Effects of amino acid substitutions in the sialylmotifs on molecular expression and enzymatic activities of α2,8-sialyltransferases ST8Sia-I and ST8Sia-VI

Shou Takashima1,2,3, Takumi Matsumoto4, Masafumi Tsujimoto2,5, and Shuichi Tsuji4

1Laboratory of Cellular Biochemistry, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; 2Laboratory of Glycobiology, The Noguchi Institute, 1-8-1 Kaga, Itabashi, Tokyo 173-0003, Japan; 3Institute of Glycoscience, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan; and 4Faculty of Pharmaceutical Sciences, Teikyo-Heisei University, 4-1 Uruidominami, Ichihara, Chiba 290-0193, Japan

Received on May 21, 2012; revised on December 7, 2012; accepted on January 4, 2013

Mouse sialyltransferases are grouped into four families according to the type of carbohydrate linkage they synthesize: β-galactoside α2,3-sialyltransferases (ST3Gal-I–VI), β-galactoside α2,6-sialyltransferases (ST6Gal-I and ST6Gal-II), N-acetylgalactosamine α2,6-sialyltransferases (ST6GalNAc-I–VI) and α2,8-sialyltransferases (ST8Sia-I–VI). These sialyltransferases feature a type II transmembrane topology and contain highly conserved motifs termed sialylmotifs L, S, III and VS. Sialylmotifs L and S are involved in substrate binding, whereas sialylmotifs III and VS are involved in catalytic activity. In addition to the conventional sialylmotifs, family and subfamily specific sequence motifs have been proposed. In this study, we analyzed the properties and functions of sialylmotifs in characterizing the enzymatic activity of mouse ST8Sia-I and ST8Sia-VI, both of which are α2,8-sialyltransferases involved in the synthesis of either ganglioide GD3 or disialic acid structures on O-glycans, respectively. The ST8Sia-VI-based chimeric enzymes, whose sialylmotif L sequences were replaced with those of ST8Sia-I and ST8Sia-IV (polysialic acid synthetase), were still active toward O-glycans. However, ST8Sia-VI-based chimeric enzymes lost expression or activity when their sialylmotif L sequences were interchanged. Amino acid substitutions in the sialylmotif S of ST8Sia-I and ST8Sia-VI also affected the enzymatic activity in many cases, indicating the crucial and functional importance of the sialylmotif S in substrate binding, which determines the substrate specificity of sialyltransferase.

Keywords: mutagenesis / sialylmotif / sialyltransferase / ST8Sia-I / ST8Sia-VI

Introduction

Sialic acids (Sia) are derivatives of the negatively charged acidic sugar neuraminic acid. Sia usually form the terminal ends of the carbohydrate groups of glycoconjugates. Because of their negative charge and their exposed positions on cell surface molecules, these acids often function as key determinants of oligosaccharide structures that mediate a variety of biological processes, including cell–cell interaction, cell migration, adhesion, metastasis and pathogen infection (Takashima and Tsuji 2011). A superfamily of glycosyltransferases called sialyltransferases catalyzes the synthesis of sialylglycoconjugates by transferring a Sia molecule from the donor substrate cytidine monophosphate (CMP)-Sia to an acceptor carbohydrate. To date, cDNA cloning of 20 mammalian sialyltransferases has been completed, and their enzymatic properties have been analyzed. These enzymes are grouped into four families according to the type of carbohydrate linkage they synthesize, namely, β-galactoside α2,3-sialyltransferases (ST3Gal-I–VI), β-galactoside α2,6-sialyltransferases (ST6Gal-I and ST6Gal-II), N-acetylgalactosamine (GalNAc) α2,6-sialyltransferases (ST6GalNAc-I–VI), and α2,8-sialyltransferases (ST8Sia-I–VI) (Tsuji 1996; Tsuji et al. 1996; Harduin-Lepers et al. 2005; Takashima 2008; Harduin-Lepers 2010; Takashima and Tsuji 2011).

