Molecular Defects That Cause Loss of Polysialic Acid in the Complementation Group 2A10*

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Michaela Windfuhr‡, Arnd Manegold‡, Martina Mühlenhoff, Matthias Eckhardt§, and Rita Gerardy-Schahn‡¶

From the ‡Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Carl-Neuberg-Straße 1, 30625 Hannover and the §Institut für Physiologische Chemie, Universität Bonn, Nußallee 11, 53115 Bonn, Germany

Polysialic acid (PSA) is a dynamically regulated post-translational modification of the neural cell adhesion molecule (NCAM), which modulates NCAM binding functions. PSA biosynthesis is catalyzed by two polysialyltransferases, ST8SiaII and ST8SiaIV. The catalytic mechanisms of these enzymes are unknown. In Chinese hamster ovary cells, ST8SiaIV is responsible for PSA expression. In the complementation group 2A10, the ST8SiaIV gene is disrupted. Investigating the molecular defects in this complementation group, seven clones with missense mutations in ST8SiaIV were found. Mutations cause replacement of amino acids that are highly conserved in α2,8-sialyltransferases. To verify the physiological relevance of identified mutations, identical amino acid substitutions were introduced into epitope-tagged variants of hamster ST8SiaIV and murine ST8SiaII and recombinant proteins were tested in vitro and in vivo. None of these constructs reconstituted PSA synthesis in 2A10 cells, although the proteins were expressed and with the exception of the cysteine variants ST8SiaIV-C356F and ST8SiaII-C371F correctly targeted to the Golgi apparatus. Interestingly, two mutations (ST8SiaIV-R277G and -M333V and the corresponding mutants ST8SiaII-R292G and -M348V) could be partially rescued if tested in vitro. Although these mutants were negative for autopolysialylation, partial reconstitution of both auto- and NCAM polysialylation was achieved in the presence of NCAM. The data presented in this study suggest a functional link between auto- and NCAM polysialylation.

Rapid changes in cellular recognition events as required in the course of development (1, 2), inflammation (3), and regeneration (4) can be realized via structural variations of plasma membrane components (e.g. by changing their glycosylation patterns) leading to modified binding capacities. One of the most intensively studied examples in this context is the neural cell adhesion molecule (NCAM). NCAM belongs to the superfamily of immunoglobulin (Ig)-like cell adhesion molecules and contains five Ig and two fibronectin type III homology domains in the extracellular part (5). NCAM mediates homo- and heterophilic binding interactions (6, 7) but, in contrast to other cell adhesion molecules, bears a second regulatory quality, which is the destabilization of cell contacts (8). Responsible for the latter function is polysialic acid (PSA), a unique posttranslational modification of NCAM (for review, see Refs. 9–11). PSA is a large homopolymer of α2,8-linked N-acetylmuramic acid. PSA addition to NCAM is developmentally (12, 13) and functionally (14, 15) regulated with maximal expression in the perinatal phase. In the adult only brain areas with persisting neurogenesis, cell migration (16), axonal growth (17), and synaptic plasticity (for review, see Refs. 18 and 19) are PSA-positive. Neuroendocrine tumors of high malignant potential, like small cell lung cancer (20, 21), neuroblastoma (22, 23), and Wilms’ tumor (24) reexpress PSA at high concentrations, and recent studies demonstrate that PSA promotes tumor growth and malignancy (20, 22, 25).

PSA synthesis in mammals involves two closely related, but independently expressed polysialyltransferases (see Ref. 26, and literature cited therein), ST8SiaII, formerly named STX, and ST8SiaIV, formerly named PST-1 or PST (for review, see Ref. 27). The modi of operation of ST8SiaII and ST8SiaIV seem to be very similar, if not identical. Minor differences have been described with respect to NCAM isofrom specificity and length of PSA chains synthesized (28, 29). A very recent study describes ST8SiaII and ST8SiaIV to have different affinities for the two PSA acceptor sites within the NCAM molecule. Co-expression of both polysialyltransferases led to maximal PSA expression in transfected cells (30).

The catalytic mechanisms used by polysialyltransferases are still obscure, and nothing is known about the structural elements that separate polysialyltransferases from other sialyltransferases. Recently, we reported an unusual autocatalytic property of ST8SiaIV termed “autopolysialylation” (31). PSA synthesis in this reaction involves N-glycosylation sites present in the enzyme. Moreover, because soluble forms of the recombinant ST8SiaIV isolated from the supernatant of transfected Chinese hamster ovary (CHO)-K1 cells were found to carry PSA and immaturely glycosylated enzyme forms were shown to be inactive, this study already demonstrated that autopolsialylation occurs at the cellular level and is a prerequisite for an

*mAb, monoclonal antibody; HA, hemagglutinin; AP, alkaline phosphatase; DTAF, dichlorotriazinyl amino fluorescein; TRITC, tetramethyl rhodamine isothiocyanate; endoNE, endo neuraminidase NE; FCS, fetal calf serum; SSC, standard saline citrate; BT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; wt, wild type; ER, endoplasmic reticulum.

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active enzyme. Later studies confirmed autopolysialylation for ST8SiaII and ST8SiaIV (30, 32), and a recent study shows that in human ST8SiaIV asparagine 74 is the major acceptor site for autocatalytically produced PSA (33).

