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Conserved Cysteines in the Sialyltransferase Sialylmotifs Form an Essential Disulfide Bond*

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The sialyltransferase gene family is comprised of 16 cloned enzymes. All members contain two conserved protein domains, termed the S- and L-sialylmotifs, that participate in substrate binding. Of only six invariant amino acids, two are cysteines, with one found in each sialylmotif. Although the recombinant soluble form of ST6Gal I has six cysteines, quantitative analysis indicated the presence of only one disulfide linkage, and thiol reducing agents dithiothreitol and β-mercaptoethanol inactivated the enzyme. Analysis of site-directed mutants showed that alanine or serine mutants of invariant Cys^{181} or Cys^{332} exhibit no detectable activity, either by direct assay or by staining of the transfected cells with *Sambucus nigra* agglutinin, which recognizes the product NeuAcα2,6Galβ1,4GlcNAc on glycoproteins. In contrast, alanine mutations of charged residues adjacent to either cysteine showed little or no effect on enzyme activity. Immunofluorescence microscopy showed that although the wild type sialyltransferase is properly localized in the Golgi apparatus, the inactive cysteine mutants are retained in the endoplasmic reticulum. The results suggest that the invariant cysteine residues in the L- and S-sialylmotifs participate in the formation of an intradisulfide linkage that is essential for proper conformation and activity of ST6Gal I.

The sialyltransferase gene family represents a group of enzymes that transfers sialic acid from its nucleotide-sugar donor, CMP-NeuAc, to carbohydrate groups of various glycoproteins and glycolipids. So far, 16 enzymes have been cloned (1–3), each of which exhibits unique specificity for its acceptor substrates and forms one of four sialic acid linkages, Neu5Acα2,6Gal, Neu5Acα2,3Gal, Neu5Acα2,6GalNAc, or Neu5Acα2,8Neu5Ac (4–6). The sialyltransferases are localized in the Golgi apparatus and are type II membrane proteins with a short cytoplasmic domain, an N-terminal signal anchor, and a large lumenal catalytic domain, characteristic of all glycosyltransferases localized to the secretory pathway (7, 8). They contain two conserved homologous regions, termed L (long)- and S (short)-sialylmotifs, which are present in the catalytic domain (9–11). Mutational analysis has suggested that the sialylmotifs are involved in binding the donor and acceptor substrates (12–14).

Although comparison of any two sialyltransferases reveals ~30% amino acid identity, there are only six amino acids that are invariant in all 16 sialyltransferases. Two of the invariant amino acids are cysteines, with one found in each sialylmotif (Fig. 1). Our previous analysis by site-directed mutagenesis of a secreted form of ST6Gal I showed that alanine substitution of either of the invariant cysteines, Cys^{181} or Cys^{332}, produced immunoprecipitated protein but no detectable enzyme activity (12–14). The results raised the possibility that these two cysteines may be involved in the formation of a conserved intrachain disulfide bond.

In this report, we provide evidence that the soluble recombinant ST6Gal I contains a single disulfide bond. Moreover, deletion of either of the conserved cysteine residues results in inactive recombinant enzyme, which fails to exit from the endoplasmic reticulum and be transported to the Golgi apparatus (15), presumably because it is improperly folded. The combined results, suggest that the invariant cysteine residues in the L- and S-sialylmotifs of ST6Gal I form an intrachain disulfide bond that is essential for maintaining an active conformation of the enzyme.

EXPERIMENTAL PROCEDURES

Construction of Expression Vector—For expression of wild type ST6Gal I and its mutants (Fig. 2), the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) was used after modifications as follows; plasmid DNA for the vector pcDNA3 was digested with *Kpn*I and *Eco*RV and then ligated using T4 DNA ligase in the presence of T7 DNA polymerase (unmodified with 3′-5′ exonuclease activity) using synthesis buffer (0.5 mM each of deoxynucleoside triphosphate, 1 mM ATP, 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1.5 mM dithiothreitol). This treatment deleted the unique restriction sites *Bam*HI and *Eco*RI, besides *Kpn*I and *Eco*RV, from the modified pcDNA3, hereafter denoted $pcDNA3_{mod}$. A dog proinsulin signal sequence was then subcloned into this modified vector as follows. The plasmid DNA for pGIR201Nhe3′ containing proinsulin signal sequence (a gift from K. Drickamer; see Ref. 16) was digested with *Nhe*I, and the smaller fragment for the insulin signal sequence was gel purified and then subcloned into *Xba*I-digested calf intestine phosphatase-treated $pcDNA3_{mod,}$ following the usual procedure (17). Prior to this treatment, the plasmid DNAs were propagated through a dam negative strain of *Escherichia coli* GM2163, the competent cells of which were made according to Hanahan's procedure (18). The clone containing the proinsulin signal sequence in the 5′-3′ orientation was selected by sequencing, hereafter termed $pcDNA_{ins}$. Two oligonucleotides were designed to incorporate into this vector $pcDNA_{ins}$, sites for nickel binding and enterokinase cleavage. The oligos (sense, 5′-CTAGCACATCATCATCATCATCATGATGATGATGATAAAGATTCTAGAGAATTCGAG-3′, and antisense, 5′-GATCCTCGAATTCTCTAGAATCTTTATCATCATCATC ATGATGATGATGATGATGTG-3′) were annealed at 37 °C and ligated with the *Xba*I- and *Bam*HI-digested $pcDNA_{ins}$, using standard molecular biological techniques (17). The resultant vector, termed $pcDNA_{ins\text{-}His,}$ was confirmed by sequencing.

For subcloning the soluble form of ST6Gal I into $pcDNA_{ins\text{-}His}$ vector, two restriction sites, *Xba*I and *Bam*HI, were introduced into its cDNA