To date, all sialyltransferases characterized in animals, ranging from insects to mammals, feature a type II transmembrane topology and usually localize to the Golgi apparatus. These sialyltransferases share common structural features, including a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region and a catalytic domain. The catalytic domain of animal sialyltransferases contains highly conserved motifs termed sialylmotifs L (long), S (short), III (third position in the sequence) and VS (very short) (Drickamer 1993; Livingston and Paulson 1993; Geremia et al. 1997; Jeanneau et al. 2004; Datta 2009). Sialylmotif L is characterized by a 45–60-amino acid region in the center of the protein, and it has been found to play a role in the binding of the donor...
substrate CMP-Sia (Drickamer 1993). Sialylmotif S is located in the C-terminal region and consists of a 20–30-amino acid stretch; it appears to be involved in the binding of both the donor and acceptor substrates (Livingston and Paulson 1993). Sialylmotif III is located between sialylmotifs S and VS and has the consensus sequence of \((H/y)Y(Y/F/W/h)E/D/q/g\) (Jeanneau et al. 2004); this motif is involved in the catalytic activity of the enzyme, and the conserved His residue directly interacts with the donor phosphate (Rao et al. 2009). Sialylmotif VS is located in the C-terminal region and is characterized by highly conserved His and Glu residues separated by 4 amino acids (Geremia et al. 1997). This motif is also involved in the enzyme’s catalytic activity (Kitazume-Kawaguchi et al. 2001). In addition to the conventional sialylmotifs, family and subfamily specific sequence motifs have been proposed (Patel and Balaji 2006; Harduin-Lepers 2010). Recently, the crystal structure of porcine \(\beta\)-galactosidase \(\alpha_2,3\)-sialyltransferase ST3Gal-I was revealed; this model stereochromically demonstrates the importance of sialylmotifs, especially the highly conserved His residue in the sialylmotif VS, which serves as the catalytic base (Rao et al. 2009).

Although all sialyltransferases characterized in animals share the common structural features as represented by sialylmotifs and many studies have been performed to elucidate the correlation between structure and enzymatic activity of sialyltransferases, it is still uncertain why each sialyltransferase has different linkage specificities and substrate preferences. In addition, these studies were mainly done using \(\alpha_2,6\)-sialyltransferase ST6Gal-I, \(\alpha_2,3\)-sialyltransferase ST3Gal-I and polysialic acid synthetases ST8Sia-II and ST8Sia-IV, and information about other sialyltransferases is limited. Therefore, to examine above issue further, we focused on ST8Sia-I and ST8Sia-VI, which we have cloned previously (Takashima et al. 2002), for better understanding of the correlation between structure and enzymatic activity of \(\alpha_2,8\)-sialyltransferases other than polysialic acid synthetases in this study. We constructed mutants of mouse ST8Sia-I and ST8Sia-VI and analyzed the properties and functions of the sialylmotifs in characterizing the enzymatic activities of these sialyltransferases.

In 1994, three groups independently cloned the ganglioside GD3 synthase, which was named as ST8Sia-I, by the expression cloning method using cDNA libraries of human WM266-4 melanoma cells (Sasaki et al. 1994), human SK-Mel-28 melanoma cells (Nara et al. 1994) and human YT lymphoid cells (Haraguchi et al. 1994), respectively. Mouse ST8Sia-I was also cloned by the expression cloning method (Yamamoto et al. 1996); however, we cloned it from a mouse brain cDNA library by means of a polymerase chain reaction (PCR)-based cloning method (Kono et al. 1996). Although ST8Sia-I is known as a ganglioside GD3 synthase, which utilizes GM3 ganglioside as an acceptor substrate, it can also utilize GM1b, GD1a, GT1b and GD3 gangliosides as acceptor substrates to some extent (Nakayama et al. 1996; Nara et al. 1996). However, its activity toward glycoproteins has not been reported. On the other hand, mouse ST8Sia-VI was identified by a BLAST (basic local alignment search tool) analysis of the mouse expression sequence tag database using the sequence of mouse ST8Sia-V, which is involved in the biosynthesis of gangliosides such as GD1c, GT1a, GQ1b and GT3 (Kono et al. 1996), as a query. The cDNA encoding the entire open reading frame of mouse ST8Sia-VI was obtained by the combination of conventional cDNA library screening (brain and heart cDNA libraries) and PCR-based approaches (Takashima et al. 2002). Human ST8Sia-VI was also cloned from the breast cancer cell line Michigan Cancer Foundation-7 by the PCR-based method (Teintenier-Lelièvre et al. 2005). Unlike ST8Sia-I and ST8Sia-V, ST8Sia-VI exhibits low activity toward glycolipids; however, it exhibits high activity toward O-glycans of glycoproteins. Here, we describe the functional importance of sialylmotifs and their constituent amino acid residues in the molecular expression of the enzyme and in exhibiting sialyltransferase activity.

