Co-expression of two distinct polysialic acids, α2,8- and α2,9-linked polymers of N-acetylneuraminic acid, in distinct glycoproteins and glycolipids in sea urchin sperm

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The 190 kDa-gp is localized in both flagellum and head of sperm. We also demonstrated that polysialogangliosides containing the α2,8-linked polyNeu5Ac are present in sperm head. Thus, this study shows two novel features of the occurrence of polySia in nature, the co-localization of polySia with different intersialyl linkages, the α2,8- and α2,9-linkages, in a single cell and the occurrence of α2,8-linked polyNeu5Ac in glycolipids. Anti-α2,8-linked polyNeu5Ac antibody had no effect on fertilization, which contrasted with the previous results that anti-α2,9-linked polyNeu5Ac antibody inhibited sperm motility and fertilization. Based on these properties, distinct functions of α2,8- and α2,9-polySia structures are implicated in fertilization.

Keywords: flagellasilain / polysialoganglioside / α2,8-polsialic acid / α2,9-polysialic acid / sea urchin sperm

Introduction

Polysialic acid (polySia) is a group of linear homopolymers of sialic acid (Sia) with a degree of polymerization (DP) of 8–400 and is widely distributed in nature ranging from bacteria to human (Troy 1996). PolySia shows a large structural diversity arising from differences in the Sia components [N-acetylneuraminic acid (Neu5Ac), N-glycoylneuraminic acid (Neu5Gc) and deaminoneuraminic acid (KDN)] and in the intersialyl linkages (α2,5glycoyl, α2,8, α2,9, α2,8/9). PolySia was first identified in capsular polysaccharides of pathogenic bacteria such as Escherichia coli and Neisseria meningitidis (McGuire and Binkley 1964; Troy 1996; Sato and Kitajima 1999). The capsular polysaccharide of N. meningitidis group B consists of α2,8-linked polyNeu5Ac residues (McGuire and Binkley 1964; Sato and Kitajima 1999), whereas that of N. meningitidis group C (NMGC) comprises α2,9-linked polyNeu5Ac residues (McGuire and Binkley 1964; Bhattacharjee et al. 1975; Sato and Kitajima 1999). In animals, the presence of the α2,8-linked polySia structure was demonstrated in the neural cell adhesion molecule (NCAM) (Finne 1982), the α-subunit of the voltage-sensitive sodium channel in mammalian brain (Zuber et al. 1992), CD36 in human and mouse milk (Yabe et al. 2003), the neuropilin-2 in mouse dendritic cell (Curreli et al. 2007), the synaptic cell adhesion molecule 1 in mouse brain (Galuska et al. 2010), fish egg glycoproteins such as polysialoglycoprotein (poly-SG; Inoue and Iwasaki 1978; Sato et al. 1993), the KDN-containing glycoprotein (Kanamori et al. 1990) and the eel electroplax sodium channel (James and Agnew 1987).

With respect to the α2,9-linked polySia structures that were first found in NMGC (Bhattacharjee et al. 1975), only two examples have been demonstrated in animal: an unidentified protein in mouse neuroblastoma cells (Inoue et al. 2003) and the sea urchin sperm flagellasilain, a major flagellar SG with a diverse molecular mass of 40–80 kDa in sea urchin sperm (Miyata et al. 2004, 2006). In particular, a new type of polySia, 8-O-sulfated Neu5Ac (Neu5Ac8S) capped α2,9-linked polyNeu5Ac was demonstrated on the O-glycans of flagellasilain (Miyata et al. 2004, 2006). In sperm, the presence of the α2,8-linked oligoNeu5Ac with a DP of up to 4 in glycolipids was reported (Ijuin et al. 1996). No polySia structure, however, has been demonstrated in glycolipids in any animal cells. Note that so-called polysialogangliosides contain four or five Neu5Ac residues, only forming a trimer of...
Neu5Ac as the longest chain. We thus sought to examine if the α2,9-linked Sia residue in flagellasialin is also expressed in glycolipids, as well as if the α2,8-linked Sia residue in glycolipids is also expressed in glycoproteins. In this study, we demonstrated that the α2,8-linked polyNeu5Ac structure occurred in sea urchin sperm. The α2,8-linked polyNeu5Ac structure was present on both glycoproteins and glycolipids. This is the first demonstration of natural occurrence of the polylia structure on glycolipids. We also demonstrated that the α2,8- and α2,9-linked polyNeu5Ac structures were linked to a 190 kDa glycoprotein (190 kDa-gp) and flagellasialin, respectively, and that these polySia-containing glycoproteins co-existed in the same single sperm by the immunofluorescence localization study, suggesting the diverse functions by different polySia structures may be operative at sea urchin fertilization.

