Glycan Recognition

Protective effects of polysialic acid on proteolytic cleavage of FGF2 and proBDNF/BDNF

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Abstract

Polysialic acid (polySia) is a linear polymer of sialic acid that modifies neural cell adhesion molecule (NCAM) in the vertebrate brain. PolySia is a large and exclusive molecule that functions as a negative regulator of cell–cell interactions. Recently, we demonstrated that polySia can specifically bind fibroblast growth factor 2 (FGF2) and BDNF; however, the protective effects of polySia on the proteolytic cleavage of these proteins remain unknown, although heparin/heparan sulfate has been shown to impair the cleavage of FGF2 by trypsin. Here, we analyzed the protective effects of polySia on the proteolytic cleavage of FGF2 and proBDNF/BDNF. We found that polySia protected intact FGF2 from tryptic activity via the specific binding of extended polySia chains on NCAM to FGF2. Oligo/polySia also functioned to impair the processing of proBDNF by plasmin via binding of oligo/polySia chains on NCAM. In addition, the polySia structure synthesized by mutated polysialyltransferase, ST8SIA2/STX(SNP7), which was previously identified from a schizophrenia patient, was impaired for these functions compared with polySia produced by normal ST8SIA2. Taken together, these data suggest that the protective effects of polySia toward FGF2 and proBDNF may be involved in the regulation of the concentrations of these neurologically active molecules.

Key words: BDNF, FGF2, NCAM, plasmin, polysialic acid, polysialyltransferase, proBDNF, SPR, ST8SIA2/STX, Trypsin

Introduction

Polysialic acid (polySia) is a unique macromolecule that modifies neural cell adhesion molecule (NCAM), principally in the vertebrate brain (Rutishauser 2008). PolySia on NCAM is composed of α2,8-linked polyNeu5Ac units with a degree of polymerization (DP) between 8 and 400 (Nakata and Troy 2005; Sato and Kitajima 2013), and is postulated to function as a regulator of cell–cell interactions due to its anti-adhesive properties (Rutishauser 2008). The majority of polySia is found in embryonic brains, but is also present in restricted areas of the adult brain, including the olfactory system and hippocampus (HIP), where neurogenesis and remodeling processes persist in the adult (Bonfanti 2006). Because of the spatio-temporal expression patterns of polySia in the brain, the biological functions of polySia have been well studied and include involvement in various neural cell activities, including adhesion, migration, growth, survival and long-term potentiation (LTP) and depression (Colley et al. 2014). In addition, polySia has been shown to influence neurite outgrowth, branching and bundle formation of axons and synaptogenesis (Colley et al. 2014). PolySia-deficient mice, including NCAM-knockout (KO) mice (Cremer et al. 1994) and polysialyltransferase ST8SIA2/STX (Angata, Chan, et al. 2004; Angata, Long, et al. 2004) and/or ST8SIA4/PST (Eckhardt et al. 2000) KO mice, display phenotypes characterized by impairments in learning, memory, circadian rhythm and social behavior (Hildebrandt et al. 2010). The relationship between polySia expression and the gene encoding the polySia biosynthetic enzyme, ST8SIA2 and a mutated ST8SIA2(SNP7) in psychiatric disorders has been
reported (Barbeau et al. 1995; Sato and Kitajima 2013). Importantly, these studies showed a decrease in polySia-expressing cells by immunohistochemistry in the HIP of brains of schizophrenic patients (Barbeau et al. 1995). An association between single nucleotide polymorphisms (SNPs) in the promoter region of the ST8SIA2 gene and schizophrenia (SCZ) in both the Japanese (Arai et al. 2006) and Chinese Han populations (Tao et al. 2007) and a relationship between SNPs in intron 4 of ST8SIA2 and autism (AD) and bipolar disorder (BD) was also reported (Anney et al. 2010; Kamien et al. 2014; Shaw et al. 2014). Despite the myriad of important functions of polySia that have been identified at both the cellular and animal levels, the underlying mechanisms that connect structure to function of polySia have not been examined at the molecular level principally because polySia has been often considered an anti-adhesive molecule with repulsive field (Yang et al. 1994; Rutishauser 2008; Sato and Kitajima 2013).

Recently, however, we and other group demonstrated that polySia binds biologically active molecules, including brain-derived neurotrophic factor (BDNF) (Kanato et al. 2008), fibroblast growth factor 2 (FGF2) (Ono et al. 2012), neurotransmitters (Isomura et al. 2011), histone H1 (Mishra et al. 2010) and thereby potentially regulating their functions. Notably, all of these molecules are intimately related to the functions and diseases that are associated with polySia. In addition, we determined that polySia-NCAM synthesized by ST8SIA2 with an amino acid mutation in the ST8SIA2(SNP7), which was identified in a schizophrenic patient (Arai et al. 2006), contains fewer and shorter-chain-length polySia molecules (Hane et al. 2012) and exhibits an impaired reservoir function for neurologically active molecules such as BDNF and FGF2 as compared with the ST8SIA2 synthesized by wild-type ST8SIA2 (Isomura et al. 2011; Hane et al. 2012). The recently demonstrated binding properties of polySia toward BDNF and FGF2 differ from those of heparan sulfate (HS) and heparin (HP) (Sato and Kitajima 2013) (Colley et al. 2014), particularly with respect to the minimum DP required for binding, oligomerization, stoichiometry and migration toward receptors after complex formation, although the binding affinities were almost the same.