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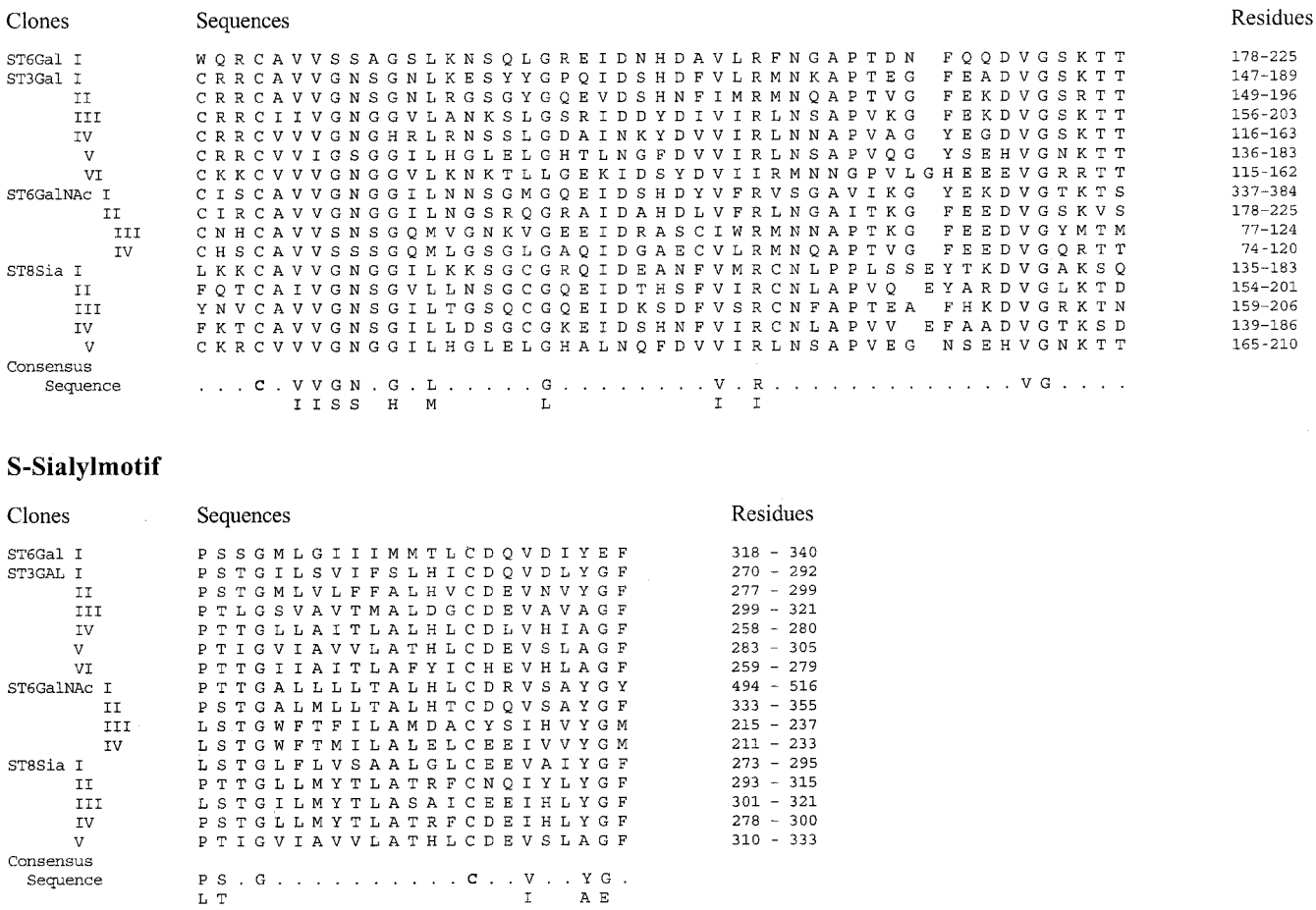

FIG. 1. **Amino acid alignment of the L- and S-sialylmotifs of all the known cloned sialyltransferases gene family.** Sialyltransferase sequences are grouped according to the linkage formed by these enzymes (for nomenclature, see Ref. 1). Sequences were aligned by the Clustal Method using the McVector 6.5 (Oxford Molecular) software program. The sequences are from the rat ST6Gal I (22), the porcine ST3Gal I (54), the rat ST3Gal II (55), the rat ST3Gal III (56), the human ST3Gal IV (57), the human ST3Gal V (58), the human ST3Gal VI (3), the chick ST6GalNAc I (11), the chick ST6GalNAc II (59), the rat ST6GalNAc III (60), the mouse ST6GalNAc IV (2), the mouse ST8Sia I (61), the rat ST8Sia II (9), the mouse ST8Sia III (62), the hamster ST8Sia IV (63), and the mouse ST8Sia V (64). The consensus sequence consists of invariant and highly conserved amino acids. The invariant cysteine residue in each of the sialylmotifs is shown in *bold type*.

by polymerase chain reaction (PCR)[1] as follows: a forward primer, 5′-GCTCTAGAATTCCAATCCTCAGTTACCACAG-3′ (nucleotides 214–236) with an internal *Xba*I site (underlined) and a reverse primer, 5′-CCAGGAGAGGATCCATAAAATGAC-3′ (nucleotides 1270–1247), with an internal *Bam*HI site were used for amplification using as template the cDNA for the rST6Gal I (for nomenclature, see Ref. 1) previously subcloned in pBluescript (19). The conditions for PCR were 94 °C for 30 s, 56 °C for 1 min, and 73 °C for 2 min for 20 cycles. Gel analysis showed the generation of a single band of 1.05 kilobases. This band was purified using Geneclean II (Bio 101, San Diego, CA), digested with *Xba*I and *Bam*HI, and then subcloned into similarly digested mammalian expression vector $pcDNA_{ins\text{-}His,}$ described above. The sequence of the resulting expression vector, termed His-spST, was confirmed by dideoxy double-stranded sequencing (20) of the entire subcloned fragment, including the restriction sites used.

For construction of the full-length form of the wild type ST6Gal I, the cDNA was released from a previously made construct in pBluescript (19) by digestion with *Bam*HI and *Xho*I and then subcloned into similarly digested pcDNA3. Sequencing was performed to check for the desired 5′-3′ orientation of this construct, termed pcDNA:ST6Gal I.

Construction of Mutants—Single point mutations were introduced in the above His-spST by a two-step PCR, following the procedure outlined previously (21). The cDNA for rST6Gal I (22), previously subcloned in pBluescript (19), was used as a template for introduction of these mutations. The mutagenic antisense oligonucleotides (substituted nucleotides underlined) used for construction of the corresponding cDNAs were 5′-CCTGGTCAGACAGCGTCATC-3′ (nucleotides 1003–984) for C332S, and 5′-TATCTACCTGGGCACACAGCGTCAT-3′ (nucleotides 1009–985) for D333A. For C181A, C332A, and R180A, the mutagenic antisense oligonucleotides used were described previously (12–14). In the first step of PCR, 25–50 pmol of each of a forward primer 5′-GCTCTAGAATTCCAATCCTCAGTTACCACAG-3′ (sense, nucleotides 214–236) and the mutant antisense oligonucleotide for the desired mutation were used to generate a megaprimer using *Pfu* DNA polymerase (Stratagene, San Diego, CA). The conditions used were 94 °C for 30 s, 56 °C for 1 min, and 73 °C for 2 min for 20 cycles. Gel analysis showed the generation of a single band for each mutation. This double-stranded DNA fragment was purified using Geneclean II and used as a forward primer in the second step of PCR. After an initial five cycles of linear amplification with the megaprimer, the reverse primer, 5′-CCAGGAGAGGATCCATAAAATGAC-3′ (nucleotides 1270–1247), was added into the reaction tube, and the reaction was continued at 94 °C for 1 min, 68 °C for 1 min and 73 °C for 3 min for 20 cycles. Products were analyzed by agarose gel electrophoresis, which showed the generation of a major single band of expected size (1.05 kilobases). This band was purified by agarose gel electrophoresis, followed by Geneclean II to separate it from the megaprimer. For the cDNAs of R180A and C181A,