CHO cells of the complementation group 2A10 exhibit a defect in the ST8SiaIV gene and are PSA-negative (34). In this study the molecular defects that inactivate ST8SiaIV in 2A10 cells have been analyzed. In a panel of 31 clones, 7 were found to be inactive due to missense mutations in ST8SiaIV. Amino acid replacements caused by the mutations in all cases concern positions that are highly conserved in the family of α,2-sialyltransferases. Five mutations inactivate ST8SiaIV completely, whereas two were found to affect mainly the autopolysialylation capacity. Mutations identified in ST8SiaIV were introduced in 2A10 cells and caused identical defects. With this study we confirm earlier data suggesting a functional link between auto- and NCAM polysialylation (31, 33).

**EXPERIMENTAL PROCEDURES**

**Materials**— Monoclonal antibodies (mAb) 735 (murine IgG2a) directed against PSA (35), 9E10 (murine IgG1) directed against the Myc-epitope (EQKLISEEDL), and KD11 (murine IgG1) directed against transmembrane forms of NCAM (36) were used after purification on protein G-Sepharose (Amersham Pharmacia Biotech). MAb 12CA5 (murine IgG2b) directed against the hemagglutinin (HA) epitope (YPYDVPDYASL) was purchased from Roche (Penzberg, Germany), and mAb M5 (murine IgG1) directed against the Flag tag (MDYKDDDDK) was from Sigma (Deisenhofen, Germany). A polyclonal rabbit anti-serum recognizing the luminal part of α-mannosidase II (37) was a kind gift of Dr. K. Moremen (University of Georgia, Athens, GA). Secondary antibodies anti-mouse Ig-alkaline phosphatase (AP) conjugate, anti-mouse Ig-dichlorotriazinyl aminofluorescein (DTAF) conjugate and anti-mouse Ig-alkaline phosphatase (AP) conjugate, anti-gift of Dr. K. Moremen (University of Georgia, Athens, GA). Secondary antibodies anti-mouse Ig-alkaline phosphatase (AP) conjugate, anti-mouse Ig-dichlorotriazinyl aminofluorescein (DTAF) conjugate and anti-mouse Ig-alkaline phosphatase (AP) conjugate, anti-gift of Dr. K. Moremen (University of Georgia, Athens, GA).

**Cell Lines**—CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). 2A10 cells represent a genetic complementation group, which is PSA-negative due to defects in ST8SiaIV (34). 31 individual 2A10 clones were isolated from chemically mutagenized CHO-K1 cells. Wild type CHO cells and 2A10 mutants were maintained in DMEM/Ham’s F12 (1:1; Seromed) supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 37 °C, 5% CO₂ incubator. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Seromed) supplemented with 10% FCS, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Northern Blot Analysis**—Total RNA was isolated from CHO-K1 and 2A10 cells by guanidinium isothiocyanate extraction and centrifugation through CsCl gradients (39). Polyadenylated RNA (mRNA) was isolated from total RNA using Oligotex beads (Qiagen) according to the manufacturer’s instructions. The purification step was carried out twice to obtain poly(A)⁺ RNA. 4 μg of poly(A)⁺ RNA were fractionated on 1% agarose, 1 M formaldehyde gels and transferred onto nylon membranes (Qiagen). Blots were hybridized with a digoxigenin-labeled antisense RNA probe transcribed from the entire coding region of the hamster ST8SiaIV cDNA (34). Hybridization was performed overnight at 65 °C in 5× standard saline citrate (5× SSC: 50 mM sodium phosphate, 7% SDS, 50% formamide) and 1% blocking reagent (Roche). Membranes were washed twice for 10 min in 2× SSC, 0.1% SDS at room temperature and once in 0.1× SSC, 0.1% SDS at 68 °C for 20 min. Membranes were incubated with anti-digoxigenin Ig-AP conjugate and bound RNA probes displayed by chemiluminescence using disodium-3-(4-methoxyisopropyl)1,2-dioxetane-3,2’-dicarbonyl (Roche) as a substrate.

**Isolation of Mutant ST8SiaIV cDNAs from 2A10 Cells by Reverse Transcription and Polymerase Chain Reaction (RT-PCR)**—Oligonucleotide primers used in this study are listed in Table I. The table also details the localization and orientation of the primers.

1 μg of poly(A)⁺ RNA was reverse transcribed with 200 units of Superscript™II RNase H⁻ reverse transcriptase (Life Technologies, Inc.) using primer ME54. Full-length cDNAs were then amplified by PCR using primers ME21 and ME22. The amplification was carried out for 40 cycles with Tor DNA polymerase (Sigma) and 80 s of elongation time. PCR products were electrophoresed on an agarose gel and cDNAs of the expected size excised and extracted using QX™ PCR DNA and gel band purification kit (Amersham Pharmacia Biotech). To increase the yield of PCR products, gel-extracted cDNAs were used as a template in a second amplification step (25 cycles) with the same primer combination. PCR products were purified by electrophoresis, extracted from the gel as described, and ligated into the vector pGEM™ T (Promega). The nucleotide sequences were determined by the dideoxy chain termination method (40) using α-[³²P]dATP (Amersham Pharmacia Biotech) and T7 DNA polymerase (Amersham Pharmacia Biotech).

**Construction of Epitope-tagged Forms of ST8SiaII and ST8SiaIV**—Wild type and mutant forms of ST8SiaII and ST8SiaIV were tested for enzymatic activity in vitro and in vivo. Therefore, two sets of epitope-tagged proteins were constructed: (i) full-length forms with N-terminal Flag-HA tags and (ii) soluble forms with C-terminal Myc-His epitopes.

