The Occurrence of Novel 9-O-Sulfated N-Glycolylneuraminic Acid-capped α2→5-Oglycolyl-linked Oligo/PolyNeu5Gc Chains in Sea Urchin Egg Cell Surface Glycoprotein

IDENTIFICATION OF A NEW CHAIN TERMINATION SIGNAL FOR POLYSIALYLTRANSFERASE*

We report the isolation and structural characterization of an oligo/polyasialic acid-containing glycopeptide fraction (designated ESP-Sia) prepared from the egg cell surface complex of the sea urchin, Hemicentrotus pulcherrimus, by exhaustive pronase treatment. The carbohydrate chains isolated from ESP-Sia were shown to consist of O-linked oligo/polyasialic acid-containing glycan units and N-linked carbohydrate chains. The present studies have revealed that the O-linked oligo/polyasialic acid-containing glycan chains derived from the ESP-Sia were similar to those present in egg jelly coat polysialylated glycoprotein in being composed of tandem repeats of N-glycolylneuraminic acid (Neu5Gc) glycosidically linked in a novel fashion through the glycolyl group, (−5-Oglycolyl Neu5Gc)n. However, they differ from the egg jelly coat in two key respects. First, the average degree of polymerization of the oligo/polyasialic acid chains of ESP-Sia is only 3; a value far lower than that found in the jelly coat glycoprotein (average degree of polymerization was about 20). Second, ESP-Sia is uniquely characterized by the presence of 9-O-sulfated N-glycolylneuraminic acid (Neu5Gc9HSO₄) residues at the nonreducing termini of the (−5-Oglycolyl Neu5Gcα→2)n chains. The terminal sialyl residues in the Neu5Gc9HSO₄α→2−(−5-Oglycolyl Neu5Gcα→2)n chains were totally resistant to exosialidases. The discovery of Neu5Gc9HSO₄, the nonreducing terminal residue of oligo/poly(−5-Oglycolyl Neu5Gcα→2)n group is especially noteworthy in that Neu5Gc9HSO₄ appears to be of limited distribution among glycoconjugates. Following the earlier discovery of oligo/polyasialic acid chains capped with KDN, i.e. KDN α→2−(−8Neu5Gcα→2)n, found in rainbow trout egg polysialoglycoproteins, it now appears that the sulfated Neu5Gc can serve a similar capping function.

Polysialic acid (polySia) is the general name for sialic acid polymers, and the most commonly occurring of these is a linear polymer of sialic acid in α2→8 linkage expressed on cell surface glycoconjugates in a wide variety of animal species ranging from neurotropic bacteria to man (1–7). There are a number of reports demonstrating that α2→8-linked polySia chains are involved in regulation of cell-cell interactions, cell adhesion, and cell recognition (3, 8–12).

Recently, we have isolated the sialic acid-rich glycoprotein from the jelly coat of sea urchin eggs and elucidated the structure of oligo/polySia chains as (−5-Oglycolyl Neu5Gcα→2)n (13). This was the first evidence for the presence of polySia chains in sea urchin glycoproteins. The interketosidic linkages in (−5-Oglycolyl Neu5Gcα→2)n chains were found to be almost resistant to Arthrobacter ureafaciens, Clostridium perfringens, and Vibrio cholerae exosialidases (14) and were distinguished from those in α2→6-linked polySia chains. The present investigation was initiated in an attempt to determine if similar oligo/polySia-containing chains exist on cell surface glycoproteins of the sea urchin Hemicentrotus pulcherrimus. To obtain a cell surface enriched preparation we utilized the procedure originally utilized to prepare a cell surface complex from Strongylocentrotus purpuratus eggs (15). This preparation consists of the egg plasma membrane, containing the vitelline layer on its outside surface and cortical granules on its inside surface. As a starting point in characterization of any oligo/polySia chains, we sought to determine the presence of α2→5-Oglycolyl-linked oligo/polySia chains.
this preparation from H. pulcherrimus was digested extensively with pronase. Upon purification and structural characterization of the glycopeptide fraction (ESP-Sia) resulting from pronase digestion, ESP-Sia was found to be composed of α2–5-O-α2–5-linked oligopolyNeu5Gc chains attached to the O-48O of core glycan unit(s). These novel chains on sea urchin egg cell surface glycoproteins are terminated by the presence of a Neu5Gc9HSO3 residue at the nonreducing end of the oligo/polySia chain.