[1] The abbreviations used are: PCR, polymerase chain reaction; SNA, *S. nigra* agglutinin; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbant assay; PBS^-, Dulbecco's phosphate-buffered saline solution; DMEM, Dulbecco's modified Eagle's medium; MES, 2-(*N*-morpholino)ethanesulfonic acid; Ni-NTA, nickel-nitrilotriacetic acid; RCA_{120}, *R. communis* agglutinin; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

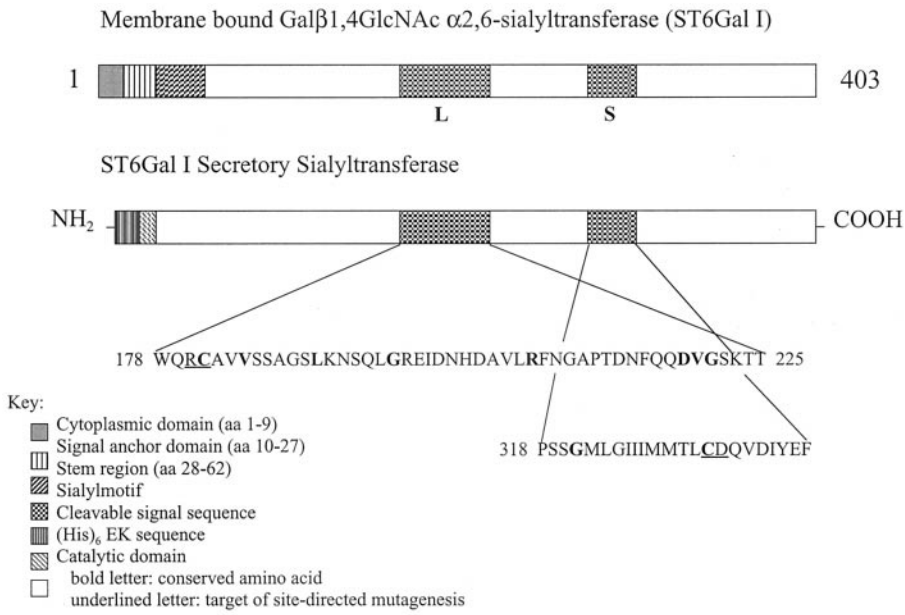

FIG. 2. **Location of the conserved cysteines in the wild type membrane form and the recombinant secreted form of ST6Gal I.** The primary structure of the wild type "membrane form" of the sialyltransferase consists of a short N-terminal cytoplasmic domain (amino acids 1–9), a signal-membrane anchor sequence (amino acids 10–27), a flexible stem region (amino acids 28–62), and a large lumenal catalytic domain (amino acids 63–403). The recombinant secreted form of the sialyltransferase, His-spST, was constructed by replacing the first 71 amino acids from the N-terminal of ST6Gal I with the cleavable signal sequence from the dog pancreatic proinsulin followed by the hexameric histidine and enterokinase cleavage site. The L-sialylmotif in this enzyme spans from amino acids 178–225 and contains one invariant cysteine at amino acid 181. The S-sialylmotif spans amino acids 318–340 and contains the other invariant cysteine at amino acid 332. The invariant amino acid residues in the sialylmotifs are shown in *bold type*. The mutated amino acid residues are *underlined*.

the gel purified product was digested with two unique restriction enzymes *Dra*III (at nucleotide 477) and *Bst*BI (at nucleotide 824), and the smaller 350-base pair fragment was purified using the MERmaid kit (Bio101, San Diego, CA). The fragment for each mutation was then subcloned into a similarly digested and purified larger fragment of His-spST. Similarly, for C332A, C332S, and D333A, the gel purified product was digested with *Bst*BI and *Bsp*EI (at nucleotide 1188), and the 330-base pair fragment was purified and subcloned. The mutation was confirmed by dideoxy double-stranded sequencing of the entire fragment that was subcloned, including the restriction sites used.

To construct the full-length form of the mutants C181A and C332A, the cDNAs were digested with *Bsp*EI (at nucleotide 1188) and *Pfl*MI (at nucleotide 477), and the smaller 711-base pair fragment for each mutant was gel purified and then subcloned into a similarly digested larger fragment of pcDNA:ST6Gal I, using standard molecular biological techniques.

Expression and Selection of Stable CHO-K1 Cell Line Transfected with His-spST—Transfection of CHO-K1 cells was performed using the calcium phosphate method as described previously (23). Cells were plated at a density of 1–2 $\times$ 10^6 cells/100-mm dish and cultured for 20–24 h before transfection with 2.0 μg of plasmid DNA/100-mm dish. During transfection, serum free DMEM/Ham's F-12 medium (Life Technologies, Inc.) was used. Stable transfectants were selected 48–60 h post-transfection with 1 mg/ml of G418 sulfate (Life Technologies, Inc.) and 0.1 mg/ml RCA_{120} (Sigma) in the medium for at least 2 weeks. Stable clones were isolated and then further selected by ELISA, using anti- rat Galβ1,4GlcNAc α2,6-sialyltransferase antibody, characterized previously (15).

For ELISA, stable clones ($\sim$ 10^4 cells/well) resistant to both G418 and RCA_{120} were individually plated in 24-well plates containing DMEM/Ham's F-12 medium (500 μl) with 5% fetal calf serum. After overnight growth, 100 μl of the medium from each stable clone were added to the wells of a 96-well microtiter plate and incubated at 4 °C overnight. The media were removed, and the plates were blocked with PBS^- containing 1% bovine serum albumin at room temperature. After washing the plates with PBS^- three times, 50 μl of anti-rat Galβ1,4GlcNAc α2,6-sialyltransferase antibody (1:500 in PBS^- containing 1% bovine serum albumin) were added and incubated at room temp for 2 h. The plates were then washed thrice with PBS^-, and 50 μl of horseradish peroxidase-conjugated anti-human IgG (Sigma; 1:1000 in PBS^- containing 1% bovine serum albumin) were added, and the plate was incubated at room temperature for 1 h. After washing the plates, the color was developed with 50 μl/well 3,3′5,5′-tetramethylbenzidine peroxidase substrate (Pierce). Color was quenched with 1 M phosphoric acid (50 μl/well), and readings were taken at 450 nm using a microtiter ELISA reader (Labsystems, Titertex Multiscan MCC/340).