To generate the N-terminally Flag-HA-tagged full-length constructs, the entire coding sequences of murine ST8SiaII and hamster ST8SiaIV were amplified by PCR without the start codons using Pfu DNA polymerase (Promega) with primers MW28 and MM52 for ST8SiaII and primers MW5 and AB6 for ST8SiaIV. PCR products were ligated into the Xhol/LuxIba sites of vector pCDNA3-Flag-HA. pCDNA3-Flag-HA is a derivative of the vector pCDNA3 (Invitrogen), obtained after ligating the FlagM5as (5'-GATCCCTTACCATGATCGACGTA-3') and HAas (5'-GGTACCTTACATGATTTTATTGCTTCATGCACTTCCCTGTGGT-3') into the KpnI/BamH I cloning sites of vector pCDNA3 and pCDNA3(−) (Roche). The cloning sites were verified by sequencing and used for epitope tagging. The resulting plasmids were named pFlag-M5as-His-tag (Flag-HA) and pFlag-M5as-His-tag (HA). The plasmids were transformed into E. coli DH5α, and the recombinant plasmids were verified by sequencing. The plasmids were used to transform mammalian cells, and the transfection efficiency was determined by the expression of β-galactosidase, which was cotransfected as a control. The expression of the recombinant proteins was verified by Western blotting using anti-Flag and anti-His antibodies. The expression levels were measured by densitometry and compared with the control vector pCDNA3-Flag-HA. The results showed that the expression levels of the recombinant proteins were similar to those of the control vector, suggesting that the epitope tagging did not affect the expression levels.

**Table I**

| Name     | Sequence (5’–3’/a) | Orientation | Target sequence             |
|----------|-------------------|-------------|-----------------------------|
| ME54     | GATGCGTAACCACCCAGGCTTGG | a           | ST8SiaII (X83562/a)         |
| ME21     | ATGGCCTGATCATAGAAACGG | s           | ST8SiaIV (Z46801/a)         |
| ME22     | TATTGCTTGACGCTACCTGCTGTT | a           | 4–21                        |
| MW28     | GCCGGTCAAGCAGCTGACCTGGGACG | a           | 1112–1128                   |
| MM32     | GCCGGTCAAGCAGCTGACCTGGGACG | a           | 4–21                        |
| MW5      | GCCGGTCAAGCAGCTGACCTGGGACG | a           | 1102–1080                   |
| MW1      | GCCGGTCAAGCAGCTGACCTGGGACG | a           | 76–93                       |
| AB6      | GCCGTTAGATTTTGGCTCATGACCTTCC | a           | 1060–1077                   |

**a** Restriction sites are bold and italicized; a, antisense; s, sense.

**b** Accession no. in GenBank™ data base.
pernatants of transiently transfected 2A10 cells. Beads were washed.

rose was used to immunoisolate epitope-tagged proteins from the su-

polysialyltransferases. MAb 9E10 covalently bound to protein G-Sepha-

plates, and 2 mM NaCl, 5 mM MgCl2) and bound secondary antibody-conjugates dis -

endoNE (100 ng of endoNE in 60

ferases were used to extract a soluble protein A-NCAM fusion protein

the bead fraction preloaded with the recombinant polysialyltrans-

amide gel electrophoresis (SDS-PAGE) and autoradiography.

[14C]Neu5Ac in a final volume of 60

6 h at 37 °C and 5% CO2, 4 ml of medium with 10% FCS were added,

mM MnCl2) and subdivided into three equal aliquots. In aliquot 1,

and incubated for 1 h with the secondary antibody (anti-mouse Ig-AP

conjugate 1:2000 in blocking solution A). Thereafter, blots were washed

radiography or used to transfer proteins onto nitrocellulose membranes

(4 weeks) on Hyperfilm MP (Amersham Pharmacia Biotech) for auto-

PAGE was performed according to Laemmli (41) in 7% or 9.5% gels

wt 2A10 7G11 8F8 9G2 wt [kb]

18 h (37 °C and 5% CO2). Transfections were carried out with 0.16 µg of

DNA, 75 µl of buffer EC, 1.76 µl of Enhancer, and 1.67 µl of Effectene®
in 400 µl of medium according to the manufacturers’ instructions. Cells

coverslips were washed three times with PBS, fixed in 4% paraform-

and pFlagHA-ST8SiaIV were transiently transfected into NIH3T3 cells

introduced into the wild type sequences of pFlag-HA-ST8SiaII, pFlag-

HA-ST8SiaIV, pMyc-ST8SiaII, and pMyc-ST8SiaIV by either subcloning

restriction fragments or site directed mutagenesis. All constructs were

confirmed by sequencing. Details concerning the construction of

mutants are available upon request.

Production of Recombinant Epitope-tagged ST8SiaII and ST8SiaIV

Variants—Soluble Myc-tagged forms of wild type and mutant ST8SiaII

and ST8SiaIV were immunoisolated from the supernatants of tran-

siently transfected 2A10 cells. Transfections were carried out with

LipofectAMINE® (Life Technologies, Inc.). Briefly, 2.4×104 cells were

seeded in 10-cm tissue culture dishes and incubated for 18 h at 37 °C,

5% CO2, 20 mM of phosphate as substrates.

mM and transmembrane domains (amino acids 1–31 in ST8SiaII, and

tor constructs allow the translation of proteins, in which the cytoplas-

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gen). Nucleotides 94–1125 of murine ST8SiaII and nucleotides 76–1077

of hamster ST8SiaIV were amplified by PCR with primer pairs MM29/

MM30 and MM26/MM31, respectively. Fragments obtained after

BamHI/XhoI digestion were cloned into the annealing vector sites. Vec-

tor constructs allow the translation of proteins, in which the cytoplas-

m and transmembrane domains (amino acids 1–31 in ST8SiaII, and

1–25 in ST8SiaIV) are substituted by the signal sequence of the Ig- light chain and which contain Myc-His epitopes at the C terminus. The

plasmids containing the wild type sequences were named pMyc-

ST8SiaII and pMyc-ST8SiaIV. All constructs were confirmed by

sequencing.

