Mutational analysis for enzyme activity of mouse Galβ1,3GalNAc α2,3-sialyltransferase (mST3Gal I)

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To determine which amino acid residues are essential for the catalytic activity of mouse Galβ1,3GalNAc α2,3-sialyltransferase (mST3Gal I), chemical modification and site-directed mutagenesis were employed against tryptophan and cysteine residues located in the predicted catalytic domain. This enzyme was strongly inhibited by N-bromosuccinimide, a specific blocking reagent for tryptophan residues, and the enzyme activity was completely lost at 3 mM, suggesting the involvement of tryptophan residues in the catalytic activity of mST3Gal I. The N-ethylmaleimide, an irreversible reagent for sulfhydryl group, significantly inhibited the enzyme activity. Seven tryptophan and six cysteine residues conserved in the cloned Galβ1,3GalNAc α2,3-sialyltransferases were separately substituted into phenylalanine and serine, respectively. The enzymatic activity assay for tryptophan mutants produced in COS cells showed a complete abolishment of the activity in all of the mutants, except that W70F and W97F retained about 60% and 40% activities of wild type, respectively. In the case of cysteine mutants, no enzyme activity was observed like tryptophan mutants, except for C139S. These results suggest that tryptophan and cysteine residues conserved in ST3Gal I are critical for its activity.

Keywords: Sialyltransferase, Chemical modification, Site-directed mutagenesis

The sialyltransferases are a family of glycosyltransferases that catalyze the transfer of sialic acid from CMP-NeuAc to the terminal position of the carbohydrate group of glycoproteins and glycolipids. To date, twenty members of the sialyltransferase gene family have been cloned and characterized. These enzymes are divided into four groups on the basis of the type of carbohydrate linkages they synthesize: β-galactoside α2,3-sialyltransferase (ST3Gal I-VI), β-galactoside α2,6-sialyltransferase (ST6Gal I and II), GalNAc α2,6-sialyltransferase (ST6GalNAc I-VI), and α2,8-sialyltransferase (ST8Gal I-VII). They are localized in the Golgi apparatus and have a domain structure similar to that of other glycosyltransferases: a short NH₂-terminal cytoplasmic tail, a hydrophobic transmembrane domain, and an extended stem region, followed by a large COOH-terminal catalytic domain.

All eukaryotic sialyltransferases contain highly conserved regions named sialylmotifs L and S have been performed using ST6Gal I as a model. The mutant analyses of the most conserved residues by site-directed mutagenesis have demonstrated that sialylmotif L participates in the binding of the common donor substrate, CMP-NeuAc, whereas sialylmotif S is involved in the binding of both donor and acceptor substrates.

Mutational analysis of two conserved cysteine residues in the sialylmotifs L and S has also suggested that they participate in the formation of an intradisulfide linkage that is essential for proper conformation and activity of ST6Gal I. Recently, the existence of a new motif (motif 3) located between the sialylmotifs S and VS has been reported and this motif with sialylmotif VS has been found to be involved in catalytic activity of human ST3Gal I. On the other hand, except for the sialylmotifs, none of studies have attempted to define or identify amino acids that are essential for the enzyme activity or active conformation.

We previously cloned two kinds of cDNA encoding mouse Galβ1,3GalNAc α2,3-sialyltransferase (mST3Gal I and mST3Gal II) by a PCR method directed to sialylmotif. They exhibit the same acceptor substrate specificities and are able to synthesize only the NeuAc α2,3Galβ1,3GalNAc

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sequence in gangliosides and glycoproteins as well as oligosaccharides, but show a different acceptor substrate preference\textsuperscript{13}. In the present study, we have performed chemical modification to obtain further information on the structure/function of ST3Gal I and found the involvement of tryptophan and cysteine residues for the enzyme activity. The site-directed mutagenesis has also been performed to identify amino acids involved in mST3Gal I activity.

Materials and Methods

Construction of mST3Gal I mutants and expression in COS-7 cells

Construction of amino acid-substituted mutants of mST3Gal I was performed using the QuikChange® XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The plasmid pcD-SRα2,3-ST\textsuperscript{11} containing the catalytic domain of mST3Gal I was used as a template DNA. The sequence of the sense and antisense mutation primers is shown in Table 1.