Treatment by Thiol-specific Reagents—ST6Gal I (0.5–0.7 μM) purified as described under "Results" was incubated in 0.1 M phosphate buffer (pH 7.5) containing varying concentration of each thiol specific reagent. After incubation for 20–30 min at room temperature, the reaction was stopped by dilution with the assay mixture, and the remaining activity was assayed immediately, as described previously (12). Control experiments were conducted under the same conditions but without thiol reagents.

Quantitation of Disulfides and Free Sulfhydryl Groups—Purified ST6Gal I, obtained from stable CHO-K1 cell line expressing the protein, was used (0.18–0.2 mg) for this assay. Quantitative estimation of the disulfide bonds were done using 5,5′-dithiobis(2-nitrobenzoic) acid, following the procedure described by Cavallini *et al.* (24). The reaction was carried out in a solution of monopotassium phosphate containing 1.6 mM EDTA, 16.6 mM HCl, 24 mmol urea at pH 7.4, in the absence or presence of sodium borohydride. The reaction was monitored at 412 nm until a constant absorbance reading was achieved.

Transient Expression of the Wild Type and Mutant Sialyltransferase—For expression of the wild type His-spST and its mutants, COS-1 cells (1–2 $\times$ 10^6 cells/100-mm dish) were transfected with $\sim$2.0 μg of plasmid DNA using LipofectAMINE™ (Life Technologies, Inc.) as described previously (12, 14). Expression of the proteins was allowed to continue for 40–60 h post-transfection before harvesting the cells. For the analysis of the soluble expressed proteins, the culture medium was collected and used for the enzyme assay as described previously (12, 14). These transfected cells were also used for metabolic labeling. The transfection experiments and subsequent analysis were repeated at least three times for each mutant using plasmid DNAs from different preparations.

For the analysis of the membrane bound proteins, the transfected COS-1 cells were harvested 40–60 h post-transfection. For sialyltransferase assays, cells were washed with PBS^- (Irvine Scientific, Irvine, CA) and lysed as previously described (19).

Pulse-Chase Labeling of Transfected COS-1 Cells and Analysis of the Transiently Expressed Proteins—Metabolic labeling of cells and immunoprecipitation of expressed proteins were performed essentially following the procedure of Colley *et al.* (25) with modification as follows. 36–48 h post-transfection, the transfected COS-1 cells were washed thrice with methionine-free DMEM (Life Technologies, Inc.) and then incubated in the same medium for 1–2 h in the 5% CO_2 incubator at 37 °C. Medium was removed, and fresh medium containing 100 μCi/ml Trans ^{35}S-label (PerkinElmer Life Sciences) was added to each dish. After 3 h of incubation in the 5% CO_2 incubator at 37 °C, the medium was removed, and the cells were washed at least thrice with DMEM containing methionine. Fresh DMEM (5 ml) containing methionine and 10% fetal bovine serum was added, and the cells were incubated for 16–18 h (chase period) at 37 °C in a humidified 5% CO_2 incubator. The radiolabeled media were then collected and used for detection of ST6Gal I protein by immunoprecipitation, as described previously (14) using affinity purified rabbit anti-rat ST6Gal I. In some experiments, the radiolabeled proteins were also analyzed by binding with Ni-NTA agarose (Qiagen). Sialyltransferase fusion protein present in the radiolabeled medium was pelleted with Ni-NTA agarose by centrifugation and detected as follows. The medium (100 -500 μl) from the transfected cells

was mixed with equal amounts of 50 mM MES (pH 6.0) containing 0.1% Triton CF-54, 25% glycerol, 0.15 M NaCl, 2 mM β-mercaptoethanol, and 100 mM imidazole. Ni-NTA agarose (20 μl) in a slurry was added, incubated by rotation at room temperature for 1–2 h, and centrifuged. The pellet was used for SDS-polyacrylamide gel electrophoresis after washing twice with the above buffer. Proteins were eluted from the pellet by boiling for 5 min in 40 μl of 1× Laemmli gel sample buffer (26) containing 10% β-mercaptoethanol and 25 mM EDTA. Ni-NTA agarose bound fusion proteins were electrophoresed on 10% SDS-polyacrylamide gels, according to the method of Laemmli (26). Radiolabeled proteins were visualized by fluorography using 2,5-diphenyloxazole/dimethyl sulfoxide (27), and the gels were exposed to Kodak XAR-5 film at −80 °C.

Western Blot—This was carried out using unlabeled medium from transfected COS-1 cells as described previously (12) with the following modifications. The sialyltransferase protein samples were separated from the transfected medium using Ni-NTA agarose, as described above. Samples were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane, following the usual technique (28). The membrane blot was developed by adsorption of the rabbit anti-rat Galβ1,4GlcNAc α2,6-sialyltransferase antibody (1:500), followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000; Amersham Pharmacia Biotech). The protein bands were finally visualized by ECL, as suggested by the supplier of the reagents (Amersham Pharmacia Biotech).

Immunofluorescence Microscopy—This was performed as described by Colley *et al.* (25) with the following modifications. Approximately 0.5×10^5 COS-1 cells were plated on poly-D-lysine coated circular (18 mm) microscope cover glasses in 500 μl of DMEM with 10% fetal bovine serum and allowed to reach 60–70% confluence by overnight incubation. Cells were transfected using LipofectAMINE™, as described by the supplier (Life Technologies, Inc.), with 1–2 μg of plasmid DNA. After 36–60 h of transfection, cells were washed twice with PBS^- and fixed in freshly prepared 2% paraformaldehyde for 35–50 min at room temperature. For detection of expressed wild type ST6Gal I and its mutants, the cells were washed twice with 0.1 M glycine and then permeabilized with 0.1% Triton X-100 for 10 min. Fixed cells were incubated for 45 min in blocking buffer at room temperature. Blocking buffer was removed, and 150 μl of a 1:100 dilution of affinity purified rabbit anti-rat Galβ1,4GlcNAc α2,6-sialyltransferase antibody in blocking buffer were added to each well, and incubation was continued for 45 min at room temperature. Cells were washed four times for 5 min with 500 μl of PBS^- at room temperature, a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma) in blocking buffer was added, and the incubation was allowed to continue for 45 min. For co-localization experiments, the cells were washed four times with PBS^- (without calcium and magnesium salts), and then a 1: 200 dilution of tetramethylrhodamine isothiocyanate (TRITC)-conjugated lectin from *Sambucus nigra* (SNA; EY Laboratories, Inc., San Mateo, CA) in blocking buffer (0.5% goat serum in PBS) containing 5% bovine serum albumin, 1 mM Ca^{2+}/Mg^{2+}, and 0.1 M lactose was added to stain the cells for detection of NeuAcα2,6Galβ1,4GlcNAc containing glycoproteins. Cells were again washed four times for 5 min each with PBS^- and then mounted on microscope slides using ~20 μl of Vectashield mounting media (Vector Laboratories Inc., Burlingame, CA). Cells were visualized on a Nikon Microphot-FXA microscope and pictures taken with Ektachrome (P1600) film.