Generation of ST8SiaII and ST8SiaIV Mutants—ST8SiaIV mutants

identified in clones of the 2A10 complementation group were intro-

duced into the wild type sequences of pFlag-HA-ST8SiaII, pFlag-

HA-ST8SiaIV, pMyc-ST8SiaII, and pMyc-ST8SiaIV by either subcloning

of restriction fragments or site directed mutagenesis. All constructs were

confirmed by sequencing. Details concerning the construction of

mutants are available upon request.

RESULTS

Mutants of the Complementation Group 2A10—CHO mu-

tants belonging to the complementation group 2A10 are char-

acterized by a loss of PSA expression. 2A10 cells were used to

isolate ST8SiaIV, the only polysialyltransferase expressed in

CHO cells (34). The molecular defects causing loss of PSA

expression in 2A10 cells were investigated in this study. First,

the presence of ST8SiaIV mRNA was analyzed. The Northern

blot in Fig. 1 shows that wild type (wt) CHO cells express two

ST8SiaIV mRNAs of about 6.5 and 2.3 kilobase pairs. In only 3

out of 31 2A10 clones were ST8SiaIV-specific hybridization

signals detectable, and only 1 clone (7G11) gave a band pattern

identical to the wild type. In clone 8F8, the upper band was

drastically reduced and migrated slightly faster than the cor-

responding wt band. In clone 2A10, a weak 6.5-kilobase pair

signal was visible. The majority of clones, however, were neg-

ative in Northern blot analysis (see, e.g., clone 9G2 in Fig. 1).

RT-PCR was used to re-investigate the presence of ST8SiaIV

mRNA in 2A10 cells. Six additional clones (2D8, 4C4, 5C3, 7F11, 9C8, and 9D8) were found to be positive for ST8SiaIV

transcripts (data not shown).

To determine the sequence of ST8SiaIV-transcripts present in

2A10 mutants, RT-PCR products were subcloned and indi-

vidual colonies were sequenced. RT-PCR and sequencing were
carried out at least twice in parallel samples to eliminate PCR artifacts. Mutations identified are listed in Table II. Single point mutations that allow the translation of full-length proteins with single amino acid exchanges were found in six clones. In clone 2D8 the simultaneous occurrence of a nucleotide transition (A997G) and transversion (G1067T) led to the amino acid exchanges M333V and C356F, respectively. The nucleotide transition C103T introduces a premature stop codon in clone 2A10. In clone 8F8 skipping of exon 3 inactivates ST8SiaIV. It is likely that an element involved in RNA-splicing (e.g. splice donor or acceptor site) is destroyed at the genomic level.

Missense mutations identified in 2A10 cells are displayed in Fig. 2. All cause the exchange of amino acids that are highly conserved in the subfamily of α2,8-sialyltransferases. Only methionine 333 is leucine in ST8SiaIII, and only glycine 281 is invariant in all sialyltransferases. Glycine 146 and glutamic acid 336 are also found in other sialyltransferases, whereas threonine 189, arginine 277, and cysteine 356 are restricted to α2,8-sialyltransferases. These positions may, therefore, contribute to the determination of α2,8 linkage specificity.

Site-directed Mutation of ST8SiaIV—From the finding that ST8SiaIV mRNA levels are drastically reduced in 2A10 clones (see Fig. 1), the possibility arises that loss of PSA expression is due to subthreshold expression of mutated proteins. To investigate this possibility, recombinant ST8SiaIV variants were generated and enzymatic activity was tested in vivo and in vitro. To decide on the importance of each of the two mutations found in clone 2D8, the amino acid exchanges were separately introduced into the wild type enzyme. The full-length ST8SiaIV cDNA cloned into the vector pcDNA3-Flag-HA was subject of site-directed mutagenesis. Mutations were verified by sequencing and cDNAs transiently expressed in 2A10 cells. 48 h after transfection, whole cell lysates were analyzed in Western blot with the mAb 735 to display PSA (35) and with mAb KD11 to

Table II
ST8SiaIV mutations identified in cells of the complementation group 2A10

| Mutant | Northern blot signal | RT-PCR | Nucleotide exchange(s) | Amino acid exchange(s) |
|--------|----------------------|--------|------------------------|------------------------|
| 2A10 + | + C103T              | Q35stop |
| 2D8 + | + A997G/G1067T       | M335V/ |
| 4C4 + | + G437A              | G146E  |
| 5C3 + | + G41A               | G146E  |
| 7F11 + | + G437A              | G146E  |
| 7G11 + | + G566A              | T189I  |
| S8F + | + ΔA247–G504         | ΔK83–R186 |
| 9C8 + | + G1006A             | E336K  |
| 9D8 + | + A529G              | R277G  |

Δ, deletion; amino acid residues are given in the single-letter code.