Results and discussion

Enzymatic activity of ST8Sia-I and ST8Sia-VI chimeric enzymes

Mouse ST8Sia-I and ST8Sia-VI are \(\alpha_2,8\)-sialyltransferases, involved in the synthesis of either ganglioside GD3 or disialic acid structures on O-glycans, respectively (Kono et al. 1996; Yamamoto et al. 1996; Takashima et al. 2002). The ST8Sia-I and ST8Sia-VI genes share a similar genomic structure, suggesting that they are evolutionally related (Takashima 2008). It should be noted that the ST8Sia-V gene also shares a similar genomic structure with the ST8Sia-I and ST8Sia-VI genes. Mouse ST8Sia-I and ST8Sia-VI consist of 355 and 398 amino acids, respectively, and they show 42% sequence identity with each other (Figure 1). The difference of the amino acid length is mainly due to the different lengths of the stem regions of ST8Sia-I and ST8Sia-VI (see the peptide region 1 in Figure 1). The catalytic domains of these enzymes, which contain sialylmotifs, seem to be almost the same size (ca. 280 amino acids; Audry et al. 2011). Although these enzymes are evolutionally related and have similar structural features, they exhibit different substrate specificity. The sialylmotifs in animal sialyltransferases are known to play an important role in exhibiting sialyltransferase activity (Drickamer 1993; Livingston and Paulson 1993; Geremia et al. 1997; Jeanneau et al. 2004; Datta 2009). Therefore, in this study, we further analyzed the properties and functions of sialylmotif sequences using ST8Sia-I and ST8Sia-VI as model enzymes. At first, we constructed chimeric enzymes of ST8Sia-I and ST8Sia-VI (Figure 2A) by swapping each of five peptide regions shown in Figure 1 and examined the effects of the replacement of sialylmotif sequences on their enzymatic activity. In this study, we used protein-A fused soluble forms of ST8Sia-I, ST8Sia-VI and their derivatives produced by CV-1 in Origin, carrying SV40 (COS)-7 cells (Takashima et al. 2002). Among these chimeric enzymes, ST8Sia-I-based chimeric enzyme PPPMP, whose peptide region 5 (Figure 1) was replaced with that of ST8Sia-VI, was not detected in the culture supernatant of COS-7 cells (Figure 2B). The discrepancies of molecular masses between ST8Sia-I-based chimeric enzymes and ST8Sia-VI-based chimeric enzymes seemed to be caused by their glycosylation state and the different length of the peptide region 1 of ST8Sia-I and ST8Sia-VI. ST8Sia-I and ST8Sia-VI have four and five potential N-glycosylation sites, respectively (Figure 1), and some of these sites were actually N-glycosylated.
Enzymatic activity of ST8Sia-VI-based chimeric enzymes toward O-glycans

It has been reported that the sialylmotif L is involved in the binding of the donor substrate CMP-Sia and the sialylmotif S is involved in the binding of both CMP-Sia and acceptor substrates (Drickamer 1993; Livingston and Paulson 1993). ST3Gal-I is known to exhibit β-galactoside α2,3-sialyltransferase activity toward the Galβ1,3GalNAc structure on O-glycans (Lee et al. 1993; Kono et al. 1997), and ST6GalNAc-II is known to exhibit GalNAc α2,6-sialyltransferase activity toward GalNAc, Galβ1,3GalNAc and Siaα2,3Galβ1,3GalNAc structures on O-glycans (Kurosawa et al. 1996; Kono et al. 2000). ST8Sia-IV, which is well known as a polysialic acid synthetase toward N-glycans of neural cell adhesion molecules, also exhibits α2,8-sialyltransferase activity toward Sia residues on O-glycans (Mendiratta et al. 2006; Foley et al. 2010). Therefore, we constructed ST8Sia-VI-based chimeric enzymes whose sialylmotif L sequence in the peptide region 2 and sialylmotif S sequence in the peptide region 4 were replaced with corresponding sequences of ST3Gal-I, ST6GalNAc-II or ST8Sia-IV (Figure 3A and B). It should be noted that the sialylmotif L sequences of ST3Gal-I, ST6GalNAc-II and ST8Sia-IV are 1 amino acid shorter than that of ST8Sia-VI (Figure 3A; Takashima 2008). Among these ST8Sia-VI-based chimeric enzymes, MMFFM, MMFMF, MFMMF, whose peptide regions 2 and/or 4 were replaced with corresponding sequences of ST3Gal-I, and MBMBM, whose peptide regions 2 and 4 were replaced with corresponding sequences of ST6GalNAc-II, were hardly detected or not detected in the culture supernatants of COS-7 cells (Figure 3C). We then analyzed whether the properly produced chimeras maintained enzymatic activity toward O-glycans. MOMMM, whose sialylmotif L sequence in the peptide region 2 was replaced with the corresponding sequence of ST8Sia-IV, exhibited a significant level of activity toward O-glycans of BSM (18% activity of wild-type ST8Sia-VI); however, other chimeric enzymes did not exhibit any activity (Figure 3D). Together with the result of the MMPMM experiments (Figure 2), these results suggested that ST8Sia family specific features of sialylmotif L are necessary for ST8Sia-VI-based chimeric enzymes to exhibit activity. Compared with MMPMM, the lower activity of MOMMM may be related to the 1 amino acid shorter sialylmotif L sequence of ST8Sia-IV when compared with ST8Sia-I.