Other Methods—The protein determination using the bicinchonic acid protein assay reagent kit (Pierce) and sialyltransferase assay, and the kinetic analysis were done essentially as described previously (12–14).

RESULTS

Selection of Stable CHO-K1 Cell Line Expressing Wild Type ST6Gal I and Purification of the Protein—The cDNA for the wild type ST6Gal I sialyltransferase, His-spST, was transfected into CHO-K1, which lacks the endogenous ST6Gal I enzyme (19). In addition to G418, we have also used *Ricinus communis* agglutinin, RCA_{120}, for selection of the transformants expressing the enzyme. It was noted that while using only RCA_{120}, the frequency of obtaining untransfected CHO-K1 resistant cells was ≤0.001%. This lectin is known to bind terminal galactose on the oligosaccharide bound to the glycoprotein. Because CHO-K1 cells have endogenous sialyltransferases other than ST6Gal I that can sialylate the terminal Gal, some untransfected cells also become resistant to RCA_{120}, although the frequency is very low. Using both G418 and RCA_{120}, not a single resistant clone was obtained using 10^6 untransfected cells. With G418 selection only, ~10–30% of the cells were resistant. In contrast, about 0.1% cells became resistant to both G418 and RCA_{120} when transfected cells were used, presumably because of increased expression of ST6Gal I. At least 30 resistant clones were selected for further screening by ELISA. The isolated resistant clones were grown individually in 48-well plates containing DMEM/Ham's F-12 medium supplemented with G418, RCA_{120}, and fetal calf serum. After about 48–36 h of growth, 100 μl of the medium from individual wells were used for ELISA using rabbit anti-rat ST6Gal I antibody to select the clone expressing the highest level of sialyltransferase, which was finally subcloned by limit dilution. ST6Gal I protein was purified from the culture medium of this clone using CDP-hexanolamine Sepharose as described previously (29). The purity of the enzyme obtained was >90% as judged by SDS-polyacrylamide gel electrophoresis (not shown).

TABLE I

Effect of thiol-specific reagents on ST6Gal I sialyltransferase activity

At least five different concentrations of each reagent were incubated with purified enzyme in 0.1 M sodium phosphate buffer (pH 7.5) at room temperature (22 °C). The reaction was quenched by dilution, and the remaining enzyme activity was assayed as described under "Experimental Procedures," using CMP-[^{14}C]NeuAc and asialo α_1-acid glycoprotein as substrates. Duplicate reactions were conducted for each test. The 50% inactivation (IC_{50}) was calculated from the graph obtained, using varying concentration of the reagent.

Reagents	IC_{50}[a]
	mM
Dithiothreitol	0.35
β-Mercaptoethanol	1.0
5,5′-Dithiobis(2-nitrobenzoate)	no inhibition at >5.0
N-Ethylmaleimide	no inhibition at >25.0
Iodoacetamide	no inhibition at >25.0

[a] The values listed are the averages of three experiments, which varied ± 10%.

Effect of Thiol Modifying Agents on ST6Gal I Enzyme Activity—The purified sialyltransferase was subjected to the thiol reducing reagents dithiothreitol and β-mercaptoethanol, as described under "Experimental Procedures." Both reagents completely abolished enzyme activity in a dose-dependent manner with 50% inhibition achieved at 0.35 mM dithiothreitol and 1.0 mM of β-mercaptoethanol (Table I). This inhibition was irreversible because no activity was regained following removal of these reducing agents by dialysis. The same results were obtained for the enzyme purified from rat liver (not shown). The results suggest that a disulfide linkage is essential for the enzyme activity. In contrast, the free sulfhydryl reagents, 5,5′-dithiobis(2-nitrobenzoic) acid (5 mM), *N*-ethylmaleimide (25 mM), and iodoacetamide (25 mM), had no effect on enzyme activity. Under the same reaction conditions, 0.5 mM ^{14}C-labeled *N*-ethylmaleimide covalently reacted with the enzyme as detected by SDS-PAGE (not shown). 5,5′-Dithiobis(2-nitrobenzoic) acid also covalently reacted with the enzyme as detected spectrophotometrically (Table II). The results demonstrate the presence of free sulfhydryls and suggest that they are not essential for enzyme activity.

Quantitative Estimation of the Number of Disulfide Linkages—The number of disulfide linkage(s) was estimated using the purified secreted form of ST6Gal I. The deduced amino acid sequence showed the presence of 6 cysteine residues in the secreted form of the enzyme. Using 13,600 M^{-1} Cm^{-1} as the extinction coefficient (30) of the 2-nitro-5-thiobenzoic acid modified enzyme, the number of cysteine residues obtained was 5.5 before reduction and 7.0 after reduction (Table II). The difference of 1.5 cysteines between nonreduced and reduced ST6Gal

TABLE II

Quantitative estimation of the number of disulfide linkage

The determination of the total number of cysteines was done before and after reduction of the protein using $NaBH_4$ at pH 7.4. The method was described under "Experimental Procedures." For the molar absorbtion coefficient of 2-nitro-5-thiobenzoic acid liberated at 412 nm, the value of Ellman (13,600 M^{-1} Cm^{-1}, ref. 30) was used. The number of cysteines was calculated according to the equation, $N = MW \times A \times V$/(molar absorption coefficient $\times$ m), where N = number of sulphydryl groups, MW = molecular weight of the protein, A = absorbance reading, V = the volume of the final solution (6 ml, in this case), and m = the weight in mg of the protein sample analyzed (24).

Protein	Amount of protein	Number of sulphydryl groups		Difference[a]	Disulfide linkage
		Before reduction	After reduction		
	mg	*mol/mol*		*mol/mol*	*mol/mol*
Bovine serum albumin	0.2	3.4	36.6[b]	33.2	17
rST6Gal 1	0.175	5.5	7.0[c]	1.5	1

[a] The value listed was the average of three experiments that varied ±5%.
[b] The theoretical value of total number of cysteine in bovine serum albumin is 35, and the total number of cystine disulfide linkage is 17 (31, 32).
[c] The theoretical value of total number of cysteine in the secreted form of rST6Gal I is six.