**EXPERIMENTAL PROCEDURES**

**Materials—**H. pulcherrimus were collected at Cape Manazuru, Japan, or were obtained from Tsushima courtesy of Drs. I. Yasumasa (Waseda University) and I. Mabuchi (University of Tokyo). Artificial seawater was purchased from J amarin (Osaka, Japan). C. perfringens sialidase was purchased from Sigma. Actinase E or pronase (specific activity, 1,000,000 Tyr units/g) was purchased from Kaken (Tokyo, Japan). Sea urchin egg jelly acidic acid-rich glycoprotein was prepared as described previously (13). Trout egg lysolecithiniglycoprotein used as a molecular mass marker (9 kDa) was prepared as described previously (17–19).

**Isolation of Egg Cell Surface Complex—**Eggs were collected from H. pulcherrimus by injection of 0.5 M KCl, washed three times with artificial seawater, and dejellied as described previously (21). Briefly, dejellied eggs from female H. pulcherrimus were suspended in the same volume of Ca2+-free sea water containing 10 mM EDTA and protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml benzamidine, and 20 μg/ml antipain) and sonicated using a Branson Sonifier 250 at 50 watts for 10 min. The egg homogenate was dialysed 5-fold with ice-cold Ca2+-free sea water, and the cell surface complex was pelleted by centrifugation at 1,000 × g for 3 min. The pellet was immediately suspended and incubated in 1× sucrose containing the protease inhibitors by gentle homogenization to degrade residual solvents. The membranal/vitelline layer complex was collected by centrifugation at 3,000 × g for 20 min.

**Preparation of Sia-rich Glycopeptide Fraction from Egg Surface Complex—**The egg surface complex (about 120 ml) was delipidated as described previously (22). Briefly, the egg surface complex was suspended in 3 volumes of 100 mM Tris-HCl buffer (pH 8.0) with subsequent addition of 8 volumes of methanol and 4 volumes of chloroform. The resulting mixture was stirred for 2 h at room temperature and centrifuged at 15,000 × g for 40 min. The pellet was resuspended in 100 mM Tris-HCl/methanol/chloroform (3:8:4) and centrifuged as above. The residual solvents were removed from the extract by evaporation with absolute ethanol. The pellet was dried under reduced pressure and weighed.

**Quantification of Acidic Acid—**The delipidated sample (22.5 g) was suspended in 800 ml of the incubation buffer (0.1 M Tris-HCl (pH 8.0), 10 mM CaCl2, and 200 mg of pronase) and 3 drops of toluene were added. The mixture was incubated with moderate shaking at 37°C for 24 h and 48 h, a 200-mg portion of fresh pronase was added. After 72 h, the digestes were centrifuged at 10,000 × g for 15 min. The supernatant fraction was dialyzed against distilled water and applied to a DEAE-Toyopearl 650 M column (3.2 × 38 cm) equilibrated with 20 mM Tris-HCl (pH 8.0). The column was eluted with a linear gradient of NaCl in 100 mM sodium gluconate at a flow rate of 1.2 ml/min.

**Chemical Treatments of ESP-Sia—**De-N-acetylation and subsequent acetylation of Neu5Gc residues in ESP-Sia were carried out as described previously (13). The resulting reaction mixture was desalted by passage through Sephadex G-25. The periodate oxidation/borylhydroxide reduction procedure followed that previously reported (23). ESP-Sia sample was hydrolyzed with 50 mM sodium acetate buffer (pH 4.8) at 80°C for 2–6 h for detecting oligoSia or with 0.1 M trifluoroacetic acid at 80°C for 1 h for detecting free Sia. The free Sia or oligoSia was separated by TLC or HPLC as described below.