There are two conserved Cys residues in the sialylmotif L of the mammalian ST8Sia family enzymes (Figure 3A; Hirano et al. 2012). One of them (e.g. C142 in human ST8Sia-IV) is thought to form a disulfide bond with the Cys residue in the sialylmotif S (C292 in the case of human ST8Sia-IV), a bond commonly observed among mammalian sialyltransferases (Angata et al. 2001; Datta et al. 2001; Rao et al. 2009; Audry et al. 2011; Hirano et al. 2012). The other Cys residue (e.g. C156 in human ST8Sia-IV) is thought to form a disulfide bond with the Cys residue in the C-terminal region (C356 in the case of human ST8Sia-IV), a bond uniquely observed among the ST8Sia family enzymes that has been shown to be necessary for the enzymatic activity of ST8Sia-IV (Angata et al. 2001). Therefore, we replaced the amino acid from the corresponding position in the sialylmotif L sequences of ST3Gal-I (Y153 in ST3Gal-I or Y200 in MFMMF) and ST6GalNAc-II (Q164 in ST6GalNAc-II or
Fig. 2. Structure of chimeric enzymes and their enzymatic activity. (A) Structure of chimeric enzymes of ST8Sia-I and ST8Sia-VI. The peptide regions of ST8Sia-I (P) and ST8Sia-VI (M) are shown by gray rectangles and white rectangles, respectively. Numbers indicate the corresponding peptide regions in Figure 1. SM-L, sialylmotif L; SM-S, sialylmotif S; SM-III, sialylmotif III; SM-VS, sialylmotif VS. (B) Western blot of chimeric enzymes produced by COS-7 cells. The protein-A-fused enzymes purified from culture supernatants of COS-7 cells were detected by western blot using the anti-protein-A polyclonal antibody. (C) Peptide:N-glycanase F (PNGase F) treatment of ST8Sia-I and ST8Sia-VI. ST8Sia-I and ST8Sia-VI have four and five potential N-glycosylation sites, respectively. These enzymes were treated with PNGase F, which removes N-glycans and analyzed by western blot. The band shift observed between before and after PNGase F treatment suggests some of N-glycosylation sites in both of ST8Sia-I and ST8Sia-VI are actually N-glycosylated. (D) Enzymatic activity of chimeric enzymes toward BSM and GM3. Error bars indicate the standard error of the mean: n = 3. NE, not examined.
Q200 in MBMMM) in MFMMM and MBMMM, respectively, with Cys and examined their sialyltransferase activity toward BSM. Unlike MFMMM, MFMMM(Y200C) was successfully produced by COS-7 cells (Figure 3C). We found that MFMMM(Y200C) but not MBMMM(Q200C) exhibited sialyltransferase activity (Figure 3D). This was probably because the...
Y200C mutation in MFMMM properly conferred the structural features of the ST8Sia family specific motif, but the Q200C mutation in MBMMM did not, even though the amino acid sequence identity of the sialylmotif L sequences between ST6GalNAc-II and ST8Sia-VI is higher than that between ST3Gal-I and ST8Sia-VI (Figure 3A). In contrast, the replacement of the sialylmotif S sequence in the peptide region 4 of ST8Sia-VI with the corresponding sequences of other sialyltransferases diminished enzymatic activity. It has been proposed that there are family specific motifs among the sialyltransferase superfamily (Patel and Balaji 2006; Harduin-Lepers 2010), and our results from sialylmotif L replacement support this hypothesis. We found that MOMMM was still functional, probably because of the functional replacement of the sialylmotif L among the same α2,8-sialyltransferase family, which MFMMM and MBMMM did not accomplish. As the sialylmotif L is involved in the binding of the donor substrate CMP-Sia, a common substrate of all sialyltransferases, the sialylmotif L sequences of different families should share common properties and structures. However, the existence of family specific motifs in the sialylmotif L impaired the functional replacement of the sialylmotif L in different sialyltransferase families. Nevertheless, sialylmotif L sequences from different sialyltransferase families also could be functionally replaceable, with optimization of the sequence by amino acid substitutions, as shown by the MFMMM(Y200C) mutant. Judging from the enzymatic activity of MFMMM(Y200C) and MBMMM(Q200C), it seems that not only the existence of two conserved Cys residues in the sialylmotif L but also other structural features are necessary for α2,8-sialyltransferases to exhibit enzymatic activity. Further study is needed to identify such structural features, including the confirmation of the presence of disulfide bond formation between C200 and C395 in these mutants.