All mutations were verified and confirmed by DNA sequence analysis. The EcoRI/XhoI fragments (973 bp) of pcD-SRα2,3-ST with the desired mutations were inserted into the corresponding sites of a pcDS vector plasmid constructed by introducing the PsI/XhoI fragment (117 bp) of pUGS into the corresponding sites of pCD-SRα\textsuperscript{12}. This vector contains the signal peptide of mouse immunoglobulin M.

For enzyme assay, the wild-type mST3Gal I and its mutants were transiently expressed in COS-7 as described previously\textsuperscript{11,12}. COS-7 cells (2 x 10\textsuperscript{5}/100 mm dish) were transfected with 2 µg of the wild-type

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mST3Gal I and its mutant plasmids using the Lipofectamine™ reagent (Invitrogen). The cell medium was harvested at 48 h post-transfection and concentrated 10-fold on Centricon 30 filters (Amicon Inc, Beverly, MA) for assaying the sialyltransferase activity and chemical modification.

Expression of the wild-type and mutants of mST3Gal I in E. coli cells

To check the expression of the desired mST3Gal I proteins with similar molecular weight, the EcoRI/XhoI fragments (973 bp) of pcD-SRα2,3-ST with the desired mutations were inserted into the corresponding sites of the expression vector pET-28a(+) (Novagen). The E. coli BL21(DE3) cells transformed with the recombinant plasmids (pET-mST3Gal I and its mutants) were grown in LB medium containing kanamycin (50 µg/ml) at 37°C with shaking until the absorbance at 600 nm was approximately 0.8. Subsequently, protein production was induced by adding isopropyl-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM and incubating at 37°C for further 3 h.

After centrifugation at 5000 × g for 30 min at 4°C, the pellets were suspended in 20 mM Tris-HCl buffer (pH 7.0), and then disrupted by sonication (30-s bursts for 5 min). The supernatant obtained by centrifugation at 12000 × g for 20 min was used as the crude enzyme source for SDS-PAGE. SDS-PAGE was performed on 12% running gel as described by Laemmli14, and the resolved proteins were visualized by Coomassie staining. A low range protein standard (Bio-Rad) was used as molecular mass marker.

Chemical modification studies

Enzyme samples were incubated in 0.1 M sodium phosphate buffer (pH 7.0) containing various concentration of N-bromosuccinimide or N-ethylmaleimide. After incubation for 30 min at room temperature, the reaction was stopped by dilution with the assay mixture, and the remaining activity was assayed immediately. Control experiments were performed under the same condition without modification reagents.

Sialyltransferase assay

ST3Gal I enzyme assay was performed by essentially the same method as described previously11-13. In a total volume of 10 µl, the assay mixture containing 50 mM sodium cacodylate buffer (pH 6.0), 10 mM MgCl₂, 50 µM CMP-[¹⁴C]NeuAc (0.9 Mbq/pmol), 0.5% Triton CF-54, 1 mM acceptor substrate Galβ1, 3GalNAcα1Bz, and 1 µl of enzyme preparation was incubated at 37°C for 1 h. For separation of the product from CMP-NeuAc, the assay mixture was applied on a silica gel 60 HPTLC plate (Merck, Germany) and then developed with ethanol/pyridine/1-butanol/acetic acid/ water (100:10:10:3:30). The radioactivity on each plate was detected and counted with the BAS2000 radioimage analyzer (Fuji Film, Japan).

Results

Effect of modification reagents on mST3Gal I enzyme activity

The mST3Gal I produced as a soluble protein was subjected to N-bromosuccinimide, a specific blocking reagent for tryptophan residues, and N-ethylmaleimide, an irreversible reagent for sulphydryl group. More than 70% of ST3Gal I activity was inhibited at 1 mM N-bromosuccinimide, and completely lost at 3 mM (Fig. 1). This result suggested that the tryptophan residues were absolutely involved in the enzyme activity of mST3Gal I. The N-ethylmaleimide inhibited enzyme activity in a dose-dependent manner and the enzyme retained about 58% of its activity at 10 mM.

