preparation of sperm head and flagellum fractions as well as for their valuable discussion.

Abbreviations

4MU, 4-methylumbelliferyl; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DP, degree of polymerization; Endo-N, Endo-N-acylneuraminidase; GLC, gas-liquid chromatography; HPLC, high-performance liquid
chromatography; mAb, monoclonal antibody; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SBP, sperm-binding protein; SDS, sodium dodecyl sulfate; SGP, sialoglycopeptide from the Actinase E digest; Sia, sialic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

References