Results
Demonstration of the occurrence of a glycoprotein containing α2,8-linked polyNeu5Ac in sea urchin sperm
The sea urchin sperm lysate was subjected to western blotting using mAb.12E3, which recognizes α2,8-linked polyNeu5Ac with a DP of ≥5 (Sato et al. 1995). mAb.12E3 reacted specifically with a 190 kDa smear glycoprotein (Figure 1A). A band at 30 kDa showed non-specific staining, because they were stained without the primary antibody. We previously showed that flagellasialin migrated as a large smear at the 40–80 kDa region, when it was detected with mAb.4F7 recognizing α2,9-linked polyNeu5Ac or with mAb.3G9 recognizing Neu5Ac8S (Yamakawa et al. 2007) at the non-reducing terminus of the α2,9-linked polyNeu5Ac chain of flagellasialin (Miyata et al. 2004, 2006; Figure 1A). With mAb.12E3, flagellasialin was not detected. These results indicate that the 190 kDa-gp is different from flagellasialin.

To determine if the α2,8-linked polyNeu5Ac is present on O- or N-glycans on the 190 kDa-gp, the blotted membrane prepared by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/electroblotting was treated with 0.1 M NaOH or with peptide:N-glycanase F (PNGase F) digestion, followed by western blotting. As a control, pig embryonic brain homogenate was analyzed by western blotting with mAb.12E3 and a smear ranging >200 kDa, which corresponds to α2,8-linked polyNeu5Ac-containing NCAM (polySia-NCAM; Sato et al. 2000), was detected (Figure 1B, Untreat). For polySia-NCAM, the staining remained unchanged following alkaline treatment (Figure 1B, NaOH), whereas it almost disappeared following PNGase F treatment (Figure 1B, PNG), consistent with the fact that polySia-NCAM contains the α2,8-linked polyNeu5Ac mainly on the N-glycans. In contrast, the staining of the 190 kDa-gp in sea urchin sperm disappeared following alkaline treatment (Figure 1B, NaOH) and was not changed by PNGase F treatment (Figure 1B, PNG). These results indicate that the α2,8-linked polyNeu5Ac chains are on the O-glycans of the 190 kDa-gp.

The blotted membrane was also subjected to endo- or exosialidase treatments before immunoblotting with mAb.12E3. Endosialidase (Endo-N) specifically cleaves α2,8-linked polyNeu5Ac with a DP of ≥5 (Sato et al. 1995; Troy 1996; Sato and Kitajima 1999), but not other known types of polySia, such as α2,9-linked polyNeu5Ac, α2,5O-glycoly-linked polyNeu5Gc or α2,8-linked polyKDN (Sato and Kitajima 1999). The exosialidases are αL-ketoside-specific hydrolytic enzymes and do not act on substituted Sia residues (Maehashi et al. 2003; Miyata et al. 2004). The staining of both polySia-NCAM and the 190 kDa-gp disappeared following exosialidase and endosialidase treatment (Figure 1, Endo and Exo). These results indicate that the polySia chain on the 190 kDa-gp is α2,8-linked polyNeu5Ac with a DP of ≥5, consisting of unsubstituted Neu5Ac residues.

Fig. 1. Detection of α2,8-linked polyNeu5Ac-containing glycoprotein derived from 1% Triton X-100 extract of sea urchin sperm by western blot analysis. (A) Sea urchin sperm lysate was subjected to SDS–PAGE and immunoblotted with mAb.12E3, mAb.4F7 and mAb.3G9, which recognizes α2,8-linked polyNeu5Ac, α2,9-linked polyNeu5Ac and Neu5Ac8S, respectively. An arrow and bars indicate the α2,8-linked polyNeu5Ac-containing 190 kDa-gp and the α2,9-linked polyNeu5Ac-containing flagellasialin, respectively. Asterisk indicates the non-specific staining. (B) Sea urchin sperm lysate (upper panel) and pig embryonic brain homogenate (lower panel) were electrophoresed and electroblotted onto the polyvinylidene fluoride membrane. The membrane was blocked with 1% BSA and treated as follows: Untreat, untreated; NaOH, incubated with 0.1 M NaOH at 37°C for 18 h; PNG, incubated with 2 μl of PNGase F at 37°C for 18 h; Endo, incubated with 0.9 mU of Endo-N at 37°C for 18 h; Exo, incubated with 100 mU of C. perfringens sialidase at 37°C for 18 h. The α2,8-linked polyNeu5Ac was detected with mAb.12E3.
Preparation and characterization of the α2,8-linked polyNeu5Ac-containing glycopeptide derived from the 190 kDa-gp