Enzymatic activity of ST8Sia-I and ST8Sia-VI mutants

The sialylmotif S is involved in the binding of both the donor substrate CMP-Sia and acceptor substrates (Livingston and Paulson 1993), and therefore, this region is thought to be important in the determination of the substrate specificity of each sialyltransferase. We expected to change the substrate specificity of sialyltransferases by the replacement of sialylmotif sequences, but this failed, probably because of the drastic change in the amino acid sequences. Therefore, we constructed ST8Sia-I and ST8Sia-VI mutants whose positionally corresponding, but different, amino acid residues in the sialylmotif S sequences were interchanged (Figure 4A). As for the several conserved amino acid residues in the sialylmotif S of these enzymes, we replaced them with Ala. Among these mutants, C286A mutant of ST8Sia-I and E333G mutant of ST8Sia-VI were not obtained (Figure 4B). In addition, the production levels of some mutants were very low. We then analyzed the enzymatic activity of these mutants. We found that the substrate specificities of ST8Sia-I and ST8Sia-VI were not changed by these amino acid substitutions, but caused drastic reductions in enzymatic activity in some cases (Table I). When the conserved amino acids—such as S273 and W295 in ST8Sia-I or S322, C335 and W344 in ST8Sia-VI—were replaced with Ala, the enzymatic activities of these mutants were lost or severely reduced. The C286 of ST8Sia-I is thought to form a disulfide bond with C137, and the C335 of ST8Sia-VI is thought to form a disulfide bond.
with C186 in this enzyme (Angata et al. 2001; Datta et al. 2001; Rao et al. 2009; Audry et al. 2011; Hirano et al. 2012). The corresponding disulfide bond is thought to be conserved among all mammalian sialyltransferases and has been shown to be necessary for the molecular expression of mouse ST6Gal-I (Hirano et al. 2012). The W295 of ST8Sia-I and W344 of ST8Sia-VI are positionally corresponding to the G293 of pig ST3Gal-I, which interacts with CMP (Figure 5A; Rao et al. 2009). The importance of Cys and Trp residues in the catalytic domain of mouse ST3Gal-I has also been shown that they are required in exhibiting enzymatic activity (Kim et al. 2010). As for the exchange of non-conserved amino acid residues, the G428E mutant of ST8Sia-I and the M326F and N337E mutants of ST8Sia-VI all lost their sialyltransferase activity. These amino acid residues may be involved in the determination of substrate specificity toward acceptor substrates in each enzyme. Amino acid substitutions other than the above residues also caused significant decreases in enzymatic activity in many cases, suggesting that the sialylmotif S was a very delicate region, important in the recognition of acceptor and/or donor substrates.