**Chemical Analyses of ESP-Sia—**ESP-Sia residues were quantitated by the thioarbituric acid method (24, 25) and the resorcinol method (26). Sia residues in ESP-Sia were determined by the mild acid hydrolysis/subsequent mild methanolysis/gas-liquid chromatographical procedure as described previously (27). The hexose content was estimated by the phenol-sulfuric acid method (28). Amino acid and amino sugar analyses were carried out after hydrolysis in 6 N HCl at 105°C for 24 h under reduced pressure. Samples were derivatized with phenylisothiocyanate before column chromatography (1). Carbohydrate analysis was carried out by the gas-liquid chromatography as described previously (29). The sulfate analysis was carried out by HPLC on a TSK-gel IC-Anion PW column using an ASO HPLC system equipped with a 980-PU pump, a 970-UV detector, and a Rheodyne injector with a 20-μl sample loop. The elution pattern was monitored by measuring at 265 nm and recorded on a Chromatograph 32 (System Instruments, Tokyo, Japan). Samples were evaporated to dryness after hydrolysis in 6 N HCl at 105°C for 12 h and dissolved in 30–50 μl of water. Samples in 1–10 μl were injected and eluted isocratically with acetonitrile/0.1% trifluoroacetic acid (4:1) containing 0.5 mM sodium-phtalate, 5.82 mM H3BO3, 1.3 mM Na2O3, and 0.9 mM sodium-glucuronate at a flow rate of 1.2 ml/min.

**Thin-Layer Chromatography**—TLC of oligoacides obtained from ESP-Sia was carried out as described previously (30). HPLC of Sialyloligosaccharides on a Mono-Q Column—The sialyloligosaccharides were resolved by HPLC on an anion exchange Mono-Q column using an Irika HPLC system as described previously (30, 31).

**Fast Atom Bombardment-Mass Spectrometry—**Fast atom bombardment-mass spectrometry spectra were recorded using a VG Analytical ZAB-2S E. FPD mass spectrometer fitted with a reflectron operated at 20–25 kV. Data acquisition and processing were performed using VG Analytical Opus software. The peracylated samples were aliquoted in methanol, and monochloroglycerol was used as matrix.

**500-MHz 1H NMR Spectroscopy—**NMR spectra of oligoSia were determined in D2O at 23°C with a Bruker AMX-500 spectrometer.

**Proton chemical shifts were expressed in parts/million relative to the methyl signal of sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4, NMR measurements were generously carried out by Drs. Y. Muto, T. Niimi, Y. Takeda, and S. Watanabe in Prof. Yokoyama's laboratory at the University of Tokyo.

**RESULTS**

**Preparation of ESP-Sia Fraction from Egg Surface Complex of Sea Urchin Eggs**

The proline digests of egg surface complex from 600 female H. pulcherrimus (600 ml of packed, dejellied eggs) were found to contain about 60 mg of Sia and were chromatographed on a DEAE-Toyopearl 650 M column (Fig. 1). TLC analysis of mild acid hydrolysates of Sia-containing fractions eluted from the columns was carried out to detect oligoSia-containing fractions. Sia-containing fractions eluting at 0.25–0.45 mM NaCl, which were positive for oligoSia on TLC analysis of mild acid hydrolysates, were collected and dialyzed against distilled water.
The Sia residue in ESP-Sia was identified as Neu5Gc. The presence of Man, GalNAc, and GlcNAc indicated the presence of both O- and N-linked glycan chains. However, peptide:N-glycanase digestion of ESP-Sia did not liberate any N-glycans, probably either because glycosylated asparagine residues were located at N-termini (32) or because N-glycanase-resistant structures. After alkaline borohydride treatment of ESP-Sia, on average, one Thr per molecular weight of 9,000 was converted to Neu5Gc monomer and seemed to be a new component, which was shown to be converted into Neu5Gc monomer when heated under more acidic conditions with 0.1 M trifluoroacetic acid for 5 h at 80 °C (data not shown). As shown in Fig. 2 (lane 4), the migration rates of G2 and G3 did not correspond to those for α2→5-GlcNAc7→O-glycolyl-linked di- and tri-Neu5Gc (lane 2) or α2→8-Linked oligoNeu5Gc (lane 1).