Production of mST3Gal I mutant proteins by site-directed mutagenesis

On the basis of the result from chemical modification studies, alignment of amino acid sequence of the catalytic domain of the cloned ST3Gal I was performed to identify the existence of tryptophan and cysteine residues. Comparison of the primary sequences of ST3Gal I revealed seven tryptophan and six cysteine residues conserved in the
catalytic domain of ST3Gal I (Fig. 2). To investigate whether these residues are involved in the mST3Gal I enzyme activity, seven tryptophan and six cysteine residues were individually substituted for phenylalanine and serine or alanine, respectively (Fig. 3). For expression of the native mST3Gal I and its mutants, the mammalian expression vector pcDS was used. By using this vector plasmid placement in-frame cDNA fragments with cloning sites just downstream from the COOH terminus of the signal peptide of mouse immunoglobulin M enables the cDNA products to be secreted as active proteins and thus the enzyme activity can be detected in the culture medium.

Activity analysis of mST3Gal I mutant proteins

To assess the effect of the mutations on the enzyme activity, wild type and mutant proteins were transiently expressed in COS-7 cells. At 48 h post-transfection, mST3Gal I activity in the culture medium was measured. As shown Fig. 4, the native form of mST3Gal I showed soluble enzyme activity amounting to 2.2 mU/ml, whereas except for C139S, the cysteine mutants C56S, C58S, C61S, C136S, C139A and C278S revealed a dramatic reduction (less than 2%) in enzyme activity, compared with the native form. The mutation of tryptophan to phenylalanine also caused more than 97% reduction in the enzyme activity, except for W70F and W97F exhibiting 57 and 38% of the activity, respectively, compared with that of the native enzyme.

The expression of mST3Gal I and its mutant proteins was checked by bacterial expression system, because there is no commercially available ST3Gal I antibody at present. All mutant proteins were expressed and exhibited similar molecular weight (about 32 kDa) to the native form of mST3Gal I, although they were accumulated in the inactive form of inclusion bodies in the cytoplasm of the E. coli cells, as in the case of Galβ1,4GlcNAc α2,6-sialyltransferase (ST6Gal I) reported in our previous study (data not shown).

Discussion

In this study, we firstly investigated the effect of seven tryptophan and six cysteine residues conserved in the catalytic domain of ST3Gal I on its enzyme activity. Chemical modification study exhibited involvement of tryptophan residues for enzyme activity. It was observed that mST3Gal I was inactivated to 95% after modification with 3 mM N-bromosuccinimide, suggesting the involvement of tryptophan at active site of the mST3Gal I or in stabilizing the functional three-dimensional structure of the enzyme. This result was consistent with the
results obtained from site-directed mutagenesis, which indicated that each mutation of five tryptophan residues (Trp^{98}, Trp^{170}, Trp^{213}, Trp^{256}, and Trp^{294}, and Trp^{298}) to phenylalanine resulted in drastic loss of the enzyme activity.

In contrast, the W70F and W97F mutants retained significant activities, which correspond to 57 and 38% of the activity, respectively, compared with that of the native enzyme. This indicated that amino acid substitutions at these two positions did not dramatically impair the function of the mST3Gal I enzyme. By using SSPro program (http://scratch.proteomics.ics.uci.edu), secondary structure prediction of the catalytic domain of mST3Gal I revealed that five tryptophan residues (Trp^{98}, Trp^{170}, Trp^{213}, Trp^{256}, and Trp^{294}) were included in α helix and the other two residues (Trp^{294} and Trp^{298}) were contained in random coils and β sheet, respectively. Previous study^2^ reported that W301F mutant of human ST3Gal I expressed in baculovirus-mediated insect cell showed 30% activity of the native enzyme, whereas W298F mutant of mouse ST3Gal I expressed in COS cells revealed about 3% activity of the native enzyme. This might be due to protein levels by different expression systems.