Among mouse sialyltransferases, there are four highly conserved amino acid residues in the sialylmotif L (Cys, Arg, Val and Gly), one residue in the sialylmotif S (Cys), two in the sialylmotif III (His and Tyr) and two in the sialylmotif VS (His and Glu) (Figure 5A). Some of these highly conserved amino acid residues in mammalian sialyltransferases have been shown to play important roles in exhibiting sialyltransferase activity or disulfide bond formation, which was required for proper molecular expression in the case of mouse ST6Gal-I (Hirano et al. 2012). The highly conserved Cys residues in the sialylmotifs L and S (C186 and C335 of ST8Sia-VI, respectively) are thought to form a disulfide bond (Angata et al. 2001; Datta et al. 2001; Rao et al. 2009; Audry et al. 2011; Hirano et al. 2012). The highly conserved His residue in the sialylmotif III (H358 of ST8Sia-VI) is thought to interact with the donor phosphate of CMP-Sia (Rao et al. 2009). The highly conserved His residue in the sialylmotif VS (H370 of ST8Sia-VI) is thought to serve as the catalytic base (Kitazume-Kawaguchi et al. 2001; Rao et al. 2009). The replacement of these highly conserved nine residues and D225 in ST8Sia-VI with Ala caused loss or decrease in the molecular expression of some mutants such as G227A, C335A and H358A (Figure 5B). In addition, even the properly expressed mutants exhibited no or severely reduced sialyltransferase activity toward BSM (Table II). Mouse ST6Gal-I has three disulfide bonds; the disulfide bond between C181 in the sialylmotif L and C332 in the sialylmotif S was shown to be necessary for the molecular expression of the enzyme; the disulfide bond between C350 and C361 was shown to be necessary for enzymatic activity; however, the disulfide bond between C139 and C403 was not necessary for enzymatic activity or for activity (Hirano et al. 2012). Similar to the case of ST6Gal-I, C186A and C335A mutations in ST8Sia-VI, which are thought to disrupt the disulfide bond formation between C186 in the sialylmotif L and C335 in the sialylmotif S, affected molecular expression of the enzyme: C186A mutation caused the band shift of the enzyme on western blot and C335A mutation caused reduced levels of molecular expression, and these amino acid substitutions also caused a loss of enzymatic activity. Furthermore, the replacement of Cys (C200 in ST8Sia-VI), which is a highly conserved residue among the sialylmotif L sequence of the ST8Sia family and is thought to form a disulfide bond with C395, with Leu, which is often observed at the corresponding position in the sialylmotif L sequences of other sialyltransferase members (Figure 5), also caused the band shift of the enzyme on western blot and a loss of enzymatic activity [see also the MFMMM(Y200C) and MBMMM(Q200C) experiment in Figure 3]. Although the presence of disulfide bonds between above Cys residues in ST8Sia-VI must be confirmed, these results suggest that the proper disulfide bond formations are critical for molecular expression and enzymatic activity of ST8Sia-VI.

In this study, we found that the replacement of some amino acid residues in the sialylmotifs affected the molecular expression of sialyltransferase and/or enzymatic activity. The importance of these residues should be stereochmically analyzed in the future when the three-dimensional structures of ST8Sia-I and ST8Sia-VI are available. Unlike the active chimeric enzymes of ST8Sia-II and ST8Sia-IV (Angata et al. 2004), which are both polysialic acid synthetases, the replacement of sialylmotifs and the introduction of amino acid substitution in the sialylmotifs of ST8Sia-I and ST8Sia-VI caused a loss of enzymatic activity in many cases, probably because the enzymatic properties of ST8Sia-I and ST8Sia-VI were originally different. However, active chimeric enzymes or mutants of sialyltransferase, which have novel substrate or linkage specificities, may be successfully obtained if the functions and the proper structure of sialylmotifs are not impaired by amino

<table>
<thead>
<tr>
<th>Table 1. Effects of amino acid substitutions in the sialylmotif S of ST8Sia-I and ST8Sia-VI on the enzymatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic activities of ST8Sia-I and ST8Sia-VI mutants were examined using BSM and GM3 as substrates. Their activity was measured three times independently: the average is shown. ND, not detected more than 1% activity. NE, not examined.</td>
</tr>
<tr>
<td>ST8Sia-I</td>
</tr>
<tr>
<td>Relative activity (%)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ST8Sia-VI</td>
</tr>
<tr>
<td>Relative activity (%)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Acid substitutions. Further analysis of sialylmotifs and the identification of important amino acid residues for sialyltransferase activity, stability and structure will help construct such engineered sialyltransferases, which are useful for developing novel drugs and biomaterials.