FGF2 is a well-characterized neurologically active molecule that can stimulate the growth of various cell types, including fibroblasts and tumor cells (Itoh 2007). Although FGF2 lacks a signal peptide sequence, it exists in the extracellular space as a soluble protein. FGF2 is expressed in large amounts in the brain at early stages of development and is involved in brain formation. Recent studies have demonstrated that FGF2 plays an important role in adult neurogenesis as a potent modulator of proliferation and differentiation of multi-potent neural progenitor cells in the adult subventricular zone (SVZ) (Mudø et al. 2009). In addition, several reports have shown that a relationship exists between FGF2 levels and the severity of several psychiatric disorders (Fumagalli et al. 2005; Gaughran et al. 2006; Turner et al. 2008, 2009; Perez et al. 2009; Graham and Richardson 2010). It is well established that FGF2 binds to HS and HP, which protect FGF2 against the trypsin cleavage of FGF2 in the extracellular space (Saksela et al. 1988). Recently, trypsin and trypsin-like proteinases were shown to present in extracellular space in brain and are involved in brain functions (Wang et al. 2008). Although HS/HP and polySia are anionic polymers that are able to bind FGF2, it remains unclear if polySia also has protective effects on the trypsic cleavage of FGF2.

BDNF is the most abundant neurotrophic factor in brain, where it promotes the growth and development of immature neurons and enhances the survival and functional maintenance of adult neurons (Barde et al. 1982; Barde 1994). BDNF levels are correlated with several disorders, including depression, epilepsy, BD, SCZ, Parkinson’s disease and Alzheimer’s disease (Kozisek et al. 2008; Buckley et al. 2011). Recently, it was reported that proBDNF is secreted extracellularly, where it is cleaved by tPA/plasmin and processed to mature BDNF. Interestingly, extracellularly processed BDNF is reported to play a pivotal role in memory (Pang et al. 2004). Although the effects of polySia and HP on processing of proBDNF to BDNF remain unknown, the binding of these polymers by BDNF has been demonstrated (Kanato et al. 2009).

Based on the binding properties of polySia and HP towards FGF2 and BDNF/proBDNF, we have analyzed the protective effects of polySia on the cleavage of these neurologically active molecules by trypsin and plasmin.

**Results**

**Effects of polySia on proteolytic cleavage of fluorescent-labeled substrates**

To evaluate the possible effects of polySia on proteases, we used fluorescent-labeled substrates of trypsin or plasmin to measure the release of 4-amino-7-methylcoumarin (AMC), a fluorescent molecule. Cleavage of the fluorescent-labeled substrates by trypsin and plasmin increased as a function of the concentration of the proteases (Figure 1, Control). In the presence of polySia, the amount of fluorescence after cleavage by trypsin or plasmin was identical to that detected in the absence of polySia (Figure 1, Control vs. polySia). The same result was obtained in the presence and absence of HP (Figure 1, Control vs. HP). These data thus show that polySia and HP did not influence the proteolytic activities of trypsin and plasmin.

**Effects of polySia on cleavage of FGF2 by trypsin**

Protective effect of polySia on FGF2 as measured by SDS–PAGE/western blotting

The protective effect of polySia on the protease activity of trypsin toward FGF2 was studied by incubating intact FGF2 in the absence (control) and presence of polySia or HP, then followed by treatment with increasing amounts of trypsin (Figure 2). At 6.4 ng trypsin, ~50% of the FGF2 was proteolytically cleaved in the absence of polySia or HP (Figure 2A, Control). The remaining undigested component was

**Fig. 1.** Effects of polySia on cleavage of fluorescence-labeled substrates by trypsin and plasmin. Trypsin (Bz-Arg-MCA, 0.5 ng/µL in PBS) or plasmin substrates (Boc-Val-Leu-Lys-MCA, 0.5 ng/µL in PBS) were incubated without (Control, ▲) or with polySia (1 µg/µL in PBS, ◆) or HP (1 µg/µL in PBS, ♦) at 37°C for 5 min and protease enzymes were then added. After incubation at 37°C for 90 min, the fluorescence (AMC) released by proteases was measured with a fluorescence spectrometer at Ex 360 nm and Em 420 nm. (A) Activity of trypsin. (B) Activity of plasmin.
attributed to protease-resistant FGF2, as was previously reported (Weltrowski et al. 2012). The protease-resistant FGF2 was not degraded even with excess trypsin (trypsin:FGF2 = 12.8 : 80 ng (1 : 5) (mol/mol)). Intact FGF2 was used in all of the following experiments.

When FGF2 was incubated in the presence of HP, the amount of FGF2 remained unchanged (Figure 2B, HP), indicating that HP completely protected FGF2 against trypsin, as reported previously (Coltrini et al. 1993; Weltrowski et al. 2012). In the presence of polySia, the trypsin cleavage of FGF2 was also inhibited (Figure 2B, polySia), although 75% of FGF2 remained undigested under the excess trypsin. Therefore, the protective efficacy of polySia towards FGF2 was estimated to be 48%, because 52% of total FGF2 were shown to be protease-resistant component.