Purification of the α2,8-linked polyNeu5Ac-containing glycopeptide. To identify the 190 kDa-gp, we sought to purify it to homogeneity. However, it was difficult to purify the intact glycoprotein by the usual column chromatographic procedures, because this glycoprotein tends to form aggregates during purification. Therefore, soluble glycopeptide fractions containing α2,8-linked polyNeu5Ac were prepared for structural analyses, because only the 190 kDa-gp contains the α2,8-polyNeu5Ac chains in sea urchin sperm glycoproteins (Figure 1). Sea urchin sperm was delipidated and subjected to exhaustive trypsin digestion. The trypsin digest was applied to a DEAE-Toyopearl 650 M column and eluted with a 0–0.6 M gradient of NaCl. Five Sia-containing fractions (Fr.1–Fr.5) were obtained as shown in Figure 2A. The yields of Sia for Fr.1–Fr.5 were: Fr.1, 6.2 mg; Fr.2, 4.4 mg; Fr.3, 4.4 mg; Fr.4, 9.2 mg; Fr.5, 20 mg. Western blot analysis using mAb.3G9 recognizing flagellasialin showed that Fr.4 and Fr.5 contained flagellasialin as a smear at 40–80 kDa (Figure 2B). This is consistent with a previous observation that flagellasialin is resistant against trypsin (Miyata et al. 2006). Our preliminary analysis suggested that the glycopeptides derived from the

![Fig. 2. Purification of α2,8-linked polyNeu5Ac-containing glycopeptides derived from trypsin digests of sea urchin sperm lysates. (A) DEAE-Toyopearl 650 M anion-exchange chromatography of trypsin digests of delipidated H. pulcherrimus sperm lysates. The sample was applied to a column equilibrated with 10 mM Tris–HCl (pH 8.0) (2.2 × 15 cm) and eluted with a linear gradient of NaCl (0–0.6 M). The concentration of NaCl was indicated by a dotted line. The elution profile was monitored for Sia by the resorcinol method (black dots, A580). The fractions indicated by bars were pooled. Fr.1, 20–40; Fr.2, 41–55; Fr.3, 56–73; Fr.4, 74–91; Fr.5, 92–118. (B) Western blot analysis of the Fr.1–Fr.5 with mAb.3G9 showed that flagellasialin was recovered in Fr.4 and Fr.5 as a smear at 40–80 kDa. Sephacryl S-100 chromatography of the Fr.2 (C) and Fr.3 (D). The early eluting Sia-containing fractions indicated by the bars were pooled. (E) Sephacryl S-100 re-chromatography of the pooled fraction in (C and D). The Sia-containing fractions were pooled as indicated by the bar and designated α2,8GP. Elution profiles were monitored for absorbance at 230 nm (open circles; A230) and for the Sia analysis by the resorcinol method (black dots; A580 in C and D). V0, void volume. Vt, total volume.](https://academic.oup.com/glycob/article-abstract/21/12/1596/1988537)
190 kDa-gp were recovered in Fr.2 and Fr.3. Therefore, Fr.2 and Fr.3 were further purified by Sephacryl S-100 chromatography to give two Sia-positive peaks (Figure 2C and D). The early eluting peak was pooled and subjected to three rounds of Sephacryl S-100 column to purify the 190 kDa-gp-derived glycopeptide as a single peak on Sephacryl S-100 chromatography (Figure 2E). Fractions under a single Sia-positive peak were pooled and designated α2,8-linked polyNeu5Ac-containing glycopeptide (α2,8GP). Based on the Sia yield for Fr.1–Fr.5, 66, 20 and 14% of the total Sia are present in flagellasialin, the α2,8GP and other SGs, respectively. This is consistent with previous observations that flagellasialin is a major SG among sperm glycoproteins (Miyata et al. 2004, 2006).