Materials and methods

Materials

BSM (type I-S), ganglioside GM3 and Triton CF-54 were purchased from Sigma (St Louis, MO). CMP-[\(^{14}\)C]N-acetyl neuraminic acid (NeuAc) (12.0 GBq/mmol and 925 kBq/mL) was sourced from GE Healthcare (Pittsburgh, PA). Peptide: N-glycanase F (PNGase F) was purchased from Takara (Shiga, Japan).

Construction of expression vectors

To construct expression vectors for each sialyltransferase mutant, PCR-based, site-directed mutagenesis was performed using PrimeSTAR HS DNA polymerase (Takara) and the mutagenic primers listed in Supplementary Table SI, with the corresponding sialyltransferase cDNA on pBluescript II SK(+) vector as a template, according to the manufacturer’s instructions. For the generation of chimeric enzymes, EcoRI and AatII sites were introduced into the sialylmotif.
L-encoding sequences of ST8Sia-I and ST8Sia-VI cDNAs, and AgeI and BalI sites were introduced into the sialylmotif S-encoding sequences of ST8Sia-I and ST8Sia-VI cDNAs, without changes to encoding amino acids. Then, the appropriate DNA fragments were swapped between ST8Sia-VI and ST8Sia-I cDNAs, or sialylmotif-encoding DNA fragments of other sialyltransferases. Mutations were confirmed by DNA sequencing, and each mutated ST8Sia-I and ST8Sia-VI cDNA was introduced into the expression vector pcDSA (Takashima et al. 2002), which encoded a soluble form of sialyltransferase fused with the IgM signal peptide and the Staphylococcus aureus protein-A IgG-binding domain.

Preparation of soluble forms of sialyltransferases
For the production of soluble forms of sialyltransferases, COS-7 cells were transfected with pcDSA vectors using the LipofectAMINE™ reagent (Invitrogen, Carlsbad, CA) and cultured as described previously (Kojima et al. 1995). The protein-A-fused sialyltransferases secreted in the medium were purified by IgG-Sepharose gel (GE Healthcare) and used as the enzyme source.

Sialyltransferase assays
Sialyltransferase assays were performed as described previously (Kono et al. 1996; Lee et al. 1999). In brief, enzymatic activity was measured in 50 mM 2-morpholinoethanesulfonic acid buffer (pH 6.0), 1 mM MgCl₂, 1 mM CaCl₂, 0.5% Triton CF-54, 100 μM CMP-[¹⁴C] NeuAc, an acceptor substrate and an appropriate amount of enzyme preparation totaling 10 μL. As acceptor substrates, 10 μg of BSM or 5 μg of GM3 was used. The enzyme reaction was performed at 37°C for 3–20 h. For the BSM substrate, the reaction was terminated by the addition of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer, and the reaction mixtures were directly subjected to SDS–PAGE. For the GM3 substrate, the reaction mixtures were applied to a Sep-Pak Vac C₁₈ column (100 mg; Waters, Milford, MA), and purified glycolipids were subjected to high-performance thin-layer chromatography (Silica Gel 60; Merck, Darmstadt, Germany). The radioactive materials were visualized and quantified with a BAS2000 radioimage analyzer (Fujiﬁlm, Tokyo, Japan).

Western blot analysis
Western blotting was carried out using the standard protocol. To detect the soluble form of sialyltransferase fused with the S. aureus protein-A IgG-binding domain, the horseradish peroxidase-conjugated chicken anti-protein-A polyclonal antibody (GenScript, Piscataway, NJ) was used (1:2000). The protein-A-fused sialyltransferase on the polyvinylidene fluoride membrane (Millipore, Billerica, MA) was then visualized with the ChemiDoc XRS System (Bio-Rad, Hercules, CA) using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Waltham, MA).

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

Funding
This work was supported in part by a grant for the “Chemical Biology Research Program” from RIKEN.

Conflict of interest
None declared.

Abbreviations
BSM, bovine submaxillary mucin; CMP, cytidine monophosphate; Gal, galactose; GalNAc, N-acetylgalactosamine; NeuAc, N-acetyl neuraminic acid; PCR, polymerase chain reaction; PNGase F, peptidase:N-glycanase F; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sia, sialic acid.

References

Analysis of sialylmotifs in mouse α2,8-sialyltransferases