Time course of FGF2 incubation
FGF2 has a short half-life (7.6 h) (Bush et al. 2001) as it readily changes conformation and is degraded by extracellular proteases (Tardieu et al. 1994). Although HS and HP protect FGF2 against proteolysis, the potential protective effect of polySia on the half-life of FGF2 is not known. We therefore examined the protective effect of polySia on denatured FGF2 at 37°C for 0–24 h and then digested the denatured samples with trypsin in the presence and absence of polySia or HP. In the absence of polySia and HP, FGF2 was increasingly degraded at 37°C with longer incubation times to a maximum of ∼50% after 24 h (Figure 3A) and the remaining amount was due to trypsin-resistant FGF2 based on the results obtained in Figure 2A and a previous report (Weltrowski et al. 2012). In the presence of polySia, no significant proteolysis of FGF2 was observed during incubation at 37°C for 0–6 h. After 24 h incubation at 37°C, however, ∼40% of FGF2 was degraded (Figure 3B). In contrast, as reported previously, the protective effects of HP on the degradation of FGF2 by trypsin were similar under all incubation conditions (Figure 3D) (Weltrowski et al. 2012). The protective effects of polySia and HP on the proteolytic cleavage of FGF2 was not observed when polySia and HP were added to FGF2 after the addition of trypsin (Figure 3C and E). These different sensitivities of FGF2 to trypsin cleavage, and the binding properties of HP and polySia toward FGF2 are distinct, as reported previously (Ono et al. 2012; Sato and Kitajima 2013; Colley et al. 2014).

Binding properties of polySia-NCAM toward FGF2
As exogenous polySia and HP did not influence the activity of trypsin using synthetic peptide as substrate (Figure 1A) and it has been shown that polySia and HS/HP differentially bind to FGF2 (Ono et al. 2012), we considered that the polySia-specific protective effects were attributable to the binding properties/affinity of polySia toward FGF2. To confirm that polySia is able to bind intact, but not heat denatured, FGF2, we analyzed the direct binding of polySia moieties on NCAM to the intact and heat-denatured FGF2 (Weltrowski et al. 2012) using a surface plasmon resonance (SPR)-based procedure (Biacore). First, we purified polySia-NCAM-Fc(WT), which contained polySia synthesized by normal human ST8SIA2(WT), and additionally prepared oligoSia-NCAM-Fc and asialo-NCAM-Fc by treating polySia-NCAM-Fc(WT) with endo-N-acetylgalactosaminidase (Endo-N) and exo-sialidase, respectively. Endo-N is an enzyme that specifically cleaves polySia to oligoSia-chains (DP = 2–3) (Hallenbeck et al. 1987; Sato et al. 1993). The oligoSia and polySia structures were confirmed using anti-diSia and anti-polySia antibodies (Figure 4A, IB: diSia and polySia).
OligoSia-NCAM-Fc was not stained by anti-polySia antibody, but did not react with anti-dsia-Gal antibody (Sato et al. 2000). Anti-polySia antibody, 12E3 recognizes polySia (DP ≥ 5) including non-reducing terminal end (Sato et al. 2000) and the intensity of immunostaining with this antibody shows the amount of polySia chain moieties. Asialo-NCAM-Fc was not stained by either Sia-specific antibody. The Fc region of each purified sample was confirmed by staining with anti-Fc antibody (Figure 4A, IB: anti-Fc). We also prepared polySia-NCAM-Fc(SNP7), which was synthesized by ST8SIA2 (SNP7) of a schizophrenia patient. The polySia structure of polySia-NCAM-Fc(WT) and polySia-NCAM-Fc(SNP7) was analyzed using anti-polySia antibody (12E3), as shown in Figure 4B (IB: 12E3), and was found to be similar based on the immunoreactivity. The maximum DPs of each polySia chain were 43 and 33, respectively, as reported previously (Hane et al. 2012).

To study the binding between polySia and FGF2, polySia-NCAM-Fc, oligoSia-NCAM-Fc and asialo-NCAM-Fc were immobilized on the Au surface via pre-immobilized protein A onto a self-assembled membrane (SAM) with FGF2 as an analyte (Figure 4C, asialo/oligo/polySia-NCAM-Fc). Based on this evaluation, intact FGF2 was shown to bind polySia (Figure 4D, polySia-NCAM), indicating that binding of FGF2 decreased with increased incubation time at 37°C from 3 to 6 h (Figure 4E and F). After 24 h of incubation, no binding was observed (Figure 4E and F). The amount of FGF2 bound by the polySia-NCAM synthesized by the mutated polysialyltransferase, SNP7, decreased to ca. 30% of that of WT (Figure 4D, WT vs. SNP7), although the immunostaining with anti-polySia antibody was nearly identical between the two molecules (Figure 4B). The Kd of polySia derived from ST8SIA2 (SNP7) was 2.2 × 10^{-4} M, indicating that its affinity for FGF2 decreased ca. 10-fold compared to that of WT. The oligoSia on oligoSia-NCAM did not bind FGF2 (Figure 4D, oligoSia-NCAM), indicating that binding of polySia-NCAM to FGF2 appears to be a property of the more extended polySia chain on NCAM (Ono et al. 2012). The heat-incubation time of FGF2 influenced on the amounts of FGF2 bound by polySia on NCAM (Figure 4E, polySia-NCAM(SNP7)), indicating that heat-denatured FGF2 is not recognized by polySia on NCAM. In addition, this property was impaired in case of polySia-NCAM synthesized by ST8SIA2 (SNP7) (Figure 4E, polySia-NCAM(SNP7)). The efficiency of polySia-NCAM binding to intact FGF2 was also evaluated by measuring the time for the half binding of FGF2 (Figure 4F). The incubation time for 50% binding of polySia-NCAM(WT) to FGF2 was ca. 3 h, but was only 1 h for polySia-NCAM-Fc(SNP7) (Figure 4F). Therefore, the polySia structure synthesized by ST8SIA2(SNP7) was presumably different from that synthesized by ST8SIA(WT) based on the binding to intact FGF2 (Figure 4F).