Strong Alkaline Treatment and Subsequent N-Acetylation—Strong alkaline treatment was employed to show that the interketosidic linkages in oligo/polySia chains of ESP-Sia are α2→5-Glycolyl. ESP-Sia was treated with 2 M NaOH as described previously (13, 33), and after N-acetylation the reaction product was analyzed by TLC. Two spots appeared by visualization of the TLC plate using the resorcinol reagent. The lower spot corresponded to Neu5Ac2→O-CH2COOH by TLC mobility and elution position on Mono-Q HPLC (data not shown). The upper one (Rf = 1.23 relative to Neu5Ac2→O-CH2COOH) eluted from Mono-Q HPLC at retention time similar to Neu5Ac2→O-CH2COOH, and the molar ratio of the upper to the lower one was estimated to be 1:2 by the resorcinol method. As is described below, ESP-Sia was verified to contain the nonreducing terminal 9-O-sulfated Neu5Gc residues on the oligo/polySia chains with average DP of 3. Thus, the upper component was tentatively assigned to desulfated Neu5Ac9GSO4glycolyl. Although limitation in the quantity of the upper spot material available prevented further rigorous structural studies. Nevertheless, all oligo/polySia chains underwent depolymerization by strong alkaline treatment, indicating that all of their interketosidic linkages in ESP-Sia were α2→5-Glycolyl-

Identification of G1 as 9-O-Sulfated Neu5Gc Monomer (Neu5Gc9GSO4)—The conversion of G1 to Neu5Gc upon treatment with acid (0.1 M trifluoroacetic acid, 80 °C, 5 h) suggested that G1 was a modified Neu5Gc that contained an acid labile

**TABLE I**

| Amino acid | Before treatment | After treatment |
|------------|-----------------|----------------|
| Asx        | 2.3             | 1.9            |
| Thr        | 1.7             | 0.52           |
| Ser        | 1.2             | 0.66           |
| Glx        | 1.2             | 0.80           |
| Pro        | 1.4             | 1.7            |
| Gly        | 0.69            | 0.42           |
| Ala        | 0.85            | 0.81           |
| Val        | 0.38            | 0.42           |
| Ile        | 0.25            | 0.20           |
| Leu        | 0.23            | 0.14           |
| Lys        | 0.17            | 0.11           |
| αABA       | 0.89            | 0.76           |

Values are molar ratios of the component amino acids, monosaccharides, and sulfate ion in ESP-Sia of the average molecular weight of 9,000.

**Chemical compositions of ESP-Sia before and after alkaline-borohydride treatment**

The chemical composition of ESP-Sia is shown in Table I. The carbohydrate content of ESP-Sia was about 89% by weight. It should be noted that ESP-Sia contained high amounts of sulfate. The Sia residue in ESP-Sia was identified as N-glycolylneuraminic acid (Neu5Gc). The presence of Man, GalNAc, and GlcNAc indicated the presence of both N- and O-linked glycan chains. However, peptide:N-glycanase digestion of ESP-Sia did not liberate any N-glycans, probably either because glycosylated asparagine residues were located at N-termini and/or C-termini (32) or because N-linked glycan chains had peptide-N-glycanase-resistant structures. After alkaline borohydride treatment of ESP-Sia, on average, one Thr per molecular weight of 9,000 was converted to α2→5-O-glycolyl-linked di- and tri-Neu5Gc (lane 2) or α2→8-Linked oligoNeu5Gc (lane 1).
substituent. When G1 was subjected to Mono-Q HR5/5 column, as described under “Experimental Procedures,” it eluted at higher NaCl concentration than the Neu5Gc monomer (Fig. 3A). This finding indicated that G1 had an additional anionic group. The presence of a sulfate group in G1 was revealed by chromatography of the acid hydrolysate of G1 (6 N HCl, 105 °C, 12 h) on a TSK gel IC-Anion PW column. Furthermore, fast atom bombardment-mass spectrometry showed molecular ion peaks, [M + Na – 2H]⁺ at m/z 426 and [M – H]⁻ at m/z 404, in the spectrum determined at the negative ion mode. Measurement of the deuterioacetyl derivative of G1 in the negative ion mode gave major molecular ions [M – H]⁻ and [M + Na – 2H]⁻ at m/z 629 and 651, respectively, corresponding to the addition of five deuterioacetyl groups and the retention of the sulfate. In the positive ion mode, the derivative G1 gave a major molecular ion at m/z 675 corresponding to [M – H + 2Na]⁺ for a sulfated Neu5Gc (data not shown). These results unambiguously established G1 to be mono-O-sulfated Neu5Gc.