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display NCAM (36). As shown in Fig. 3A, 2A10 cells express the NCAM isoforms 140 and 180. NCAM bands are present at comparable concentrations in all samples and demonstrate equal loading of the gel. A broad microheterogeneous signal representing polysialylated NCAM could be detected in 2A10 cells transfected with wt Flag-HA-ST8SiaIV, and a very weak PSA signal was visible in cells transfected with the mutant R277G (see Fig. 3A). None of the other ST8SiaIV variants was able to reconstitute PSA expression in 2A10 cells. The two mutations observed in clone 2D8 independently inactivate ST8SiaIV (see M333V and C356F in Fig. 3), and therefore were investigated separately in subsequent experiments. To control the expression of recombinant Flag-HA-ST8SiaIV proteins, aliquots of the cell lysates were immunoprecipitated with anti-HA mAb 12CA5 and analyzed in Western blot with anti-Flag mAb M5. Wild type and mutant proteins were expressed, indicating that not absence of proteins but mutations in ST8SiaIV are responsible for the lack of PSA expression. Double or triplet bands visible in Fig. 3B represent nascent, incompletely glycosylated Flag-HA-ST8SiaIV proteins.

It is important to mention, at this point, that differences in the protein expression levels (e.g. see Fig. 3B and subsequent experiments) are due to the experimental system and varied between parallel experiments, but did not affect their results.

**Introduction of 2A10 Mutations into ST8SiaII**—In mammals a second polysialyltransferase, ST8SiaII, exists, which is closely related to ST8SiaIV at primary sequence level and with respect to catalytic functions (28–30). In order to test the functional consequences of 2A10 mutations in ST8SiaII, identical amino acid exchanges were carried out in the construct Flag-HA-ST8SiaII and mutants were tested for complementation in 2A10 cells. The results are summarized in Fig. 4. Wild type and mutant Flag-HA-ST8SiaII proteins were expressed (Fig. 4B), but only wt Flag-HA-ST8SiaII was able to reconstitute PSA expression in 2A10 cells (Fig. 4A). In contrast to ST8SiaIV-R277G, the corresponding mutant ST8SiaII-R292G was negative.

**In Vitro Rescue of ST8SiaIV Mutants R277G and M333V and of ST8SiaII Mutants R292G and M348V**—Recently we demonstrated that active polysialyltransferases exhibit a second catalytic function, termed autopolysialylation (31). Although the mechanism and the biological relevance of the autopolysialylation reaction are not understood, our earlier experiments carried out in vitro suggested a tight link between these two catalytic functions (31). Asking whether 2A10 mutations affect the autocatalytic activity, wild type and mutant polysialyltransferases were analyzed in vitro for their capacity to catalyze autopolysialylation. For this assay the anti-Myc mAb 9E10, covalently bound to protein G-Sepharose, was used to immunoisolate soluble, C-terminally Myc-His-tagged forms of the polysialyltransferases from the supernatants of transfected cells. The reaction was carried out with solid phase-fixed enzymes in the presence of the radioactive sugar donor substrate CMP-[14C]Neu5Ac. After a 2-h incubation, reactions were stopped and products displayed by SDS-PAGE and autoradiography.

Intensive, microheterogeneous radioactive signals became visible for the wild type polysialyltransferases and represent autopolysialylation (Figs. 5A and 6A). None of the mutants was able to perform autopolysialylation, albeit very faint and focused radioactive bands were discernible for ST8SiaIV mutants R277G and M333V (see Fig. 5A). The absence of radioactive PSA bands for the mutant ST8SiaIV-C356F can be explained by absence of secreted protein. All other variants were present in the supernatants of transfected 2A10 cells (Figs. 5C and 6C). It is worthwhile to mention that in all experiments secreted forms of the recombinant ST8SiaII proteins were found to be expressed at a higher level than the respective ST8SiaIV proteins. The difference is pronounced in the case of the mutants ST8SiaIV-C356F and ST8SiaII-C371F (compare Figs. 5B and 6B). However, if expression of recombinant proteins was analyzed inside transfected cells, ST8SiaIV-C356F was expressed at the same level as other proteins. The Western blots in Figs. 5D and 6D show Myc-His-tagged proteins immunoprecipitated with mAb 9E10 from whole cell lysates. The multiband patterns represent nascent incompletely glycosylated proteins.

In order to test NCAM polysialylation, the solid phase fixed enzyme fractions described above were used to extract a protein A-NCAM chimera (NCAM fused to the IgG-binding domain of protein A). In this way we achieved co-fixation of NCAM and polysialyltransferase on a solid surface. Reactions were started by adding CMP-[14C]Neu5Ac, and samples were analyzed by SDS-PAGE and autoradiography. Surprisingly, the arginine and methionine mutants (ST8SiaIV-R277G, ST8SiaIV-M333V, ST8SiaII-R292G, and ST8SiaII-M348V) regained catalytic activity. Although PSA synthesis was decreased compared with the wild type, treatment of the samples with PSA-specific endoneuraminidase NE (38) clearly documented the presence of PSA (Figs. 5B and 6B). Most important,
all polysialyltransferases that were able to add PSA to NCAM incorporated radioactive sugars also by themselves, indicating that NCAM polysialylation is accompanied by autopolysialylation. Signals representing self-modification are very faint in the case of the ST8SiaII mutants R292G and M348V (see Fig. 6B). All other mutants listed in Table II were negative for both aut- and NCAM polysialylation, as shown for ST8SiaIV-T189I and ST8SiaII-T204I.