Characterization of polySia-containing O-glycans from the α2,8GP. The α2,8GP was analyzed by mild acid hydrolysis/DMB (1,2-diamino-4,5-methylenedioxybenzene) derivatization/fluorometric anion-exchange high-performance liquid chromatography (HPLC) to detect the α2,8-linked polyNeu5Ac structure. As reported previously (Miyata et al. 2004), we can distinguish the α2,9-linked polyNeu5Ac from the α2,8-linked polyNeu5Ac with this method (Figure 3A). The α2,8-linked polyNeu5Ac gave the comb shape-like peaks for oligo/polySia structures with a DP of up to 15 (Figure 3B). Based on co-elution experiments using DMB derivatives of authentic α2,8- and α2,9-linked polyNeu5Ac, the α2,8GP contained the α,2,8-linked polyNeu5Ac structure, but not the α2,9-linked polyNeu5Ac structure. These oligo/polyNeu5Ac were sensitive to the exosialidase treatment, and all the oligo/polyNeu5Ac peaks disappeared (Figure 3C). Furthermore, α2,8-linked polyNeu5Ac-specific endosialidase digestion resulted in the disappearance of polyNeu5Ac with a DP of >7 (Figure 3D), indicating the presence of the α2,8-linked polyNeu5Ac structure in the α2,8GP. These properties of the α2,8GP in the sensitivity toward exo- and endosialidase treatments coincide with those of the 190 kDa-gp.

As shown in Figure 1, the α2,8-linked polyNeu5Ac structure was present on the O-glycan chains of the 190 kDa-gp. The O-glycans were released by alkali-borohydride treatment of the α2,8GP and subjected to anion-exchange HPLC (Figure 4). The untreated α2,8GP was eluted as a single peak at 0.25–0.5 M NaCl (Figure 4, open circle). Based on the elution positions of authentic α2,8-linked polyNeu5Ac chains, the α2,8-linked polyNeu5Ac of α2,8GP before (B) and after (C) treatment with exosialidases and α2,8-linked polyNeu5Ac-specific endosialidase (D).

Fig. 3. Detection of polyNeu5Ac structure by a mild acid hydrolysis/DMB/fluorometric anion-exchange HPLC method. (A) Co-chromatography of DMB derivatives of authentic α2,8- and α2,9-linked polyNeu5Ac. DMB derivatives were applied to a Mono Q anion-exchange chromatography and eluted with 10 mM Tris–HCl (pH 8.0) with a gradient from 0 to 0.4 M NaCl as indicated by a dotted line. The elution was monitored by a fluorescence detector (excitation 373 nm and emission 448 nm). These oligo/polyNeu5Ac are separated from each other for peaks with DP of up to 6, whereas peaks with DP ≥7 are co-eluted. The early and the late eluting peaks of each doublet are oligomers of α2,8- and α2,9-linked Neu5Ac, respectively (Miyata et al. 2004). The number at each peak represents the DP. HPLC of DMB derivatives of the α2,8GP before (B) and after (C) treatment with exosialidases and α2,8-linked polyNeu5Ac-specific endosialidase (D).
(Figure 4B), the α2,8GP is suggested to contain various numbers of Sia residues ranging from 10 to 80 residues and an average of 25. By the alkali-borohydride treatment, the resulting O-glycans were eluted at the lower NaCl concentration regions as a lot of peaks corresponding to the oligo/polySia with DP 2–40 (Figure 4, closed circle). These results suggest that the α2,8GP contains more than one O-glycans and that 2–40 Sia residues are linked to the O-glycan chain.

Amino acid sequence analysis of the α2,8GP. The α2,8GP was hydrolyzed under 0.1 N trifluoroacetic acid (TFA) at 80°C for 2 h to release the Sia residues. The hydrolysate was subjected to a reverse-phase HPLC as described under Materials and methods. Of several peak components, one peak component was successfully analyzed to give the 19 amino acid sequence: EQGXERLXXLLXLIXEAPE (X, unidentified). GalNAc-Thr-phenylthiohydantoin amino acid was detected at the cycle of X (Supplementary data, Figure 1S). This peak component was subjected to matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) analysis and a molecular mass of 3131 was obtained. Provided that all the X residues were GalNAc-Thr residues, the molecular mass for the peak was [M + H]+ = 3132, identical to the observed value. These results suggest that the obtained component is EQGTERLTTLLITAPE, where all five T residues are modified by GalNAc residues. This structural feature of the component is consistent with the observation that the α2,8GP contains a few O-glycans on the peptide. Searches of various databases for this amino acid sequence resulted in no homologous sequence, and this component is thus suggested to be derived from a novel glycoprotein.

