A Human STX cDNA Confers Polysialic Acid Expression in Mammalian Cells*

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Polysialic acid, or PSA, is a term used to refer to linear homopolymers of α(2,8)-sialic acid residues displayed at the surface of some mammalian cells. PSA is typically linked to the neural cell adhesion molecule N-CAM, where it can modulate the homotypic adhesive properties of this polypeptide. PSA expression is developmentally regulated, presumably through mechanisms involving regulated expression of sialyltransferases involved in PSA biosynthesis. Several different sialyltransferase sequences have been implicated in PSA expression, although the precise roles of these enzymes in this context remain unclear. One such sequence, termed STX, maintains approximately 59% amino acid sequence identity with another sialyltransferase (PST-1, from hamster; PST, human) that is known to participate in PSA expression. While a murine STX fusion protein can catalyze the synthesis of a single α(2,8)-sialic acid linkage in vitro, the ability of STX to participate in PSA expression in vivo has not been demonstrated. We show here that STX transcripts are present in a PSA-positive, N-CAM-positive human small cell carcinoma line (NCI-H69/F3), but are absent in a variant of this line (NCI-H69/E2) selected to be PSA-negative and N-CAM-positive. To functionally confirm this correlation, we have cloned a human cDNA encoding the human STX sequence, and show, by transfection studies, that human STX can restore PSA expression when expressed in the PSA-negative, N-CAM-positive small cell carcinoma variant. We furthermore show that STX can confer PSA expression when expressed in a PSA-negative, N-CAM-positive murine cell line (NIH-3T3 cells), or when expressed in PSA-negative, N-CAM-negative COS-7 cells. These observations imply that STX, like PST-1/PST, can determine PSA expression in vivo. When considered together with the correlation between STX expression and PSA expression in vivo in the brain, these results suggest a regulatory role for STX in PSA expression in the developing central nervous system and small cell lung carcinoma.

Sialyltransferases represent a family of terminal glycosyltransferases that catalyze the attachment of sialic acid to carbohydrates of many glycoproteins and glycolipids (1). Sialic acids are key determinants of carbohydrate structures involved in a variety of biological processes and are widely distributed on many cell types (2-4). Homopolymers of sialic acids in α(2,8) linkage (polyasialic acid, PSA) also abbreviated as polysia) have a more restricted spatio-temporal tissue distribution pattern than the more commonly found α(2,6)- and α(2,3)-linked sialic acid residues. For example, PSA is expressed by neuronal tissues, in the heart and the developing kidney, and in association with malignant transformation, such as in small cell lung carcinoma (SCLC) (5-8). Furthermore, PSA has been reported to be associated with only two proteins, the neural cell adhesion molecule (N-CAM) and sodium channel α subunits (9, 10). Changes in the amount of PSA on N-CAM modulates the adhesive properties of N-CAM, and also affects the cell surface properties of other molecules like some integrins, N-cadherin, and G4/NcCAM (11-14). These effects are presumed to be due to the unusual physicochemical properties of this very large, abundant, negatively charged, and linear cell surface poliglycan (14).

A requirement for N-CAM as an acceptor molecule in PSA synthesis is implied from several studies (15, 16), although this has not been demonstrated directly. Other studies suggest that PSA biosynthesis involves the concerted activity of two or more specific sialyltransferases (16, 17). This includes a requirement for one or more α(2,3)-sialyltransferases to create α(2,3)-linked sialic acid moieties, that in turn are the presumed acceptor substrate for subsequent addition of α(2,8)-linked sialic acid moieties (18, 19). It is possible that PSA synthesis then proceeds through a two-step process involving the addition of a single α(2,8)-linked sialic acid residue to the α(2,3)-linked sialic acid by one distinct α(2,8)-sialyltransferase (an “initiase” reaction), followed by the addition of multiple α(2,8)-linked sialic acid residues that yield PSA by a second distinct α(2,8)-sialyltransferase (a “polymerase” reaction). This possibility is supported by in vitro experiments indicating that at least three different α(2,8)-sialyltransferases (ST8Sial, GD3 synthase, Refs. 20-22; ST8Sial, STX, Ref. 23; ST8SialI, Ref. 24) can catalyze the attachment of a single α(2,8)-linked sialic acid residue to terminal α(2,3)-sialic acid linkages.