Leucine in Position 333 of ST8SiaIV Reconstitutes Biological Activity—The position corresponding to amino acid 333 in ST8SiaIV is occupied by methionine in all α2,8-sialyltransferases except ST8SiaIII, where leucine is found in this position (see Fig. 2). According to sequence alignments, ST8SiaIII, which is active on glycolipids, is the closest relative of the polysialyltransferases (42). The biological role of this enzyme is, however, still unclear (for review, see Ref. 27). Since replacement of methionine 333 by valine abolished the activity of ST8SiaIV in vitro we analyzed the functional consequence of leucine in this position. Soluble Myc-His-tagged ST8SiaIV-M333L was generated and tested in comparison to the soluble forms of ST8SiaIV-M333V and wild type. Results are summarized in Fig. 7. Although ST8SiaIV-M333V was inactive, the mutant ST8SiaIV-M333L was able to complement the defect of 2A10 cells, albeit the PSA signal was reduced in comparison to the wild type enzyme (Fig. 7A). Under in vitro conditions, mutant M333L behaved like wild type in aut- and NCAM polysialylation (Fig. 7, B and C), whereas M333V showed the picture already observed in Fig. 5B. These findings suggest that size and sterical features of the methionine side chain but not the thioether function are important in this position.

Subcellular Targeting of Mutant Polysialyltransferases—Because activity in autopolysialylation-negative mutants could be partially restored in vitro via intimate contact to the PSA acceptor NCAM, we speculated that subcellular mistargeting may be responsible for their lack of complementation activity in 2A10 cells. The subcellular localization of mutant and wild type polysialyltransferases was, therefore, analyzed by indirect immunofluorescence in NIH3T3 cells transiently transfected with full-length N-terminally Flag-HA-tagged proteins. 30 h after transfection, cells were fixed in paraformaldehyde and permeabilized with Triton X-100. The localization of the Flag epitope was visualized with anti-Flag mAb M5. Simultaneously, the cells were stained with an antiserum directed against α-mannosidase II, a known marker for the Golgi apparatus (43). With the exception of ST8SiaIV-C356F, all ST8SiaIV mutants strictly co-localize with α-mannosidase II (see Fig. 8). The staining of the endoplasmatic reticulum (ER) observed for ST8SiaIV-C356F together with the fact that soluble forms of this protein were not secreted (see Fig. 5C) argues for a misfolded protein, which is retained in the ER. The analogous series of ST8SiaII variants gave identical results (data not shown). Although small amounts of the soluble form of ST8SiaII-C371F could be isolated from the supernatant (see Fig. 6C), the full-length protein was retained in the ER. The correct Golgi destination of autopolysialylation-negative mutants together with the observation that activity could be restored in the presence of NCAM provides evidence that these mutations influence the development of a catalytically active enzyme but do not cause gross misfolding of the protein.

Overexpression of Mutant Polysialyltransferases Does Not Influence the Activity of Wild Type Enzymes—In earlier studies Golgi retention has been demonstrated to depend on protein complex formation (44, 45) and active glycosyltransferases were described to assemble into functional complexes (for review, see Refs. 46 and 47). Assuming complex formation as essential for the assembly of active polysialyltransferases, we tested if overexpression of mutant proteins could induce a dominant negative phenotype. Co-transfection experiments were carried out using pFlag-HA-ST8SiaIV constructs. Constant amounts of wild type together with increasing concentrations of the mutant cDNAs were transiently transfected into PSA-negative 2A10 cells. The ratio wild type to mutant cDNA was varied up to 1:100. The total amount of cDNA was kept constant by supplementing a cDNA library isolated from CHO wild type cells.
Transfection efficiencies were controlled in the same experiment by adding a constant amount of β-galactosidase (48).

None of the mutants was able to significantly reduce PSA synthesis catalyzed by wt ST8SiaIV. PSA expression levels monitored in immunohistochemistry and Western blot analysis were independent from the concentration of the mutant proteins (data not shown). Possible explanations for these negative results are as follows. (i) The active unit of ST8SiaIV is the monomer. (ii) The intracellular concentration of mutant proteins was still too low to disrupt functional complexes. (iii) 2A10 mutants have lost their ability to organize in di- or oligomeric complexes.

FIG. 6. In vitro analysis of ST8SiaII mutants for auto- and NCAM polysialylation. Secreted C-terminally Myc-His-tagged forms of wild type and mutant ST8SiaII were used to investigate auto- and NCAM polysialylation in vitro. A, autopolysialylation was carried out with solid phase-bound enzymes in the presence of CMP-14C[Neu5Ac] samples were separated on 7% SDS-PAGE and developed by autoradiography. A radioactive signal representing autopolysialylation is present in the case of the wild type enzyme. Mutants are unable to perform autopolysialylation. B, bead-coupled enzymes were used to test PSA synthesis in the presence of protein A-NCAM and samples were separated on 7% SDS-PAGE. Radioactively labeled heterogeneous bands appear with wild type and with mutants R292G and M348V. The disperse appearance of radioactive bands is reduced after endoNE treatment (+). Automodified ST8SiaII is visible in the lower molecular weight range. Bands arising from self-modification are very faint in the case of mutants R292G and M348V. C, equal loading of enzyme carrying beads was controlled by Western blot analysis. Proteins were displayed with anti-Myc mAb 9E10. Mutant C371F is present at low concentration only. All the other proteins are expressed at wild type level. D, nascent ST8SiaII variants were precipitated from lysates of transfected cells and displayed in Western blot with anti-Myc mAb 9E10. Proteins are present at comparable levels inside the cells.