Localization of the α2,8-linked polyNeu5Ac-containing 190 kDa-gp in sperm

Sea urchin sperm was fractionated into the flagellum and head parts as described in Materials and methods. Western blotting...
of these fractions using mAb.12E3 showed that the 190 kDa-gp was detected in the flagellum, while only faint staining was detected in the head (Figure 5A). In addition, chemical detection of the polySia was performed by mild acid hydrolysis/DMB derivatization/fluorescent anion-exchange HPLC. The oligo/polySia structures with a DP of up to 13 were detected in the whole sperm lysate (Figure 5B). Notably, both the α2,9- and the α2,8-linked polyNeu5Ac structures with DPs of 3 were observed in the flagellum fraction (Figure 5C), although we could not discriminate the α2,9-linked polyNeu5Ac from the α2,8-linked polyNeu5Ac when their DPs were >4. The oligo/polySia structures were also detected in the head fraction, although their amounts were very low (Figure 5D). Note that the 10 times higher quantities of the head fraction than those of the whole sperm lysate and flagellum fractions were analyzed. By co-elution experiments using the DMB derivatives of authentic α2,8- and α2,9-linked polyNeu5Ac, it was confirmed that the polySia structures in the head fraction consisted of α2,8-linked polyNeu5Ac (Supplementary data, Figure 2S). The α2,9-linked polyNeu5Ac structures were not detected in the head fraction.

Detection of the α2,8-linked polyNeu5Ac structures in the sperm lipid fraction

Western blotting with mAb.12E3 detected the α2,8-linked polyNeu5Ac structures only in the 190 kDa-gp in the flagellum glycoprotein fraction (Figure 5A). On the other hand, the α2,8-linked polyNeu5Ac structures are also present in the head fraction in addition to the flagellum fraction, although less present in the head fraction (Figure 5D). These results indicate that α2,8-linked polyNeu5Ac-containing components are present in the sperm head. We thus examined if the α2,8-linked polyNeu5Ac structures were present in the lipid fraction. The lipid fraction prepared from sperm was subjected to mild acid hydrolysis/DMB derivatization/fluorescent anion-exchange HPLC (Figure 6A). The major and the second major peaks corresponded to the α2,8-linked diNeu5Ac (2) and Neu5Ac8S (S1), respectively. Although the amounts were very low, polyNeu5Ac structures with DPs of up to 16 were also detected (Figure 6A, inset). The positions of elution were all identical to those for the authentic 2,8-linked polyNeu5Ac. Peaks with DPs >5 disappeared with α2,8-linked polyNeu5Ac-specific endosialidase digestion (Figure 6B). These results demonstrate the occurrence of the α2,8-linked polyNeu5Ac with DPs ranging from 2 to at least 16 in sperm lipids, most probably in the head fraction. The α2,9-linked polyNeu5Ac structures were not detected in the lipid fraction.

Immunolocalization of the α2,8-linked polyNeu5Ac structure in sperm

To localize the α2,8-linked polyNeu5Ac structures in sperm, immunofluorescence localization on whole sperm was examined using mAb.12E3. No non-specific immunofluorescence was observed in all specimens, using mouse IgM as isotypic immunoglobulin controls for mAb.12E3 (data not shown). As shown in Figure 7A, the α2,8-linked polyNeu5Ac were localized over the entire sperm flagellum and head, although more intense immunostaining was observed in head. Confocal fluorescence microscopic observations (Figure 7B) also showed that α2,8-linked polyNeu5Ac was localized more in head than in flagellum. In 0.2% Triton X-100-treated sperm, most immunofluorescence signals were lost (Figure 7C). The same results were obtained for sperm treated with 0.2% Nonidet P-40 (data not shown).

Effects of the anti-α2,8- and α2,9-linked polyNeu5Ac antibodies on fertilization

In our previous report (Miyata et al. 2004, 2006), we showed that the anti-α2,9-linked polyNeu5Ac antibody, mAb.4F7, inhibited sperm motility through an increase of intracellular Ca2+ concentration, eventually inhibited fertilization, whereas the anti-α2,8-linked polyNeu5Ac antibody, mAb.12E3, had no effect on sperm motility. In this study, we examined the effect of mAb.12E3 on fertilization. Preincubation of sperm with mAb.12E3 had no effect on fertilization. Therefore, the α2,8-linked polyNeu5Ac structure appears not to be important for fertilization events.