Alternatively, a single α(2,8)-sialyltransferase may operate to directly catalyze PSA synthesis on a glycoconjugate template containing a terminal α(2,3)-linked sialic acid. This notion is supported by the recent demonstration that expression of a single α(2,8)-sialyltransferase gene, termed PST-1 (25), or PST (26), is sufficient for the expression of PSA in N-CAM-positive, PSA-negative mammalian cell lines (CHO-2A10, NIH-3T3, and COS-HN-6, a COS cell line expressing with human N-CAM-140 cDNA, Ref. 25; COS-1 cells and HeLa cells transfected with an

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U33551.

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1 The abbreviations used are: PSA, polysialic acid; bp, base pair(s); SCLC, small cell lung carcinoma; N-CAM, neural cell adhesion molecule; RACE, rapid amplification of cDNA ends; kb, kilobase(s); mAb, monoclonal antibody.
N-CAM expression vector, Ref. 26). In principle, both such modes of PSA synthesis may exist, although this remains to be confirmed, since it has not been possible to detect a PSA expression vector that contains a 5'-linked sialic acid in vitro.

The STX gene represents a member of the sialyltransferase gene family whose developmentally regulated expression patterns correlate well with PSA expression in certain tumors (L, 24, 27). A role for this sequence in PSA expression remained circumstantial, however, since initial efforts failed to demonstrate an enzymatic activity associated with the (rat) STX polypeptide (27). Subsequent efforts have demonstrated that a recombinant (mouse) STX protein can catalyze the synthesis of a single- or 2,8-linked sialic acid linkage in vitro (23). Nonetheless, a definitive demonstration that STX participates in PSA expression, in vitro or in vivo, has not been accomplished.

We show here that STX expression correlates with PSA expression in a PSA-negative human small cell lung carcinoma (SCLC) cell line (NCI-H69/F3) (8), whereas STX transcripts are absent from a variant of this line selected to be PSA-negative (NCI-H69/E2) (8). Transfection of the PSA-negative variant SCLC line with an STX expression vector restores PSA expression in that line, and in other PSA-negative cell lines. The observations imply that transcriptional regulation of STX can regulate PSA expression, suggesting that STX shares overlapping enzymatic activity with PST-1/PSST, and imply that determination of PSA expression by STX can be independent of N-CAM expression.

**EXPERIMENTAL PROCEDURES**

Cell Lines—Two N-CAM-positive, non-SCLC sublines differing in PSA expression (NCI-H69/F3 and NCI-H69/E2) have been described (8). NIH-3T3 and COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in Eagle's medium containing 10% fetal calf serum with penicillin-streptomycin. Molecular Cloning of a Full-length Human STX cDNA—A cDNA library was constructed using the mammalian expression vector pCDM8 (Invitrogen) and poly(A)* RNA from the PSA-positive cell line (NCI-H69/F3), according to Ref. 26. A 580-bp fragment corresponding to bp 69–649 of a human STX cDNA (1) (5' primer, GCCCAAGCTTGTGCTACTAGGT; 3' primer, GCCGCGAGCTCAGTTTCAAGGCC), the NCI-H69/F3 cell cDNA library as a template, and standard PCR conditions (Taq polymerase; 95°C for 30 s, 65°C for 30 s and 72°C for 90 s, 35 cycles). The resulting 580-bp PCR product was subcloned into pCR® II (Invitrogen) and sequenced. The insert was subcloned into pCDNAI (Invitrogen), and C-terminal and N-terminal inserts were subcloned into pCDNAI-CAT (Invitrogen), and subsequently assaying extracts prepared from the transfected cells for chloramphenicol acetyltransferase activity (29). Immunochemical Analysis—Transfected cells were analyzed by fluorescence-activated flow cytometry (33). Cells were stained and washed at 4°C in staining medium (RPMI 1640 medium, 2% fetal calf serum, 0.1 mM HEPES, pH = 7.4). PSA expression was assessed with the mouse monoclonal anti-PSA antibody mAb 735 (used at 5 μg/ml) (34), from Dr. B. Bitter-Suermann (Institute of Medical Microbiology, Mainz, Germany). Rabbit anti-human N-CAM antisemur was used at a saturating concentration (Dako). Cell-bound antibodies were detected with fluorescein-conjugated goat anti-mouse or anti-rabbit antibodies (Sigma).