I is consistent with a single disulfide bond. Alternatively, the difference could be accounted for by multimerization of the enzyme by interchain disulfide bonds. Bovine serum albumin, which was used as control, showed the presence of 17 disulfide linkages in accord with the experimental value, obtained by x-ray crystallography and NMR (31, 32).

The Soluble Form of ST6Gal I Is a Monomer—It was shown earlier that the intracellular membrane bound ST6Gal I exists as both monomer and dimer (33). While the monomer was active, the dimer was found to be inactive, although it could act as a galactose binding lectin (33). To establish whether the secreted form of this enzyme also exists in both monomer and dimer form, His-spST was expressed in COS-1 cells. As described under "Experimental Procedures," the ^{35}S-radiolabeled ST6Gal I was analyzed by nonreducing and reducing SDS-PAGE followed either by purification from the medium using Ni-NTA resin (using the His tag sequence) or by immunoprecipitation with polyclonal antibody to the enzyme. As a control, medium of COS-1 cells transfected with vector alone was used for comparison. As shown in Fig. 3 (*A* and *B*), both before and after reduction with 10% β-mercaptoethanol, the Ni-NTA resin bound enzyme showed a single band with a molecular mass of ~39 kDa, as predicted for the monomer. The other band was for nonspecific binding of a protein, plausibly serum albumin, which was also present in the medium of cells transfected with vector only. Similar results were observed for the immunoprecipitated enzyme (Fig. 3, *C* and *D*). Thus, it does not appear that the soluble recombinant ST6Gal I forms dimer or higher multimers via interchain disulfide bonds. Indeed, a slight shift in mobility between the reduced and nonreduced enzyme (Fig. 3, *A* and *B*) provides additional evidence for the presence of an intradisulfide linkage.

Analysis of Site-directed Mutants of ST6Gal I—Analysis of alanine (or serine) mutants of the two invariant cysteines (Cys^{181} and Cys^{332}) and their adjacent charged amino acids described previously (12, 14) was extended to further probe the role of these residues. In the present study, each mutant cDNA was placed in the expression vector $pcDNA_{ins\text{-}His}$ as described under "Experimental Procedures." To assess the effect of the mutations on the enzymatic activity, the cDNAs for the wild type ST6Gal I and the mutant proteins R180A, C181A, C332A, C332S, and D333A were transiently expressed in COS-1 cells. SDS-PAGE of immunoprecipitated radiolabeled proteins showed that all mutant sialyltransferases were expressed and exhibited similar molecular mass compared with that of the wild type sialyltransferase. Fig. 4*A* is shown here as a representation of mutants. We observed that the level of expression of cysteine mutants was low compared with that of the wild type. It is possible that these mutant proteins were degraded rapidly after expression. Medium containing the sialyltransferase was also used to assess the enzymatic properties of the mutant sialyltransferases. Endogenous levels of sialyltransferase activity in COS-1 cells "mock" transfected with the expression vector, $pcDNA_{ins\text{-}His,}$ were typically less than 2% that of the expressed wild type sialyltransferase. The sialyltransferase assay using similar amount of protein (as judged by SDS-PAGE) showed that the cysteine mutants C181A, C332A, and C332S were enzymatically inactive. In contrast, mutants of the adjacent charged residues, R180A and D333A, were active, exhibiting 53 and 45% of the activity, respectively, compared with that of the wild type enzyme (Fig. 4*B*). As previously reported for the alanine mutant of Arg^{180} (13), kinetic analysis of the alanine mutants of Arg^{180} and Asp^{333} revealed no significant change in K_m values for either the donor substrate CMP-NeuAc or the acceptor substrate asialo α_1-acid glycoprotein (Table III). The fact that the mutation of these adjacent charged residues has little effect on the kinetics of the sialyltransferase suggests that the invariant cysteine residues may not be directly involved in substrate binding.

Evaluation of Sialyltransferase Activity and Subcellular Localization in Transfected COS-1 Cells—One explanation for the secreted C181A and C332A sialyltransferase mutants being inactive is that they were unstable. To further evaluate the activity and subcellular localization of the mutants, pcDNA3 constructs of the full-length enzymes containing the N-terminal signal anchor were prepared for transfection into COS-1 cells. Following transfection, activity was examined indirectly by detecting the formation of the NeuAcα2,6Galβ1,4GlcNAc product of the enzyme using TRITC-conjugated SNA, which binds this structure on glycoproteins and glycolipids (34, 35). The sialyltransferase protein was detected by immunofluorescence, using FITC-conjugated secondary antibody. As shown in Fig. 5, the vector transfected COS-1 cells did not stain with either SNA or sialyltransferase antibody, consistent with the fact that COS-1 cells lack ST6Gal I enzyme activity. In contrast, the wild type ST6Gal I transfected cells were stained for both the sialyltransferase product (red TRITC stained) and protein (green FITC stained), as previously demonstrated by Colley *et al.* (36). Expression of the protein in transfected cells was variable, ranging from low to very high. Fig. 5 shows transfected COS-1 cells with two different expression levels of ST6Gal I. As reported earlier (36), for cells expressing moderate levels of the wild type ST6Gal I protein, staining was localized primarily to a perinuclear crescent on one side of the nucleus, consistent with the localization to the Golgi apparatus. For cells expressing high levels of the wild type protein, FITC staining of the Golgi region was intense but was also found diffusely localized throughout the cytoplasm in reticular structures characteristic of endoplasmic reticulum. All cells expressing the wild type ST6Gal I also stained positive with the TRITC-conjugated SNA, indicating the formation of the Neu5Acα2,6Galβ1,4GlcNAc product by the enzyme (Fig. 5).