Discussion

In this study, two novel features of naturally occurring polySia are described for the first time. The first one is the co-localization of the α2,8- and α2,9-linked polySia structures in the same sea urchin sperm. The α2,8-linked polyNeu5Ac is linked to O-linked glycans of the 190 kDa-gp as well as to glycolipids, whereas α2,9-linked polyNeu5Ac is attached to O-linked glycans of flagellasin, a major SG in sea urchin sperm (Miyata et al. 2004, 2006). The second novel finding of this study is the occurrence of α2,8-linked polyNeu5Ac with a DP of ≥16 in glycolipids. The presence of the
urchin sperm (Ijuin et al. 1996). However, no polySia structure has been demonstrated in glycolipids in any animal cells. The amount of such polysialogangliosides is too small for detection by conventional thin-layer chromatography colorimetric detection methods. Fluorometric analysis enabled us to detect them (Sato et al. 2000).

Previous study showed that sea urchin eggs contain α2,5-O-glycolyl-polyNeu5Gc structures in polySia-gp (Kitazume et al., 2008). Current efforts in our laboratory are being made to identify α2,8- and α2,9-polySialyltransferases in sea urchin gene databases (Harduin-Leper et al., 2008). An increasing number of examples of the occurrence of polySia in animal cells have been demonstrated as described in Introduction. In all these polySia-containing glycoproteins, other than NCAM, the polySia residues appear to function not only as anti-cell adhesion components. Rather, there must be more diverse functions of the polySia for those glycoproteins. In this regard, it is noteworthy that an SG from sea urchin egg jelly

Based on the amino acid sequence of the glycopeptide fragment (α2,8GP) of the 190 kDa-gp, this is a different glycoprotein from flaggellasialin. Unfortunately, we could not identify the 190 kDa-gp by database search using the amino acid sequence. Western blotting with mAb.12E3 and mAb.4F7 indicates that both glycoproteins are more abundant in flagellum rather than in head. We previously demonstrated that flaggellasialin is concentrated in membrane microdomains (lipid rafts), which are characterized as low-density, detergent-insoluble membrane, of sperm and that the α2,9-linked polyNeu5Ac structure of flaggellasialin may be involved in regulation of intracellular calcium ion concentrations that affect sperm motility (Miyata et al. 2006). Most recently, it has been shown that flaggellasialin is involved in induction of a calcium ion-dependent efflux system in motile sperm (Kambara et al. 2011). On the other hand, we could not find any effects of anti-α2,8-linked polyNeu5Ac antibody on sperm motility or fertilization rate. Therefore, these two different polySia-containing glycoproteins may have distinct functions from each other in sperm. Future studies should focus on the function of α2,8-linked polyNeu5Ac structure, and it would be interesting to consider a pH-dependent calcium regulation in sperm, which was already demonstrated for α2,5 glycolyl-linked polyNeu5Gc of egg jelly SG (Hirohashi and Vacquier 2002).

A lot of knowledge has been obtained about the function of the α2,8-linked polyNeu5Ac structure on vertebrate NCAM (Rutishauser 2008). The α2,8-polyNeu5Ac structure on NCAM negatively regulates cell–cell and cell–extracellular matrix interactions by inhibiting homophilic binding of NCAM due to its bulky, polyanionic nature, and subsequently it facilitates neural cell migration (Troy 1996; Rutishauser 2008). Recently, a novel function of α2,8-linked polyNeu5Ac in NCAM as a reservoir for the brain-derived neurotrophic factor has been postulated (Kanato et al. 2008). An increasing number of examples of the occurrence of polySia in animal cells have been demonstrated as described in Introduction. In all these polySia-containing glycoproteins, other than NCAM, the polySia residues appear to function not only as anti-cell adhesion components. Rather, there must be more diverse functions of the polySia for those glycoproteins. In this regard, it is noteworthy that an SG from sea urchin egg jelly
increases the intracellular pH of sperm and facilitates the egg jelly-induced acrosome reaction when sperm meet SG during fertilization (Hirohashi and Vacquier 2002). In sea urchin, the fucose sulfate polymer (FSP), a proteoglycan-like molecule, exists in egg jelly (SeGall and Lennarz 1979; Alves et al. 1997). FSP binds to its receptor named REJ 1 on sperm, leading to the induction of the acrosome reaction (Vacquier and Moy 1997). It is important to understand how either FSP-induced acrosome reaction.