Effects of polySia on cleavage of BDNF and proBDNF by plasmin

Protective effects of polySia on BDNF/proBDNF proteolysis of plasmin as determined by SDS–PAGE/western blotting

BDNF and proBDNF were used as substrates for plasmin, a processing enzyme for the extracellular conversion of proBDNF to BDNF and proDomain (Pang et al. 2004). Initially, we incubated BDNF in the presence and absence of polySia and then treated the samples with plasmin. As shown in Figure 5A, BDNF was not cleaved significantly with plasmin, even when treated with a large excess of enzyme, indicating that native BDNF is resistant to plasmin. We then used proBDNF that is reported to be a substrate of plasmin (Pang et al. 2004). Incubation of proBDNF with plasmin resulted in the cleavage and formation of mature BDNF, which increased with increasing levels of enzyme (Figure 5B).

Protective effects of polySia on the plasmin-mediated proteolysis of proBDNF as determined by SDS–PAGE/western blotting

Because BDNF was resistant to cleavage by plasmin (Figure 5A) and that mature BDNF processed by plasmin from proBDNF could be measured by immunostaining (Figure 5B), we sought to determine if polySia inhibits the plasmin-mediated processing of proBDNF to BDNF and proDomain. To evaluate the potential inhibitory effect of polySia on the plasmin-mediated proteolysis of ProBDNF, we incubated proBDNF without (control) and with polySia or HP followed by increasing amounts of plasmin. After separation of the samples by SDS–PAGE, all bands were quantitatively analyzed by densitometry. The intensity of bands in each lane was set to 100% and compared with the intensity of bands in the proBDNF control (Figure 6A, control), proBDNF was significantly protected from processing to BDNF but only in the presence of polySia (Figure 6A, polySia). Nearly 75% of the proBDNF remained protected by polySia from proteolysis by plasmin (Figure 6A, polySia, and B). In contrast, ca. 60% of proBDNF remained in the control and HP samples, indicating that polySia inhibits the BDNF processing activity of plasmin (Figure 6B). In contrast, HP had no effect on the plasmin-mediated conversion of proBDNF to BDNF (Figure 6A, HP and B), indicating that HP, in contrast to polySia, did not protect proBDNF against proteolysis by plasmin.

Binding properties of polySia-NCAM toward BDNF, proBDNF and proDomain

Based on the above findings that polySia did not inhibit the activity of plasmin (Figure 1), but did inhibit the processing of proBDNF to BDNF (Figure 6A and B), and that BDNF is not cleaved by plasmin (Figure 5A), we hypothesize that polySia has a binding affinity for proBDNF. Although such an affinity has not been previously reported, the binding of polySia to BDNF was described earlier (Hane et al. 2012). To determine if polySia can bind proBDNF, we analyzed the direct binding between polySia on NCAM and proBDNF, BDNF and proDomain by Biacore analyses using the procedure described in Figure 4C. When BDNF was applied on the immobilized polySia-NCAM(WT) surface, BDNF bound the surface in a dose-dependent manner (Figure 7A, polySia-NCAM(WT), BDNF); however, the sensorgram appears to be biphasic: at 0–9 nM BDNF, BDNF was dose-dependently associated; at 19 nM and higher, a larger number of BDNF molecules rapidly associated to polySia-NCAM-Fc, and then gradually dissociated even under the flow of the same concentration of BDNF. Although BDNF also bound to polySia-NCAM(SNP7) and oligoSia-NCAM, this biphasic profile was unique to polySia-NCAM(WT). When calculated with the sensorgrams for 2–9 nM BDNF to compare the binding degrees with one another, a dissociation constant for polySia-NCAM (WT) was Kd = 4.1 × 10^{-10} M, which was a similar value for polySia-NCAM(SNP7) (Kd = 5.9 × 10^{-10} M), and a lower value for oligoSia-NCAM (Kd = 1.2 × 10^{-9} M). To gain a further insight into the binding properties of BDNF, we then compared the sensorgrams for polySia-NCAM(WT) and polySia-NCAM(SNP7) using the sensorgram for oligoSia-NCAM as a negative control (Figure 7B, polySia-NCAM, BDNF). The binding of BDNF to polySia moieties expressed on polySia-NCAM(WT) in Figure 7B showed a biphasic profile similar to that in Figure 7A. Collectively, these results show that BDNF is
able to bind both polySia and oligoSia structure. On the other hand, the polySia moiety expressed on polySia-NCAM-Fc(SNP7) could not bind to BDNF, although immunostaining profiles with 12E3, an anti-polySia antibody which recognizes the non-reducing terminus of polySia chains, were nearly identical for both polySia-NCAM(WT) and polySia-NCAM(SNP7). This finding indicates that the structure of polySia synthesized by ST8SIA2(SNP7) has impaired BDNF binding, but that it does not affect the oligoSia-BDNF binding.

The binding of polySia-NCAM to proBDNF (Figure 7A, polySia-NCAM(WT) was nearly the same as the amount of oligoSia-NCAM bound to proBDNF (Figure 7A, oligoSia-NCAM(WT), proBDNF). However, the polySia chain synthesized by ST8SIA2(SNP7) did not bind to proBDNF, providing further evidence of the likely structural difference between the two polysialylated structures synthesized by the two distinct polySTs. To confirm the contribution of the polySia chain on NCAM to mediate the binding toward proBDNF, we re-
analyzed the sensorgram of oligoSia-NCAM as a negative control. As shown in Figure 7B, the binding of the polySia chain to proBDNF was observed but was not concentration dependent (Figure 7B, polySia-NCAM(WT)). Notably, polySia on polySia-NCAM(SNP7) inhibited the binding of proBDNF (Figure 7B, polySia-NCAM(SNP7)), whereas the binding of proBDNF by oligo/polySia structure required higher concentrations of proBDNF (39–58 nM). The binding of proDomain cleaved from proBDNF by plasmin was not observed (Figure 7A, pd-BDNF).