**RESULTS AND DISCUSSION**

Recent molecular cloning work demonstrates that a sialyltransferase sequence termed PST-1, or PST, (for polysialyltransferase) can yield cell surface PSA expression when expressed in PSA-negative cell lines (25, 26). Although these data implicate PST-1/PST in PSA expression, an in vitro enzymatic correlation for these observations is not yet available. Among all known sialyltransferases, PST-1/PST is most similar to one termed STX (59% overall amino acid sequence identity; hamster PST-1 or human PST versus rat STX). STX is expressed primarily in the fetal and newborn brain, but not in the adult brain, and thus correlates with the temporal sequence of PSA expression in that tissue. These considerations suggest a role for STX in the developmental regulation of PSA expression in the central nervous system. However, efforts to assign an enzymatic activity to the rat STX sequence have not met with success (27). More recent work indicates that, in vitro, mouse STX can utilize radiolabeled CMP-sialic acid to incorporate radiolabeled sialic acid into sialylated, N-linked glycoproteins (23). Indirect evidence suggests that α(2,8)-sialic acid linkages were formed by STX in these experiments, although there is currently no direct evidence that STX participates in PSA biosynthesis or expression.

**Differential Expression of Human STX in SCLC Cell Lines**—We have described an N-CAM-positive, PSA-positive small cell lung carcinoma cell line (NCI-H69/F3), and the isolation and characterization of clonal variants of this line that remain N-CAM-positive, but that are deficient in PSA expression (8). One such PSA-negative line (NCI-H69/E2) and the PSA-positive control line NCI-H69/F3 were used to explore the role of PST-1 and PST in PSA expression (8). Flow cytometry analyses confirm that the NCI-H69/F3 line expresses N-CAM and PSA, whereas the variant line NCI-H69/E2 is deficient in cell surface PSA expression, yet remains positive for N-CAM expression (Fig. 1A). The major carrier of PSA in the NCI-H69/E3 line is N-CAM, as reported previously (8).

Northern blot analyses indicate that the PSA-positive line NCI-H69/F3 is deficient in transcripts corresponding to PST-1/PST (although PST-1 transcripts are observed in human heart; Fig. 2B), indicating that this gene does not participate in PSA expression in these cells. Likewise, this cell line is deficient in GD3 synthase and mST8Sia III transcripts (data not shown). However, these analyses demonstrate that the STX expression could be independent of N-CAM expression. Nonetheless, a definitive demonstration that STX participates in PSA expression, in vitro or in vivo, has not been accomplished.
transcript is easily detectable in the PSA-positive line (Fig. 2A).

The STX transcript in the PSA-positive SCLC line (Fig. 2A) is similar in size (~6.0 kb) to the human (5.7 kb, human heart; data not shown and Ref. 1) and rat (5.5 kb, Ref. 27) STX transcripts and corresponds roughly to broad range (1.7–6.7 kb) of STX transcripts observed in the embryonic mouse (24). By contrast, STX transcripts are absent from the PSA-negative variant SCLC line (Fig. 2A). These observations suggest that PSA expression in this pair of cell lines is controlled by transcriptional regulation of the STX gene.

Primary Structure of a Human STX cDNA—We sought to confirm an essential role for STX in PSA expression in these cell lines by expressing the STX polypeptide in the PSA-negative line, and asking if this would convert the variant to a PSA-positive state. A human cDNA encompassing the entire coding region of the human STX polypeptide was therefore isolated and characterized (see "Experimental Procedures").