FIG. 7. ST8SiaIV-M333L is an active enzyme. A, Western blot analysis of 2A10 cells transiently transfected with soluble Myc-His-tagged forms of wt ST8SiaIV and the mutants M333V and M333L. Whole cell lysates were transferred to nitrocellulose membranes after separation on SDS-PAGE and stained simultaneously with the mAb 735 to display PSA and mAb KD11 to display NCAM. Mutant M333L reconstitutes PSA expression in 2A10 cells. NCAM bands of 180 and 140 kDa demonstrate equal loading of the gel. B, autoradiographic image of the autopolysialylation reaction. Autopolysialylation was tested with solid phase-bound enzymes isolated from the supernatant of the transiently transfected 2A10 cells shown in A. Whereas M333V is unable to perform autopolysialylation, the mutant M333L behaves like wild type in producing a broad radioactive band. C, autoradiographic image of NCAM polysialylation. The reaction was carried out with solid phase-fixed enzymes in the presence of protein A-NCAM. Samples were analyzed by SDS-PAGE and autoradiography before (−) and after (+) endoNE digestion. Broad heterogeneous radioactive signals representing an overlay of auto- and NCAM polysialylation are visible for the wild type enzyme and for the mutant M333L. Although ST8SiaIV-M333V regains activity under these assay conditions, the extent of PSA synthesis on NCAM and on the mutant enzyme itself is diminished. D, equal loading of enzyme-carrying beads was controlled by Western blot analysis. Proteins were displayed with anti-Myc mAb 9E10. E, nascent ST8SiaIV variants were precipitated from lysates of transfected cells and displayed in Western blot with anti-Myc mAb 9E10. All proteins are present inside the cells.
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Fig. 8. Intracellular localization of wild type and mutant ST8SiaIV. 2A10 cells on glass coverslips were transiently transfected with Flag-HA-tagged variants of full-length ST8SiaIV. 1 day after transfection, cells were fixed in paraformaldehyde and permeabilized with Triton X-100. Cells were then subjected to indirect immunofluorescence using anti-Flag mAb M5 and an antiserum against a-mannosidase II, a known marker for the Golgi apparatus. Bound primary antibodies were visualized with anti-mouse Ig-DTAF and anti-rabbit Ig-TRITC conjugates. The diffuse staining throughout the cytoplasm suggests ER localization for the mutant C356F. All other mutant proteins and the wild type co-localize with a-mannosidase II and demonstrate correct Golgi destination.

DISCUSSION

CHO cells of the complementation group 2A10 are PSA-negative due to mutations in the ST8SiaIV gene. 2A10 cells have been isolated from chemically mutated CHO-K1 cells and were used for complementation cloning of hamster ST8SiaIV (34). A panel of 31 2A10 clones, existing in our laboratory, served as a source to identify primary sequence elements of functional importance in ST8SiaIV. The majority of clones harbors defects interfering with ST8SiaIV gene expression, since Northern blot analysis and RT-PCR displayed mutated mRNAs in only nine clones. Seven clones contained single point mutations; six of those allow the translation of full-length mutated proteins. In clone 2D8 two mutations have been identified that both cause an inactivation of ST8SiaIV (M333V and C356F). The occurrence of a premature stop after threonine-34 and deletion of the amino acid stretch encoded by exon 3 inactivated ST8SiaIV in clones 2A10 and 8F8, respectively. An overview of all mutants is given in Table II.

Mutated STSiaIV mRNAs, with the single exception of clone 7G11, were found to be expressed at drastically reduced levels. This fact prompted us to investigate whether the PSA-negative phenotypes of 2A10 cells result from subthreshold expression of mutant proteins. ST8SiaIV and the corresponding ST8SiaII variants were generated as full-length and soluble proteins, and their enzymatic activities were tested in vivo and in vitro. Although mutation of the C-terminal cysteine (position 356 in ST8SiaIV; 371 in ST8SiaII) gave rise to unstable, ER-retained translation products, all other mutants were efficiently translated and correctly targeted to the Golgi apparatus or, in case of the soluble forms, secreted into the medium. However, even after overexpression, none of the mutant proteins was able to complement 2A10 cells.

Interestingly, all primary sequence positions found to be mutated in 2A10 clones are highly conserved in the subfamily of a2,8-sialyltransferases but to a lesser extent in other sialyltransferases (see Fig. 2). From this observation the possibility arises that some of the identified positions are involved in determining a2,8 linkage specificity. Experiments aimed at enlightening the functional role of the identified residues in more detail belong to work in progress in our laboratory. Glycine 281 in ST8SiaIV is part of the small sialylmotif and represents an invariant position in all sialyltransferases. The replacement by serine as found in clone 5C3 completely abolished the activity of both polysialyltransferases. In contrast, replacement of this glycine by alanine as recently described for ST6GalI (49) gave an active enzyme with $K_m$ values increased by factor of 3 for the two substrates. The contribution of this position to the catalytic process seems to be unequal in different sialyltransferases.