Discussion

Glycosaminoglycan chains, including HP and HS, are well-studied anionic molecules that can bind FGF2 and protect it from proteolysis in extracellular spaces (Coltrini et al. 1993; Weltrowski et al. 2012). In contrast, polySia is an anionic molecule found principally in the brain, and has been postulated to have repulsive properties (Rutishauser 2008) (Figure 8). For this reason, the protective effect of polySia on the proteolytically degradation of FGF2 and other biologically active molecules has not been previously studied. We recently demonstrated, however, by gel-filtration, native-PAGE and SPR-based analyses that polySia can bind to FGF2 and BDNF (Kanato et al. 2008; Ono et al. 2012; Sato and Kitajima 2013; Colley et al. 2014) and that the binding properties of polySia towards these molecules are different from those of HS. This new information stimulated the importance of analyzing the protective effect of polySia toward FGF2 and BDNF/proBDNF because the polysialyl moiety on NCAM has not been previously analyzed as a defensive molecule to protect FGF2 and BDNF/proBDNF against proteolysis as has been shown for HS.

FGF2

As shown in Figure 2, HP was completely protective towards trypsin cleavage of FGF2 while polySia was only ca. 50% protective. The protective effect of polySia might be conformation dependent, because heat-denatured FGF2 was readily cleaved by trypsin in the presence of polySia (Figure 3B). In contrast, HP completely protected FGF2 from proteolytic cleavage even after heat denaturation (Figure 3D). These results show that the binding properties between polySia and FGF2 differ from those between HP and FGF2. In this regard, we previously demonstrated that the binding sites of FGF2 by these molecules are different, although the dissociation constants of polySia and HS toward FGF2 are nearly identical (Ono et al. 2012; Colley et al. 2014). These data suggest that the binding sites on FGF2 for HP are resistant to heat denaturation, whereas those for polySia are heat sensitive. The results obtained by Biacore analyses are consistent with this conclusion, as heat-denatured FGF2 showed decreased binding toward polySia. Thus, it appears that polySia may have the possibility to sense the stability of FGF2 via subtle changes in the conformation of FGF2, and that unlike HP, only intact FGF2 can interact with polySia chains (Figure 8). We previously reported that FGF2 in the complex with polySia readily make a complex with HS by exchanging polySia with HS when HS is provided, whereas FGF2 in the complex with HS does not make a complex with polySia even when polySia is additionally provided (Ono et al. 2012). This finding indicates that polySia might serve as a primary pool of intact FGF2. Because expression of polySia is principally restricted to the embryonic brain and areas within the adult brain where neurogenesis is ongoing (Rutishauser 2008), the polySia moiety on NCAM may serve as a reservoir of intact FGF2, since the developing brain would presumably require large amounts of FGF2. Based on the present results, we conclude that polySia may be an effective therapeutic molecule for the repair of wounded areas due to its ability to bind intact FGF2, as has been demonstrated for HP.

In the present study, the dissociation constant of polysialylated NCAM towards FGF2 was estimated to be 8.3 × 10⁻¹⁰ M. This value was determined by subtracting the contribution of asialo-NCAM, whose structure is identical with that of polySia-NCAM, with the exception of polySia chains. As the K_D value of colominic acid, a linear polymer of sialic acid DP’s ranging from ~2 to >100, was measured to be 1.5 × 10⁻⁴ M, the polySia chain(s) on N-linked glycan chains in NCAM increased the binding affinity of this biologically active molecule for FGF2 by 20-fold. Although the underlying mechanism remains unclear, we have postulated that it may involve a conformational change mediated by the polySia structure (Nagae et al. 2013). Because the binding of oligoSia to FGF2 was not observed, the polySia chain thus appears to be a critical determinant for binding to intact FGF2.

BDNF/proBDNF

BDNF is an important neurologically active molecule that is reported to be extracellularly processed from proBDNF by plasmin (Pang et al. 2004). In the present study, we found that polySia inhibited the processing of proBDNF and that BDNF production was significantly decreased compared with the control and HP (Figure 6B). Based on the reported results that extracellularly processed BDNF affects the LTP in HIP (Pang et al. 2004) and that polySia-NCAM is involved in the LTP in CA1 in HIP (Eckhardt et al. 2000; Muller et al. 2000), polySia chains on NCAM may be involved in LTP via the regulation of proBDNF processing. Notably, we found that polysialylated NCAM bound to proBDNF but only at high concentrations of proBDNF and that the degree of binding was slightly higher compared with that by oligoSia chains on NCAM (Figure 7A, proBDNF). Interestingly, the binding was present but not stable in case of polySia chain (Figure 7B, polySia-NCAM(WT), proBDNF), while stable in case of oligoSia chain (Figure 7A, oligoSia-NCAM, proBDNF). The binding affinity of oligoSia- and polySia-moieties toward proBDNF was the same.
(\(K_D = 1.3 \times 10^{-9}\) M). These findings indicate that the non-reducing terminal end of oligo/polySia chains on NCAM may be sufficient to bind proBDNF, although the overall structure of the polySia chains on the Ig5 domain of NCAM has not been determined. Relative to this point, it is noteworthy that oligoSia-NCAM, which contains diSia to tetraSia chains because it is reactive with S2-566 (anti-diSia-