In sea urchin sperm, the disialoganglioside, Neu5Aco2,8Neu5Aco2,6Glcβ1Cer, is a major glycolipid component, while oligosialoglycolipid with tri- and tetrameric Neu5Ac residues are minor (a few percent) components (Ijuni et al. 1996). There are far fewer polysialogangliosides with α2,8-linked polyNeu5Ac in the 190 kDa-gp or α2,9-linked polyNeu5Ac in flagellasilalin in the sperm is involved in the FSP-induced acrosome reaction.

In sea urchin, polysialogangliosides, such as α2,8- and α2,9-linked polyNeu5Ac, are involved in the interaction with the egg 350 kDa sperm receptor, which recognizes α2,8-linked polyNeu5Ac with a DP of ≥16 in sperm. We do not know the biologic function of such a tiny amount of polysialoglycolipids. However, they tend to accumulate in the lipid rafts, which are known as a hot spot for ligand-receptor interactions and signal transduction (Simons and Toomre 2000). Thus, it would be important to search for a binding counterpart of the polysialogangliosides in egg components in the future. In this regard, it is noted that more than 50% of the major gangliosides are enriched in the lipid rafts (Ohta et al. 1999) and that the gangliosides in the sperm lipid rafts are involved in the interaction with the egg 350 kDa sperm binding protein at fertilization (Ohta et al. 2000; Maehashi et al. 2003). Furthermore, it should also be noted that artificial polysialoglycolipids are reported to enhance membrane fluidity of liposomes (Janas et al. 2001; Timoszyk et al. 2004). Since the polysialogangliosides appear to be localized in the sperm head, these glycolipids may facilitate rearrangement of the membrane proteins on the sperm surface upon sperm activation (Neill and Vacquier 2004), membrane fusion at exocytosis of acrosome reaction and sperm–egg plasma membrane fusion.

**Materials and methods**

**Materials**

Hemicentrotus pulcherrimus were collected from Chiba coast or purchased from the Fisheries at Fukushima, Japan, and maintained in aquaria in the Center for Education and Research of Field Sciences, Shizuoka University, Japan. Sperm were collected as undiluted semen (dry sperm) and maintained in aquaria in the Center for Education and Research of Field Sciences, Shizuoka University, Japan.

Materials

Hemicentrotus pulcherrimus were collected from Chiba coast or purchased from the fisheries at Fukushima, Japan, and maintained in aquaria in the Center for Education and Research of Field Sciences, Shizuoka University, Japan. Sperm were collected as undiluted semen (dry sperm) and kept on ice (Miyata et al. 2004). An mAb.4F7, which recognizes α2,9-linked oligo/polyNeu5Ac, was prepared as described previously (Miyata et al. 2006). mAb.12E3, which recognizes α2,8-linked oligo/polyNeu5Ac with DP ≥5, was generously gifted by Dr. Tatsunori Seki (Tokyo Medical University, Japan) and prepared as described previously (Sato et al. 1995; Sato and Kitajima 1999). A goat IgG (Beckman Coulter, Inc., CA), a mouse IgG (Inter-Cell Technologies, Inc., Jupiter, FL) and a mouse IgM (Wako Pure Chemical Industries, Ltd, Japan) were used as immunoglobulin isotype controls. Peroxidase-conjugated anti-mouse IgM was purchased from Zymed, San Francisco.

**Purification of the α2,8-linked polyNeu5Ac-containing glycopeptides from trypsin digests of sea urchin sperm**

Unless otherwise stated, all purification procedures were carried out at 4°C. Sperm (200 mL) were suspended in 600 mL of acetone to prepare the acetone powder as described (Miyata et al. 2004). The lipid was extracted from the powder with 300 mL of chloroform/methanol (2:1, v:v) and with subsequent 300 mL of chloroform/methanol (1:2, v:v). The lipid-extracted powder was then washed with 300 mL of ethanol, suspended with 2 L of 50 mM Tris–HCl (pH 7.5) and treated with 0.22 g of trypsin (Sigma, St. Louis, MO) at 25°C for 20 h. The same amount of trypsin was added every 20 h and total time of trypsin digestion was 60 h. The reaction mixture was then centrifuged at 18,000 × g for 30 min, and the supernatant was subjected to ethanol precipitation at final ethanol concentration of 70%. After centrifugation at 5000 × g for 20 min to remove DNA, the supernatant was 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 eluents were analyzed for the Sia amount as described (Sato et al. 2004). The fractions (41–73) eluted with 0.17–0.28 M NaCl were pooled and reapplied to a DEAE-Toyopearl 650 M column. The fractions eluted with 0.17–0.28 M NaCl were pooled and applied to a Sephacryl S-100 gel filtration column (0.8 × 106 cm) eluted with 0.1 M NaCl. The Sia-containing fractions were then pooled and further purified by DEAE-Toyopearl 650 M chromatography and Sephacryl S-100 gel filtration. The single peak containing α2,8-polySia glycopeptides was obtained and named as α2,8GP.