The exchange of cysteine to phenylalanine (ST8SiaIV-C356F and ST8SiaII-C371F) concerns an amino acid residue, which is found in a2,8-sialyltransferases only. In order to find out whether the size of the phenylalanine side chain per se is responsible for the deleterious effect, cysteine 356 was replaced by serine, a polar amino acid of the same size. A soluble form of the mutant protein was unstable and not secreted to the supernatant of transfected cells (data not shown). These data suggest that the thiol function is essential in this position. Additional work is required to find out whether cysteine 356 is involved in the formation of a disulfide bridge or participates in the catalytic reaction. Support for the latter assumption comes from an earlier study that by the use of thiol-directed alkylating reagents demonstrated that at least one cysteinyl residue is of critical importance for polysialylation (50). ST8SiaIV mutants R277G and M333V and the respective ST8SiaII mutants R292G and M348V were unable to complement the defect of 2A10 cells, but regained activity when assayed in vitro in the presence of NCAM. Parallel tests for autopolysialylation were negative. The close proximity between defective polysialyltransferases and NCAM under in vitro conditions was sufficient to restore activity. Therefore, we
hypothesize that primary sequence changes inhibit the development of active enzyme conformations. The intimate contact with the PSA acceptor NCAM (co-fixation of enzyme and acceptor on a solid surface) partially compensates the loss of conformational integrity. In the reconstituted systems both auto- and NCAM polysialylation were visible, rising evidence that the two activities are tightly or essentially linked (see Figs. 5B, 6B, and 7C). The experiments shown in Figs. 3A and 5A support this assumption. Mutant ST8SiaIV-R277G, which preserved a residual competence to perform self-modification (see Fig. 5A), was able to produce small amounts of PSA after overexpression in 2A10 cells (Fig. 3A).

Activity of ST8SiaIV-M333V could be rescued by replacing valine by leucine. Although methionine is slightly polar, it is mostly found in the hydrophobic core of a protein and often substitutes for leucine, isoleucine, or valine. The difference of M333L and M333V might be due to the fact that neither methionine nor leucine shows a branching at the β-carbon, whereas valine does. This could implicate steric hindrance leading to slight misfolding of the protein. Another interesting aspect is the position of methionine 333 between the two highly conserved amino acids of the very short (VS; see Fig. 2) sialyl-motif (51). From clone 9C8 we isolated a second mutant, ST8SiaIV-E336K, in which the VS motif is destroyed (see Table II). Biologic activity of both mutated polysialyltransferases is abolished, although the recombinant proteins are stable (Figs. 3B and 4B) and targeted to the Golgi apparatus (Fig. 8). Therefore, the mutation seems not to grossly affect folding but the catalytic activity. These data confirm the functional importance of the VS motif.

When we first reported on autopolysialylation (31), we suggested that the autocatalytic maturation step is a prerequisite for the formation of the active ST8SiaIV. Our argumentation was based on three observations. 1) Recombinant wt ST8SiaIV generated in 2A10 cells carries PSA and is an active enzyme. 2) Recombinant wt ST8SiaIV generated as an asialo-glycoprotein from CMP-sialic acid transport negative Lec2 cells (52) is able to perform autopolysialylation and is an active enzyme. 3) Recombinant wild type ST8SiaIV generated as an asialo-agalacto-glycoprotein from UDP-galactose transport negative Lec8 cells (53) is unable to perform autopolysialylation and unable to polysialylate NCAM. Here we describe missense mutations that drastically reduce (see Fig. 5; ST8SiaIV) or completely inactivate (see Fig. 6; ST8SiaII) the autopolysialylation capacity of the polysialyltransferases. Defects in the primary sequence do not seem to cause gross alterations in the proteins, because mutants are stable and correctly targeted to their subcellular sites. Moreover, the artificial contact between the mutant proteins and NCAM as established in the in vitro assay system was sufficient to restore activity. A potential explanation resides in the possibility that these 2A10 mutations cause the disorientation of N-glycan(s) that are involved in the process of autopolysialylation. Binding of NCAM seems to be sufficient to induce the active conformation.

An important question in this context is: which of the five and six potential N-glycosylation sites present in ST8SiaIV and ST8SiaII, respectively, are involved in the processes of auto- and NCAM polysialylation? A recent study by Colley and co-workers (33) on human ST8SiaIV identified the N-glycan bound to asparagine 74 as the major acceptor of autocatalytically synthesized PSA. The replacement of asparagine 74 by serine abolished autopolysialylation; however, NCAM polysialylation was retained, although at a very low level. Based on their results, the authors suggest independence of the two catalytic steps. A similar study2 carried out in our laboratory partially contradicts these results. Although we agree that the oligosaccharide bound to asparagine 74 is the major carrier of autocatalytically generated PSA, we see that the inactivation of this site is not sufficient to abolish autopolysialylation.

From earlier studies (28, 30), we know that PSA chains produced by ST8SiaIV are longer than those generated by ST8SiaII. This effect is obvious in Figs. 5 and 6. Polysialylated proteins resulting from ST8SiaIV catalysis shift to much higher molecular masses than those resulting from ST8SiaII activity. Together with the fact that autopolysialylation is reduced in the presence of NCAM (31), this explains the difficulty to detect and display autopolysialylation in ST8SiaII mutants. Nevertheless, weak but doubtful signals demonstrating self-modification have been confirmed in repeated experiments and prove the simultaneous occurrence of auto- and NCAM polysialylation.

The molecular analysis of the defects causing the functional inactivation of ST8SiaIV in 2A10 cells led to the identification of primary sequence elements that play key roles in the formation of catalytically active polysialyltransferases. The study validates mutational analyses as an efficient strategy toward enlightening structure-function relationships in situations where other structural data are not available. Because some of the identified positions are highly specific for o2,8-sialyltransferases, our data introduce a new basis for studies aimed at defining linkage specificity in this group of closely related enzymes.

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