The eukaryotic ST3Gal I contained six cysteine residues conserved in its catalytic domain, and among them three cysteines (Cys^{56}, Cys^{81}, and Cys^{61}) located at N-terminus of its catalytic domain were in close proximity with one another (Fig. 2). Because these three cysteines are not present in other sialyltransferases known until now, moreover, it may be worth studying their roles in the enzymatic functions of ST3Gal I. In the present study, we firstly revealed that mutation of these cysteines to serine resulted in almost complete loss of the enzyme activity. Cys^{139} and Cys^{181} in the catalytic domain of mST3Gal I are the residues found within the highly conserved sialylmotifs L and S, respectively, which are invariably present in all the cloned sialyltransferases^4-7^.

Previous studies have shown that alanine substitution of either of the invariant two cysteines yielded inactive enzyme^7,9^, Similarly, our present result revealed that C139A and C278S also caused almost complete loss of the enzyme activity. However, mutation of Cys^{139} to serine had a slight effect on enzyme activity, which corresponds to 20% reduction relative to native enzyme. It is difficult at this stage to explain this difference between C139A and C139S mutants, without comparative analysis of protein expression level in COS-7 cells.

The sialylmotif L, including Cys^{181} in ST6Gal I is found to be crucial for the binding of the common donor substrate, CMP-NeuAc, whereas sialylmotif S, including Cys^{332} is critical for the binding of both donor and acceptor substrates^8,9^, It is also reported that these cysteine residues (Cys^{181} and Cys^{332}) in the sialylmotifs L and S of ST6Gal I participate in the formation of an inter-disulfide linkage that is essential for production and maintenance of active conformation of ST6Gal I^10^, Like ST3Gal I, ST6Gal I also contains six cysteine residues (Cys^{139}, Cys^{181}, Cys^{332}, Cys^{350}, Cys^{361}, and Cys^{403}) in its catalytic domain and the replacement of Cys^{350} (C350S) or Cys^{361} (C360S) with serine yield inactive enzyme, indicating that these two relatively close cysteine residues are critical for in vivo enzyme activity^16^.

Recent study has shown that the replacement of Cys^{336} (C836S) or Cys^{339} (C839S) with serine in the O-linked N-acetylgalcosaminyltransferase (OGT) results in more than 90% reduction in the enzyme activity^17^, Considering that these two cysteine residues are in close proximity with one another, these results may be consistent with our present finding that mutation of these cysteines (Cys^{56}, Cys^{78}, and Cys^{61}) to serine resulted in almost complete loss of enzyme activity.

By using DIPro program (http://scratch.proteomics.ics.uci.edu), prediction of disulfide bond in the catalytic domain of mST3Gal I revealed the existence of two disulfide bonds: one between Cys^{56} and Cys^{136} and a second between Cys^{61} and Cys^{139}. A number of Golgi glycosyltransferases are known to form disulfide-bonded dimers and the dimers of most of these enzymes are catalytically active and are generated by disulfide bonds between cysteine residues in their stem regions and/or catalytic domains^16^, Previous study^18^ has shown that active form of GM2 synthase is a disulfide-bonded dimer, which is generated through intercatalytic domain disulfide bonds formed in an anti-parallel manner between Cys^{80} and Cys^{82} with Cys^{412} and Cys^{529}.

In this study, we found that mST3Gal I activity was significantly inhibited by N-ethylmaleimide, an agent that reacts with free cysteines, but its effect was not significant in C139S mutant. Our findings in this study, combined with the previous results mentioned above provide an evidence that disulfide bonds different from that formed in ST6Gal I, including
dimmer formation, can be formed between cysteine residues in the catalytic domain of ST3Gal I, although it is difficult at this stage to predict which cysteine residues are involved in direct disulfide linkage in three-dimensional structure of ST3Gal I.

It is important to check expression levels of mutant proteins, because the introduced point mutations can induce some conformational change of the protein that affects its expression, resulting in the enzyme inactivation\textsuperscript{19-21}. In this study, we could not confirm whether mST3Gal I and its mutants were produced as a properly folded active enzyme in COS-7 cells, due to lack of commercially available ST3Gal I antibody\textsuperscript{15}. But, our present results provide useful information on the structure and function of ST3Gal I; however, the crystal structure is required for the precise understanding of the enzymatic function.

Acknowledgements

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References