The ¹H NMR data obtained for G1 and signal assignments are shown in Fig. 4A. When compared with Neu5Gc, the H-8, H-9, and H-9’ in G1 resonated at lower magnetic field (H-8, δ 3.78 ppm; H-9, δ 3.82 ppm; H-9’, δ 3.61 ppm) for Neu5Gc (taken from Refs. 34 and 35)). The largest downfield shift was observed for H-9, and the spectrum of G1 was different from that of 8-O-sulfated Neu5Gc, previously identified in Anthocidaris crassipina (38), where H-8 signal resided at the lowest magnetic field. To further examine the position of sulfate ester on Neu5Gc residue, periodate oxidation/borohydride reduction of ESP-Sia was carried out (29). The GLC analysis of the reaction products showed that all of the Sia residues in ESP-Sia were converted into 2-keto-3,5-deoxy-α-galacto-5-N-glycolyleheptonic acid, the C₇ analogue of N-glycolyneuraminic acid. Based on these data, it was concluded that G1 was 9-O-sulfated Neu5Gc (Neu5Gc9H5SO₄). This is the first example of a sulfated ester at the C-9 position of sialic acid.

Separation of Sulfated oligoSia and Nonsulfated oligoSia by Mono-Q HPLC—G2 and G3 were separately subjected to HPLC on Mono-Q HR5/5 column. Unexpectedly, G2 gave two major peaks (Fig. 3B). One of them (designated as (Gc)₂) eluted at almost the same position as that of the α₂–8-linked Neu5Gc dimer chromatographed as a reference. The other one (designated as GX2) was eluted at a higher NaCl concentration than the Neu5Gc dimer. G3 also gave two major peaks (Fig. 3C). One of them (designated as (Gc)₃) eluted at the same position as the α₂–8-linked Neu5Gc trimer used as a reference, and the other (GX3) eluted at a higher NaCl concentration than the Neu5Gc trimer. Compositional analysis of GX2 and GX3 revealed that molar ratios of Neu5Gc to sulfate were 2:1 for GX2 and 3:1 for GX3, indicating that these were mono-O-sulfated oligomers.

Determination of the Position of Sulfated Neu5Gc Residues in Sulfated oligo polySia Structures—(Gc)₂, GX2, (Gc)₃, and GX3 were separately treated with C. perfringens sialidase (14), but under the reaction conditions employed Neu5Gc₂–5-O-glycolyl Neu5Gc (Gc)₂, Neu5Gc₂–5-O-glycolyl Neu5Gc, (Gc)₃, and Neu5Gc₂–5-O-glycolyl Neu5Gc₂–5-O-glycolyl Neu5Gc, (Gc)₃, were completely hydrolyzed to Neu5Gc (lane 2 and lane 6). Contrary to this, GX2 and GX3 were totally resistant to the

![Fig. 2. Thin-layer chromatogram of the mild acid hydrolysate of ESP-Sia.](http://www.jbc.org/fig2.png)

![Fig. 3. Mono-Q HR5/5 HPLC separation of G1 (A), G2 (B), and G3 (C) derived from mild acid hydrolysis of ESP-Sia.](http://www.jbc.org/fig3.png)
FIG. 4. 500-MHz $^1$H NMR spectra of sulfated Neu5Gc (G1) (A); Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc ((Gc)$_\alpha$) (B); Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc ((Gc)$_\alpha$) (C); Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc ((Gc)$_\alpha$) (D); and Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc$\alpha_{2-3}$SO$_\alpha$glycolyly Neu5Gc (G3) in D$_2$O at 23°C (E). In A, a downfield proton signal at 4.43 ppm exhibited a large coupling constant of 11.8 Hz, which was interpreted as a geminal coupling constant of methylene protons, i.e. H-9s. In the correlated spectroscopy of G1, the cross peaks due to the magnetic transfer from the resonance at 4.43 ppm were observed at 3.85 ppm (strong) and at 4.05 ppm (weak) (spectrum not shown). The resonance at 3.85 ppm was assignable to H-9 based on the coupling constant of 11.8 Hz. Accordingly, the 4.05 ppm signal was assignable to H-8 having a weak coupling with the vicinal H-9 (1 Hz). H-7 was also shown to resonate at 3.85 ppm from the following observations: (i) the peak area at 3.85 ppm comprised two protons; (ii) the magnetic transfer from the resonance at 4.05 ppm was seen only at 3.85 ppm; and (iii) $J$, value was large.
were respectively assigned to H-3eq of the reducing terminal Neu5Gc residue. These resonances at 3.76 and 2.67 ppm were respectively thought to be the down-field shifted H-3ax and H-3eq of the sulfated nonreducing terminal Neu5Gc residue. Other resonances observed in Fig. 4 (D and E) were again superimposable. Based on these results, GX3 was determined to be Neu5Gc9HSO3a2→5-O-glycolyl-Neu5Gc2→5-O-glycolyl-Neu5Gc.