**SDS–PAGE and immunostaining**

The head and flagellum fractions of sperm were prepared as described (Miyata et al. 2004). Whole sperm, head and flagellum fractions were subjected to SDS–PAGE and immunoblotting as described (Sato et al. 2000). Alkaline treatment, PNGase F treatment and exo- and endosialidase treatment of the blotted membrane were performed as described (Sato et al. 2000). All membranes were further treated with 10 mM sodium phosphate buffer (pH 7.2)/0.15 M NaCl (PBS), containing 0.05% Tween 20, and 1% bovine serum albumin (BSA), and then incubated with mAb.12E3 (Sato et al. 1995; 0.36 mg/mL) at 4°C for overnight. As the secondary antibody, peroxidase-conjugated anti-mouse IgM (1:5000 dilution) was used at 37°C for 1 h and the color development was performed as described (Sato et al. 2000).

**Chemical detection of polySia**

For analyses of polySia structure, a mild acid hydrolysis/fluorometric anion-exchange HPLC analysis (Sato et al. 1999) was performed. The samples (1 μg as Sia) were treated with 0.01 N TFA at 50°C for 1 h, followed by derivatization with DMB (Dojindo, Kumamoto, Japan). The DMB derivatives were treated with exo- or endosialidase at 37°C for 20 h as described (Sato et al. 2000).
Analysis of sialoglycopeptide alditols obtained by alkaline borohydride

The α2,8GP (100 μg as Sia) was hydrolyzed in 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 sialoglycopeptide alditols thus obtained were subjected to anion-exchange 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 5 mM Tris–HCl (pH 8.0).

Amino acid sequencing of α2,8GP

The α2,8GP (85 μg as Sia) was hydrolyzed in 0.1 N TFA at 80°C for 2 h to remove Sia residues. The acid hydrolysate was then applied to a reverse-phase HPLC. The column (Wakopak Handy ODS, 4.6 × 150 mm) was equilibrated with solvent A (0.1% aqueous TFA) and eluted at 0.2 mL/min with solvent A at 0–10 min and with gradients of solvent A/solvent B (0.09% TFA in acetonitrile) 100:0 to 40:60 (v/v) at 10–130 min. The elution profile was monitored by absorbance at 210 nm. The obtained peptides were sequenced by the automated Edman degradation method on Procise HT (Applied Biosystems, Foster City, CA) or by MALDI-TOF MS as described (Yasuakawa et al. 2005).

Immunofluorescence

Hemicentrotus pulcherrimus sperm was suspended and fixed in calcium-free sea water [444 mM NaCl, 9 mM KCl, 40 mM MgCl₂, 22 mM MgSO₄, 5 mM NaHCO₃ (pH 8.2)] containing 3.7% paraformaldehyde and deposited onto poly-L-lysine-treated coverslips. After 15 min, the coverslips were washed three times with PBS. For detergent treatment, the coverslips were treated with 0.2% Triton X-100 in PBS at room temperature for 40 min or with 0.2% Nonidet P-40 in PBS at room temperature for 10 min, before blocking. The blocking was performed by treatment of the coverslips with 3 mg/mL BSA, 0.1 mg/mL normal goat IgG in PBS for 1 h. After washed with PBS, the coverslips were incubated at 4°C for overnight with 20 µg/mL of mAb.12E3. Isotype negative control also ran with normal mouse IgM. After washed three times with PBS, the coverslips were incubated with Alexa 488-conjugated goat anti-mouse IgM antibodies at room temperature for 1 h. After washed three times with PBS and dipped into water, the coverslips were mounted with PermaFluor™ Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA) on a glass slide. Cells were observed with an Olympus BX51 microscope equipped with epifluorescence as well as with diffraction interference microscope. They were also observed with an Olympus FV1000-D laser-scanning confocal microscope equipped with an IX81 microscope.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org.

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Conflict of interest

None declared.

Abbreviations

α2,8GP, α2,8-linked polyNeu5Ac-containing glycopeptide; BSA, bovine serum albumin; DMB, 1,2-diamino-4,5-methyle-dodecyl sulfate; TFA, trifluoroacetic acid.

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