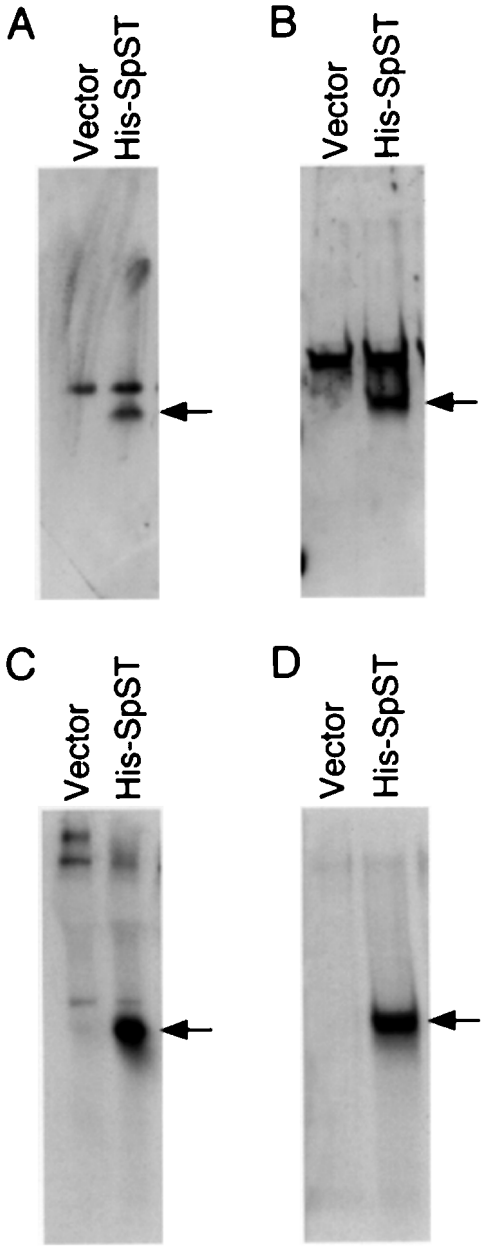

FIG. 3. **SDS-PAGE analysis of the Ni-NTA agarose bound His-spST.** The metabolic labeling of COS-1, transfected cells with the wild type cDNA, was carried out as described under "Experimental Procedures." The medium collected 48–60 h post-transfection was used for the analysis after precipitation with Ni-NTA agarose (*A* and *B*) or immunoprecipitation (*C* and *D*). Precipitated proteins either untreated (*A* and *C*) or treated (*B* and *D*) with 10% β-mercaptoethanol were run on a 10% SDS-polyacrylamide gel. The radiolabeled proteins were visualized by fluorography. The ST6Gal I specific protein band produced by the expression vector His-SpST had an apparent molecular mass of ~39 kDa (see *arrow*) with and without reduction.

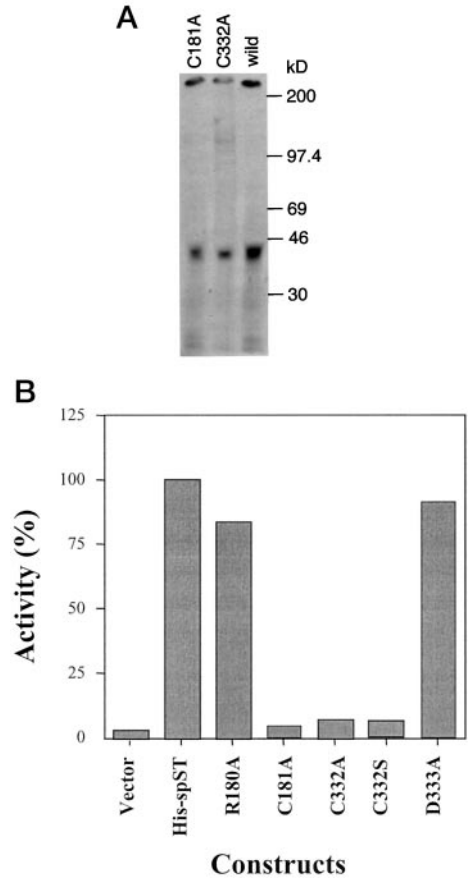

FIG. 4. **Analysis of His-spST and its mutants.** In *A*, immunoprecipitation of the wild type ST6Gal I and its mutants is shown. Metabolic labeling of COS-1 cells using Trans ^{35}S-Express protein label containing [^{35}S]methionine and immunoprecipitation of expressed proteins with rabbit anti-rat ST6Gal I were performed essentially as described previously (12). The medium from cells 48–60 h post-transfection with wild type and mutant cDNAs was used for the immunoprecipitation. Immunoprecipitated, radiolabeled proteins were electrophoresed on a 10% SDS-polyacrylamide gel and then visualized by fluorography. As shown, the mutants C181A and C332A moved similarly with the wild type enzyme (His-spST) in the SDS-polyacrylamide gel, indicating no gross rearrangement because of the mutation. In *B*, the sialyltransferase activity for different mutants has been shown. The cDNAs of the wild type ST6Gal I and the mutants were expressed in COS-1 cells, and the medium was collected 48–60 h post transfection. This was used as a source of enzymes, after concentrating using Amicon filters. The sialyltransferase assays were carried out using CMP-[^{14}C]NeuAc as donor and asialo α_1-acid glycoprotein as acceptor, as described under "Experimental Procedures."

TABLE III

Kinetic constants for the wild type ST6Gal I and its Cys mutants

The kinetic analysis was performed using Ni-NTA agarose bound proteins, expressed in the medium of COS-1 cells transfected with cDNAs of the wild type secretory sialyltransferase and mutants (see "Experimental Procedures" and description in the text).

ST6Gal I constructs	Apparent K_m values[a]			
	CMP-NeuAc	−fold	Acceptor (ASGP)[b]	−fold
	μM		μM	
Wild type	50	1.0	330	1.0
Mutants				
R180A[c]	110	2.2	260	0.8
D333A	120	2.4	428	1.3

[a] The apparent K_m values listed are the averages of three experiments that varied ±0.5 −fold. As mentioned earlier (12), the values (apparent) were determined from the *x* intercept ($-1/K_m$ apparent), using the Cricket Graph program (Cricket Software, Malvern, PA).

[b] Asialo α_1-acid glycoprotein (ASGP) was prepared by mild acid hydrolysis of α_1-acid glycoprotein (Sigma), following the procedure of Schmid (65).

[c] This was reported earlier (13).

The TRITC-conjugated SNA staining of the intracellular organelles is due to the permeabilization procedure used for staining the cells.

In contrast to the wild type enzyme that was primarily localized to the Golgi apparatus, the cysteine mutants, C181A and C232A, were localized diffusely throughout the cytoplasm, with more intense staining of the membrane surrounding the nuclear area, characteristic of localization to the endoplasmic reticulum (Fig. 5). This staining pattern was seen for all levels of expression. Despite the fact that the level of expression of the mutant proteins appeared to be the same as that of the wild