The STXFL clone maintains a continuous 375-amino acid open reading frame (Fig. 3) encompassing segments termed sialylmotifs, which are highly conserved among members of the mammalian sialyltransferase superfamily (27). The human STX protein maintains a 17-amino acid hydrophobic sequence near its NH₂ terminus. This segment is flanked by charged residues, and thus corresponds to a "signal anchor" sequence predicting a type II transmembrane orientation characteristic of glycosyltransferases (35). Human STX differs from the rat enzyme at 8 positions (rat residues Ala-68, Leu-75, Ser-78, Gln-155, Thr-188, Gly-232, Ala-257, and Asn-308), for an overall 98% amino acid sequence identity between the rat and human enzymes (1). Similarity to the mouse STX sequence cannot be determined since its DNA and derived protein sequence are not available (23). Human STX shares an overall amino acid sequence identity of 59% with hamster PST-1 human PST (25, 26), 31% with human GD3 synthase (20), and 36% with mouse STBα III (24), three other mammalian sialyltransferases implicated in the synthesis of α(2,8)-linked sialic acid linkages. There are local regions of sequence identity among these enzymes that are substantially higher than these values, including the region encompassing the L and S sialylmotifs (Fig. 3) (data not shown).

Expression of PSA in Cell Lines Transiently Transfected with the Human STX cDNA—To determine if human STX can restore PSA expression to the PSA-negative, STX-negative SCLC variant, the human STX cDNA was installed in a mammalian expression vector and was introduced into these cells by transfection (see "Experimental Procedures"). A substantial fraction (~30%) of the STX-transfected variant cells stain brightly (Fig. 1B) with the monoclonal antibody (mAb 735) specific for PSA (34). The fraction of positive cells observed in this experiment is similar to the fraction of antigen (sialyl Lewis x)-positive cells seen when (sialyl Lewis x-negative) NCI-H69/E2 cells are transiently transfected with a control fucosyltransferase expression vector (pCDNAI-Fuc-TII; Ref. 33) (data not shown). By contrast, the PSA-negative line remains PSA-negative following transfection with the control vector pCDNAI (Fig. 1B), as do cells transfected with the STX expression vector and stained with a negative control antibody (Fig. 1B). These results indicate that the human STX sequence can confer PSA expression in this N-CAM-positive cell line and are consistent with the conclusion that loss of PSA expression in this variant is a consequence of virtually complete loss of STX transcripts by consequence of virtually complete loss of STX transcripts by
Effective PSA synthesis and expression requires N-CAM, H69/F3 (8). These observations have been taken to mean that has been directly demonstrated on the SCLC cell line NCI-3. The catalytic specificity of human STX overlaps with that of isnotidiosyncratic to the SCLC variant line and suggests that only (adds sialic acid in nonamer-2,3) and the transfectants were assayed for PSA expression. More than 25% of the STX-transfected COS-7 cells stained with the predicted amino acid sequence. The predicted signal anchor sequence is underlined.

Fig. 3. Human STX cDNA and predicted amino acid sequences. The DNA sequence of the coding region of the human STX is shown above the predicted amino acid sequence. The potential asparagine-linked glycosylation sites are underlined. Amino acids underlined with a dotted line correspond to consensus sequences identified previously for mammalian sialyltransferases (the “L” and “S” sialylmotifs; Refs. 1 and 20).

is not idiosyncratic to the SCLC variant line and suggests that the catalytic specificity of human STX overlaps with that of hamster PST-1, which is also able to direct PSA expression in the absence of N-CAM expression. PSA expression in the absence of N-CAM expression.

The nature of the glycoconjugates that display STX-determined PSA expression on the cell lines we have used will require detailed structural analysis. Our experiments also do not allow us to know if STX participates in PSA expression as an “initiase” only (adds sialic acid in α,2,8 linkage to an α,2,3-linked sialic acid precursor), or as a “polymerase” only (adds sialic acid in α,2,8 linkage to an α,2,8-linked sialic acid precursor), or if both of these reactions are catalyzed by STX. A full biochemical resolution of this question, as it relates to STX, and to the other α,2,8-sialyltransferases implicated in PSA expression, will require the development of an in vitro assay for PSA synthesis.

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