Oligo Polyasialylated Glycan Chains—The carbohydrate composition of ESP-Sia suggested the presence of both N- and O-linked glycan chains. The ESP-Sia sample treated with alkaline borohydride was subjected to preparative TLC as described under “Experimental Procedures.” Analysis of the TLC showed that almost all Sia-containing components migrated from the origin, suggesting that oligopolyasialyl groups were attached to the liberated O-linked glycan chains. The N-glycan chains, still linked to peptide, remained at the origin in this TLC system (data not shown). Detailed structural analysis of the core glycan chains is now under way.

DISCUSSION

Polyasialylated glycoproteins from a wide variety of sources have been studied in recent years, but the contributions of polysialylation to glycoprotein functions are only beginning to be understood (2, 3). The discovery of the new types of oligo/polySia residues in different biological materials is expanding the long list of naturally occurring oligopolySia chains. Indeed, the recent discovery of unusual structural features in the oligo/polySia chains containing (→5-O-glycolyl)-Neu5Gc2→5-O-glycolyl Neu5Gc residues having a mean chain length of approximately 20 Neu5Gc residues in a sialic acid-rich glycoprotein isolated from the jelly coat of sea urchin eggs (13) led us to search for related proteins on the egg cell surface.

In this study, we first showed that the sea urchin egg cell surface glycopeptides (ESP-Sia) released by exhaustive pronase digestion of the cell surface complex have O-linked oligo/polySia-glycan chains and (→5-O-glycolyl)-Neu5Gc2→5-O-glycolyl Neu5Gc common basal structures that were characterized thoroughly by chemical and physical methods. The main differences between jelly coat-derived oligopolySia chains and egg cell surface-derived oligopolySia chains appear to be (a) the lower degree of polymerization of the latter (average DP was about 3) and (b) the presence of sulfate groups on (→5-O-glycolyl)-Neu5Gc2→5-O-glycolyl Neu5Gc chains in the latter. Perhaps the most prominent feature of oligopolySia chains found in ESP-Sia is that the α2→5-O-glycolyl-linked oligopolyNeu5Gc chains are capped at their nonreducing termini by the 9-O-sulfated Neu5Gc residues, i.e. Neu5Gc9HSO3a2→(→5-O-glycolyl)-Neu5Gc2→5-O-glycolyl Neu5Gc. This appears to be the first report of the occurrence of sulfated polySia groups in a glycoprotein. Although generally present in relatively very low amounts, O-sulfated sialic acids are not uncommon in nature, particularly in gangliosides. It has been reported also that they are constituents of gangliosides from lower animals and of 8-sulfated Neu5Gc and 8-O-sulfated Neu5Ac in A. crassipina. A number of modified sialic acids have previously been isolated from sialoglycoconjugates, but this is the first report of the occurrence of 9-O-sulfated N-glycolylneuraminic acid in nature.

The Neu5Gc9HSO3 residues in oligopolySia-glycoprotein of sea urchin eggs are not randomly incorporated into the oligo/polySia chains; they occur exclusively as the Neu5Gc9HSO3-capped oligoNeu5Gc structures, i.e. Neu5Gc9HSO3a2→(→5-O-glycolyl)-Neu5Gc2→5-O-glycolyl Neu5Gc. In the ESP-Sia, it should be emphasized that direct experimental evidence for these capped structures are: (a) the presence of Neu5Gc9HSO3 at the nonreducing end that blocked the action of exosialidases; (b) the isolation of a series of monosulfated...
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oligoSia from nonenzymatic mild acid hydrolysates of ESP-Sia that was consistent with the structures in the form of Neu5Gc9HSO3(α2→(5-O-glycolyl)Neu5Gc9HSO3)n, and Neu5Gc9HSO3α2→(5-O-glycolyl)Neu5Gc9HSO3, other than Neu5Gc9HSO3α2→(5-O-glycolyl)Neu5Gc detected in the mild acid hydrolysate of ESP-Sia. It is notable that mild acid hydrolysis released Neu5Gc, Neu5Gc9HSO3, and both capped and uncapped oligoNeu5Gc, i.e. (5-O-glycolyl)Neu5Gc2→(n) and Neu5Gc9HSO3α2→(5-O-glycolyl)Neu5Gc2→(n), from ESP-Sia. Although we could not determine that every oligo/polySia chain in ESP-Sia is capped by Neu5Gc9HSO3, our results are compatible with the possibility that part of the sulfate groups at the nonreducing termini of the oligo/polySia chains in egg surface glycoprotein, from which ESP-Sia was derived, may have been lost during isolation and purification procedures.