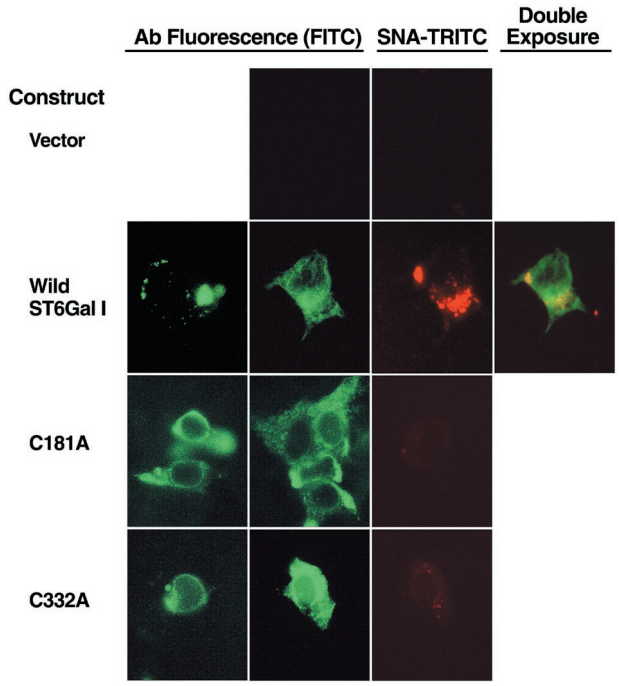

FIG. 5. **Fluorescence microscopy of ST6Gal I expressed in transfected COS-1 cells.** Immunolocalization detection of the product of ST6Gal I using SNA was accomplished by transfecting the COS-1 cells with cDNAs coding for the full-length wild type ST6Gal I and the corresponding alanine mutants of Cys^{181} and Cys^{332}. 48–60 h post-transfection, the cells were washed, permeabilized, and stained with FITC-conjugated (*green fluorescence*) secondary antibody after adsorption of the anti-ST6Gal I antibody to check the expression of the ST6Gal I protein. The enzyme activity of the wild type ST6Gal I and its mutants was indirectly examined by detecting the product NeuAcα2,6Galβ1,4GlcNAc-R structure on the glycoproteins by co-staining the cells with SNA-TRITC (*red fluorescence*).

type enzyme, as judged by the FITC staining intensity, TRITC-conjugated SNA gave very weak staining, if any, which was often indistinguishable from background staining, of the cells transfected with the cysteine mutants. Thus, the cysteine mutants appear to produce an inactive sialyltransferase.

DISCUSSION

In this report we have investigated the role of the conserved cysteine residues of the sialyltransferase ST6Gal I, Cys^{181} and Cys^{332}. Sialyltransferase mutants replacing either invariant cysteine with Ala or Ser results in the production of sialyltransferase polypeptide when expressed in COS-1 cells either as a soluble recombinant form of the enzyme (residues 71–403) or as the full-length membrane bound form (Fig. 3). However, none of these cysteine mutants exhibit activity. In contrast to the Golgi localization of the wild type sialyltransferase, the full-length mutants accumulate primarily in the endoplasmic reticulum, suggesting that they are retained as improperly folded proteins (see reviews in Refs. 37 and 38). These results do not distinguish between the possibility that the sialyltransferase is initially produced as a properly folded active enzyme, which is then rapidly denatured, and the possibility that the enzyme never reaches an active conformation. However, in either case it appears that the two conserved cysteines, Cys^{181} and Cys^{332}, are critical for production and maintenance of an active conformation of the sialyltransferase.

In total, the full-length wild type sialyltransferase contains seven cysteine residues, and the recombinant secreted enzyme studied here contains only six of these cysteines, because of deletion of Cys^{24}, which is present in the transmembrane domain. Quantitation of free cysteines using 2-nitro-5-thiobenzoic acid (Table II) showed a difference of 1.5 mol/mol before and after reduction, consistent with the presence of a single disulfide bond. Although reduction of the enzyme using either dithiothreitol or β-mercaptoethanol abolishes enzyme activity, agents that react with free cysteines had no effect on activity. These results, combined with the fact that site-directed mutagenesis of either of the two conserved cysteines, Cys^{181} and Cys^{332}, produces an inactive enzyme, provides strong evidence that these conserved cysteines are involved in the formation of the disulfide bond, which is essential for enzyme activity. Because each of the two sialylmotifs carry one of these two cysteines, the disulfide bond would physically link them in close proximity in the active conformation of the enzyme as proposed previously (13, 14). Site-directed mutagenesis of conserved amino acid residues in the two sialylmotifs suggested that the S-sialylmotif is involved in binding the acceptor substrate and that they are both involved in binding the donor substrate CMP-NeuAc (14). The existence of a disulfide bond joining the two sialylmotifs provides further credence for how the two motifs would participate in binding the substrates of ST6Gal I. Although only ST6Gal I was studied in this report, we propose that a disulfide bond between the corresponding invariant cysteines would similarly join the sialylmotifs in each of the 16 sialyltransferases cloned to date.

Although there is little sequence homology between sialyltransferases and other families of glycosyltransferases, they all have a common topology with a short N-terminal cytoplasmic domain, a transmembrane signal anchor sequence, and a lumenal stem region and catalytic domain (8, 25, 39). Within each homologous glycosyltransferase family there are regions of high conservation (8, 13, 39), and conserved cysteines have been reported for β1,4-galactosyltransferases (8), β1,3-galactosyltransferases (8), fucosyltransferases (40–43), *N*-acetylgalactosaminyl-transferases (44–46), and *N*-acetyl-glucosaminyltransferases (47, 48). However, examination of the pattern of conserved cysteines in these families does not reveal an obvious pattern of disulfide bonds as a conserved structural motif across all glycosyltransferases.

The cysteines of the *N*-acetylglucosamine-β1,4-galactosyltransferase (or β4GalT1) have been studied most extensively of any glycosyltransferase to date. Early chemical modification studies with the bovine enzyme (49, 50) suggest that two of the five cysteines are involved in a disulfide linkage (residues 134 and 247). Similar conclusions were reached in studies involving site-directed mutagenesis of cysteines of the bovine and human galactosyltransferase, followed by expression in *E. coli* (51, 52). The recent crystal structure of the *N*-acetylglucosamine-β1,4-galactosyltransferase (53) revealed two pairs of cysteine residues, residues 134 176 and residues 247 and 266, were close enough (~5 Å) to permit disulfide bond formation. The apparent discrepancy between the crystal structure data and the earlier studies is not entirely clear. However, the site-directed mutagenesis studies are not necessarily inconsistent with the crystal structure data. Expression of wild type galactosyltransferase in *E. coli* typically produces partially active enzyme requiring purification of active fractions and/or denaturation and renaturation steps prior to analysis (51, 52). Thus, deletion of a cysteine that participates in a disulfide bond need not abolish activity providing that at least part of the enzyme can assume an active conformation in its absence. Moreover, there are now seven members reported for the β4GT family (8). Of the five cysteines in the human and bovine β4GalT1, only one, Cys^{129} (corresponding to the bovine Cys^{134}), is found in human β4GalT7. Thus, for one member of the β4GalT family, neither of the disulfide bonds is required for proper folding and maintenance of activity.

Analysis of glycosyltransferase sequences has revealed the possibility of protein motifs that cross glycosyltransferase families (39). Although it may ultimately be shown that these enzymes as a class will have similar peptide folds, it is premature to speculate that this is the case, based on the sequence information and limited structure-function analysis currently available. As additional glycosyltransferase structures become available, it will be of interest to determine whether common structural motifs are associated with their common function.

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