Fig. 6. Protective efficacy of polySia toward the processing of proBDNF by plasmin. (A) Processing of proBDNF by plasmin. ProBDNF (30 ng) was incubated with polySia or HP (0.4 µg) at 37°C. After reaching equilibrium, 0–3.2 ng of plasmin was added to the mixtures, which were further incubated at 37°C for 150 min. All samples were separated by SDS–PAGE (12.5% polyacrylamide gel) and blotted onto a PVDF membrane. ProBDNF, truncated BDNF and mature BDNF were detected with anti-BDNF antibody (0.1 µg/mL). A typical result is shown in the right panel. The compositions (average values) of proBDNF (black), truncated BDNF (gray) and mature BDNF (white) are shown in the bar graph (left panel). (B) Inhibitory effect of polySia toward the processing of proBDNF to BDNF. *p < 0.05.
Fig. 7. Biacore measurement of binding of BDNF/proBDNF toward polysialo/oligoSia structure on polySia/oligoSia-NCAM. (A) Sensorgrams of molecule binding by polySia-NCAM(WT), polySia-NCAM(SNP7) and OligoSia-NCAM. The sensorgram of asialo-NCAM-Fc was used as a negative control. BDNF/proBDNF/proDomain (pd-BDNF) (0–111 nM) in HBS-EP was injected over the polySia/oligoSia/asialo-NCAM-immobilized sensor chip surfaces at a flow rate of 20 µL/min. After 120 s, HBS-EP was flowed over the sensor surface to monitor the dissociation phase. (B) Sensorgrams of molecule binding by polySia-NCAM(WT) and polySia-NCAM(SNP7) using that of oligoSia-NCAM-Fc as a negative control.
Gal), but not with 12E3 (anti-polySia with DP ≥ 5) (Figure 4A), has been reported to occur frequently in adult brain (Sato et al. 2000; Inoko et al. 2010). Because the binding of oligo/polySia-NCAM to proBDNF was observed at specific concentrations of proBDNF, this suggests that proBDNF might have more than one conformation, and that the conformation at higher concentration may have a greater affinity for oligo/polySia and would therefore be protected from processing by plasmin. It is therefore possible that oligo/polySia chains may regulate the concentration of proBDNF. Unlike polySia, HP did not protect proBDNF from processing (Figure 6). The differential regulation of the processing of proBDNF via HP or polySia may explain why different anionic macromolecules are abundantly present in the brains of various species. Proteolytically cleaved BDNF can be bound by polySia (Figure 7A and B), whereas proDomain had no such affinity. Based on the binding of oligoSia to BDNF (Figure 7A, oligoSia-NCAM), BDNF appears to have at least two binding sites for polySia-NCAM; one recognizing polySia chains and the other recognizing oligoSia chains. Interestingly, the binding properties of polySia toward BDNF at low and high BDNF concentrations are different and biphasic (Figure 7B, polySia-NCAM(WT), BDNF), indicating that there might be other BDNF-binding regions in polySia-NCAM and this may be a potential mechanism to release bound BDNF from polySia-NCAM.

PolySia chain synthesized by ST8SIA2 from a schizophrenic patient (ST8SIA2/STX(SNP7))

SNP-7 (g421a) leads to the change of amino acid, E141K of ST8SIA2/STX and in the present study we have confirmed that polySia chain on polySia-NCAM synthesized by ST8SIA2(SNP7) had lower binding property toward FGF2 and no binding property toward BDNF. In addition, we have shown that polySia-NCAM(SNP7) does not bind proBDNF and inhibits the binding toward NCAM (Figure 7). The difference between this study and the previous study examining the binding of polySia to BDNF and FGF2 is the negative control used for the subtraction of the sensorgrams (Hane et al. 2012). The previous study used NCAM-Fc derived from a Mock-transfectant; however, to evaluate the contribution of the polySia chain toward the binding of FGF2 and BDNF, asialo-NCAM-Fc is a more meaningful negative control, as the underlying NCAM structure is identical except for the length of polySia chains. Although it has already been shown that the DP of polySia derived from SNP-7 is decreased by 75% (WT: DP = 43, SNP-7: DP = 33) (Hane et al. 2012), polySia staining by 12E3 was only marginally different between polySia-NCAM(WT) and polySia-NCAM(SNP7) (Figure 4B). This indicates that the non-reducing terminal end, which is recognized by 12E3, is highly similar between these molecules. The finding that a decrease of DP, even of only 25%, markedly

Fig. 8. Newly proposed properties of polySia moieties on NCAM. PolySia has been widely considered to be an anionic molecule exhibiting repulsive field that functions as a negative regulator of cell–cell interactions. However, polySia has recently shown to bind and serve as a reservoir of neurologically active molecules, such as BDNF, FGF2 and dopamine (Sato and Kitajima 2013), and is therefore considered to both repulsive and attractive fields toward these molecules. In the present study, polySia was newly demonstrated to modulate the activities of proteases. PolySia protects the tryptic cleavage of FGF2 by selectively binding intact FGF2. In addition, polySia was shown to inhibit the plasmin-mediated processing of proBDNF to mature BDNF by binding to plasmin through the oligoSia structure or non-reducing terminal end of polySia chains. These new properties appear to be intimately related to the structure of polySia chains on polySia-NCAM, as indicated by the impairment of these properties in polySia synthesized by mutated ST8SIA2/STX from a schizophrenic patient. This figure is available in black and white in print and in color at Glycobiology online.
impairs the binding of polySia to intact FGF2 suggests that the length of the polySia chain is the most important feature for sensing intact FGF2. It was previously demonstrated that the minimum DP of polySia required for FGF2 binding was 17 (Ono et al. 2012), and that for BDNF was 12 (Kanato et al. 2008).