A pertinent question concerns the origin and composition of ESP-Sia prepared from the cell surface complex. This complex, when prepared from S. purpuratus, was shown to consist of the plasma membrane, vitelline layer, and cortical granules. ESP-Sia is a mixture of glycopeptides released by pronase digestion of the egg cell surface complex. The glycopeptides were shown to contain both sulfated oligo/polySia-containing O-linked glycan and N-linked sulfated glycan chains. However, it is important to note that ESP-Sia is structurally distinct from the sialic acid-rich glycoprotein of the jelly coat. In fact, ESP-Sia is more abundant than the egg jelly sialic acid-rich glycoprotein, being present at a molar level that is 100 times greater per egg.

The presence of a sulfated glycoprotein, from which ESP-Sia was derived, on egg cell surface raises the possibility that the egg receptor for sperm is a 350-kDa glycoprotein (21, 40–44) that function sequentially in the initiation and elongation reactions (16). Likewise it will be most interesting to understand how (5-O-glycolyl)Neu5Gc2→(n), chains are formed in vivo. It also remains an important challenge to determine how the control of the final oligo/polySia structure, i.e. the average length of oligo/polySia chains, is achieved and how far it is correlated with the functional requirements of the oligo/polySia glycoconjugates.

Another fundamental question that needs to be answered in future is the manner by which elongation of oligo/polySia chains is terminated. It was presumed that the incorporation of an unusual component at the nonreducing terminus of a given oligo/polySia chain can function as a terminating signal, preventing further chain elongation by polysialyltransferase. The isolation and characterization of a minor 9-O-sulfated Neu5Gc from ESP-Sia raised our interest in the chemistry and biological implications of the possible termination mechanism for biosynthesis of oligo/polySia chains. Perhaps one of the most prominent findings in the current study is the occurrence of the capped structures of oligo/polySia chains, Neu5Gc9HSO3α2→(5-O-glycolyl)Neu5Gc2→(n). This together with our previous finding (45) and more recent study on the biosynthetic mechanism (46) of the KDN-capped structure of α2→8-linked oligo/polyNeu5Gc, i.e. KDNα2→(8-Neu5Gc2→(n), in salmonid fish egg polysialoglycoproteins give insight into the diverse nature of termination of oligo/polySia chain elongation in vivo. Thus, the current findings, coupled to earlier studies (16), lead us to propose the following scheme for multiple stages in polySia chain synthesis (R represents core glycan unit):

Initiation (dSia formation): Sia→R + CMP→Sia*→Sia*→Sia→R

REACTION 1.

Elongation (oligo/polySia formation):

Sia→Sia→Sia→R + nCMP→Sia*→(Sia*)n→Sia→R

REACTION 2.

To date, however, only limited information is available about the termination mechanism of polysialyl chain elongation. The present finding of 9-O-sulfation of the nonreducing terminal sialic acid residues of oligo/polySia chains provides some insight into the mechanism of termination of the oligo/polySia chains in the sea urchin egg surface glycoprotein.

Termination (substitution of stopper signal):

(Sia)n→Sia→Sia→R + HSO3→Sia(Sia)n→Sia→R

REACTION 3.

In the case of KDN- and sulfated Sia-containing oligo/polySia, great resistance was observed toward hydrolytic removal of their sialyl residues by the bacterial exosialidases tested. Thus one attractive possibility is that these signals serve as a protective mechanism against attack by bacteria. Another possibility, based on the recent finding of sulfated sialic acid as a component of the egg receptor for sperm (unpublished observations), is that this sulfated sugar residue is involved in sperm-egg recognition.

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