FGF2 is an important factor for maintaining a basal number of neural stem cells in the HIP (Woodbury and Ikezu 2014). As the impairment of polySia in polysialyltransferase-KO mice results in a decreased number of neural stem cells in the SVZ and subgranular zone (Angata, Chan, et al. 2004; Angata, Long, et al. 2004), polySia might be involved in this phenomenon. The binding of polySia-NCAM to proBDNF was quite specific for the oligoSia structure (Figure 7A and B), but the affinity was markedly reduced for polySia-NCAM synthesized by ST8SIA2 (SNP7), even though this molecule should contain regions of low DP, such as oligoSia units. Based on the sensorgram shown in Figure 7B, polySia-NCAM(SNP7) may adopt a structure that inhibits binding to proBDNF. BDNF and proBDNF have distinct functions, and an imbalance between BDNF and proBDNF is involved in depression, fear and psychiatric disorders (Martinowich et al. 2007). The finding that schizophrenic brains have a reduced number of polySia-expressing cells in the HIP (Barbeau et al. 1995), together with the fact that mice with impaired polySia, such as ST8SIA2-KO mice (Krocher et al. 2015), exhibit fear behaviors and reduced neural stem cell numbers (Angata, Chan, et al. 2004; Angata, Long, et al. 2004), and that polySia-NCAM derived from ST8SIA2(SNP7) has altered structure and molecule binding features (Isomura et al. 2011; Hane et al. 2012), supports the present results that polySia is a dynamic molecule with novel properties in addition to the regulation of cell–cell interactions (Figure 8).

In summary, we have identified three new properties of polySia moiety on polySia-NCAM: (i) protective effect on the tryptic cleavage of intact FGF2 via the specific binding of longer or extended chain of polySia, (ii) protective effect on plasmin-mediated processing of proBDNF by the specific binding of oligo/polySia and (iii) impairment of these binding affinities for polySia-NCAM synthesized by schizophrenic type ST8SIA2(SNP7).

Materials and methods

Materials

Recombinant human FGF2 was obtained from PeproTech (Paris, France). Recombinant BDNF, proBDNF and proDomain were purchased from Alomone Laboratories (Jerusalem, Israel). These recombinant molecules were expressed in Escherichia coli and were not glycosylated. Trypsin, plasmin, Clostridium perfringens exo-sialidase and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Missouri). Fluorescence-labeled substrates for trypsin (Bz-Arg-MCA), plasmin (Boc-Val-Leu-Lys-MCA) and were obtained from the Peptide Institute (Osaka, Japan). Colominic acid, o2,8-linked polyNeu5Ac (average DP = 43), which is chemically and immunologically identical with the polySia structure on NCAM, HP, α-MEM and phenylmethylsulfonyl fluoride (PMSF) were purchased from Wako (Osaka, Japan). Anti-BDNF antibody was purchased from Santa Cruz (CA). Peroxidase (POD)-labeled anti-rabbit IgG was obtained from Cell Signaling (Danvers). POD-labeled anti-mouse IgG+IgM and anti-human IgG+IgM+IgA were purchased from American Qualex (CA). Polyvinylidene difluoride (PVDF) membrane and Ultra YM-100 were obtained from Millipore (MA). Enhanced chemiluminescence western blotting Detection Reagent, SIA Kit Au, Amine Coupling kit and HBP-EP buffer were obtained from GE Healthcare (Buckinghamshire, England). Serum Free Medium (Cosmedium) was purchased from Cosmohio (Tokyo Japan). Protein A resin was the product of Nomadic Bioscience (Okayama, Japan). Anti-polySia antibody, 12E3, which recognizes oligo/polyNeu5Ac structure (DP ≥ 6) and endo-N-acylemuranidase (Endo-N), which cleaves oligo/polySia structure (DP ≥ 5), were generously provided by Dr. Tatsunori Seki (Tokyo Medical University) and Dr. Frederic A. Troy (University of California, Davis), respectively.

Measurement of fluorescence-labeled substrates

To 350 µL trypsin substrate (Bz-Arg-MCA, 0.5 ng/µL in PBS) or plasmin substrate (Boc-Val-Leu-Lys-MCA, 0.5 ng/µL in PBS) solution, 70 µL PBS solution, polySia (1 µg/µL in PBS) or HP (1 µg/µL in PBS) were added and the resulting mixture was incubated at 37°C for 5 min. The reaction mixture was then mixed with 700 µL trypsin solution (0.3–2.5 ng/µL) and incubated at 37°C for 90 min. The reactions were stopped by boiling for 3 min. The fluorescence (AMC) was measured at Ex 360 nm and Em 420 nm using a fluorescence spectrometer (FluoroMax3, Horiba, Japan).

Measurement of protein cleavage by SDS–PAGE/ western blotting

For measuring protein cleavage, 80 ng FGF2 or 10 ng BDNF/30 ng of proBDNF was incubated with 0.8 and 0.4 µg of polySia or HP, respectively. After reaching equilibrium at 37°C, 0–1.6 ng of trypsin or plasmin were added to the reaction mixtures, which were then incubated at 37°C for 90 or 150 min. In the case of heat-denatured FGF2 incubation, FGF2 was pretreated by incubation at 37°C for 6 or 24 h. The reaction was stopped by adding Laemmli buffer and boiling the resulting mixture for 3 min. All samples were analyzed by SDS–PAGE (12.5% polyacrylamide gel) and the separated proteins were blotted onto a PVDF membrane, which was then blocked with PBS containing 0.05% Tween 20 and 1% BSA at 25°C for 1 h. The membrane was incubated with the primary antibody, anti-FGF2 antibody (0.1 µg/mL) or anti-proBDNF antibody (0.1 µg/mL), at 4°C overnight. The membrane was reacted with secondary antibody, peroxidase-conjugated rabbit IgG antibody (1/5000 dilution), by incubation at 37°C for 1 h, color development and densitometric analysis were then performed as previously described (Sato et al. 2000). All reactions were performed with low-adherence plastic tubes and tips. All reactions were performed triplicate.

Purification of polySia-NCAM-Fc, oligoSia-NCAM-Fc and asialo-NCAM-Fc

A Chinese Hamster Ovary cell line stably secreting NCAM-Fc was transfected with either pcDNA-stx (wt) or pcDNA-stx (SNP7) using Lipofectamine. Stable cell lines secreting polySia-NCAM-Fc were established by culturing the transfected cells in the presence of G418. The non-serum culture medium containing secreted polySia-NCAM-Fc was collected and mixed with 100 mM Tris–HCl (pH 8.0), 1 mM PMSF and protease inhibitor cocktail. All procedures were conducted at 4°C in the presence of protease inhibitor between pH 7 and 8. The medium was centrifuged at 900 × g and the obtained supernatant was loaded on a protein A Sepharose column, which was then washed with 10 mM phosphate buffer (pH 7.2) at 4°C. PolySia-NCAM-Fc was eluted from the column with 10 mM phosphate buffer (pH 7.2) at 37°C. The sample was concentrated using a YM-100 Microcon device and the protein concentration of the sample was measured by BCA assay. OligoSia-NCAM-Fc was prepared by adding Endo-N, which cleaves polySia endogenously to oligoSia (DP ≥ 2–3) (Hallenbeck et al. 1987), at a final concentration of 0.9 µM/mL at 37°C for 20 h. Asialo-NCAM-Fc was prepared by the addition of exo-sialidase in sodium acetate buffer (pH 5.5) at a final concentration...
of 100 mM/mL at 37°C for 16 h. The enzyme-treated samples were reloaded onto the protein A Sepharose column and purified. The complete depletion of sialic acid from asialo-NCAM-Fc was confirmed by DMB-HPLC. PolySia-NCAM-Fc, oligoSia-NCAM-Fc and asialo-NCAM-Fc purified were analyzed by SDS-PAGE/western blotting using anti-polySia (12E3) (2 µg/mL), S2-566 (1 µg/mL) and anti-Fc antibodies (0.5 µg/mL) at 4°C. As the secondary antibody, either peroxidase-conjugated anti-mouse IgG+M (1/5000 dilution) or anti-rabbit IgG antibodies (1/4000 dilution) were applied to the membranes, which were then incubated for 60 min at 37°C and color development was performed using standard reagents.

Measurement of FG2, BDNF, proBDNF and proDomain binding by Biacore analysis

For SPR measurements, an Au sensor chip containing a surface-activated SAM was placed on the sensor chip support using the sensor chip assembly unit, and was then set in a Biacore 3000 instrument (Hane et al. 2012; Ono et al. 2012). After priming the system with water for 7 min, 0.1 mg/mL protein A solution was injected into the system at a flow rate of 10 µL/min for a total of 7 min. The loading with protein A was repeated once under the same conditions, and immobilized streptavidin was then monitored by measuring the resonance unit (RU) value, which typically reached 1300–1850 RU for protein A. To destroy excess activated groups, 1 mM ethanolamine was injected into the system for 7 min. After washing the sensor chip with HBS-EP (0.01 M HEPES, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA and 0.0005% Surfactant P20), purified polySia-NCAM-Fc, oligoSia-NCAM-Fc or asialo-NCAM-Fc (0.1 mg/mL in 500 mM HBS-EP) was injected into the system to allow immobilization on the Au surface. Immobilization of polySia-NCAM-Fc was monitored based on the observed RU values, which typically reached ~850–1300 RU. Asialo-NCAM-Fc was used as a negative control. For analysis of the interactions between immobilized polySia-NCAM-Fc and FG2, heat-denatured FG2, which was prepared as described above, BDNF, proBDNF and proDomain at varying concentrations (0, 1, 8, 3.6, 7.2, 14, 28, 56, 110 and 170 nM) in HBS-EP were injected over the polySia-NCAM-immobilized sensor chip surface at a flow rate of 20 µL/min. After 120 s, HBS-EP was flowed over the sensor surface to monitor the dissociation phase. Following 180 s of dissociation, the sensor surface was fully regenerated by the injection of 10 µL of 3 M NaCl. All values were analyzed using BIACore evaluation software.

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Conflict of interest statement

None declared.

Abbreviations

AMC, 4-amino-7-methylcoumarin; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; FG2, fibroblast growth factor 2; HIP, hippocampus; NCAM, neural cell adhesion molecule; PMSF, phenylmethylsulfonyl fluoride; POD, peroxidase; PVDF, polyvinylidene difluoride; RU, resonance unit; SAM, self-assembled membrane; SCZ, schizophrenia; SPR, surface plasmon resonance; SVZ, subventricular zone.

References


