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Overexpression of α2,3 sialyltransferase in neuroblastoma cells results in an upset in the glycosylation process

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Glycosylation is key posttranslational modification for membrane-bound and secreted proteins that can influence both the secondary structure and the function of the protein backbone. In order to investigate the effect of altered cellular glycosylation potential, we have generated a number of clonal cell lines over-expressing the α2,3(N) sialyltransferase enzyme (ST3N). In general, there was a decrease in total sialyltransferase (ST) enzyme activity in the clones transfected with the ST3N cDNA, with this decrease being inversely proportional to the quantity of the mRNA coding for the enzyme. The ST3N enzyme was, however, functional and there was an increase in both MAA lectin staining and the expression of polysialic acid, which is attached to the NCAM protein backbone primarily via an α2,3 linkage. These results suggest that the overexpression of a sialyltransferase may upset the sialylation potential of the cell.

Keywords: **Sialyltransferase, glycosylation, polysialic acid, clonal cell lines**

Abbreviations: **PSA, polysialic acid; ST, sialyltransferase; ST6N, α2,6(N) sialyltransferase; ST3N, α2,3(N) sialyltransferase; NCAM, neural cell adhesion molecule; PST and STX, designation for two cloned genes encoding α2,8 polysialyltransferase activities**

Introduction

Glycosylation is a key posttranslational modification that can modulate the structure and function of the protein backbone. Specifically, the negatively charged sialic acid (NeuNAc) residue has a particular influence on the protein [1] and in the nervous system, for example, it has been demonstrated to influence diverse functions including neurotransmitter receptor function [2] and cell–cell interaction [1]. The transfer of NeuNAc residues to oligosaccharide chains is catalysed by the sialyltransferase (ST) family of enzymes [3]. NeuNAc is attached to the penultimate galactose residues of N-linked oligosaccharides in two primary linkages: α2,3 and α2,6. The α2,3 linkage is the predominant form in the nervous system while the α2,6 linkage is found primarily in peripheral tissues such as liver and kidney [4].

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NeuNAc can also be linked in α2.8 homomeric linkages to form a polysialic acid (PSA) chain [1]. PSA is expressed predominantly on the neural cell adhesion (NCAM) protein where it serves to influence the strength of cell–cell interaction [5]. During the embryonic stage of development, the expression level of PSA is high, thus preventing the premature formation of synaptic connections. At a time coincident with synapse formation, there is a precipitous decrease in PSA expression, thus permitting NCAM-NCAM homophilic binding. PSA is also re-expressed coincident with plastic changes in the CNS when there may be modifications of synaptic connections [6–9].

Because of its pivotal role, particularly in nervous system function, it is important to understand the factors that control ST enzyme activity and the consequent NeuNAc expression levels. Previous studies have demonstrated that the control of both ST expression and activity is multifactorial. Corticosteroids exert a tissue-specific regulatory effect on ST activities both in neuronal cells [4,10] in addition to other tissues [11,12]. Other factors that modulate ST activities include

heavy metals [13], divalent cations [14], enzyme glycosylation [15,16] and second messenger system activation [17–20].

At the level of the Golgi, there is a complex interaction between individual glycosyltransferase enzymes. There is, for example, a competition between the two ST enzymes (ST3N and ST6N) that transfer NeuNAc to the penultimate galactose residue of an N-linked oligosaccharide chain [21]. An increase in ST6N activity results in an up-regulation of α2,6-linked NeuNAc and a parallel decrease in α2,3-linked sugar [21,22]. The expression levels of these two STN enzymes also influences the expression of the PSA epitope [1] which can be attached to the core N-linked oligosaccharide, primarily via an α2,3-linked NeuNAc residue [23–25]. In the present study, we have generated stably transfected rat neuroblastoma cell lines in order to investigate the effect of over-expression of the ST3N enzyme on (i) the pattern of cellular sialoglycoprotein (SGP) expression and (ii) the expression of the PSA epitope.

Materials and methods

Materials

The B104 rat neuroblastoma cell line [26] was a kind gift from Prof. Konrad Sandhoff, University of Bonn. The anti-NCAM antiserum was purchased from Affiniti Ltd., U.K., the anti-ST3N antibody was a kind gift from Prof. Eric Berger, University of Zurich and the anti-PSA antibody was generated from the 5A5 hybridoma cell line (NICHD).

Methods

The B104 rat neuroblastoma cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin (2 U/ml) and streptomycin (0.25 mg/ml) and maintained at 37°C in a humidified atmosphere containing 9% CO_2 as previously described [27]. The cells were routinely harvested every 3–4 days upon reaching confluency and seeded at a density of 2.2×10^6 cells per 75 cm^2 tissue culture flask. Two individual ST3N cDNA clones were used in the study. The rat ST3Gal III (ST3N) cDNA, which was a kind gift of Prof. Eric Berger (University of Zurich), was expressed in the pcDNA I plasmid [28]. Because this construct did not contain any antibiotic resistance markers, the cells were co-transfected with a trace amount of the pcDNA III plasmid (Stratagene) that codes for the neomycin resistance gene [27]. A second ST3Gal-III clone (in pcDNA III) was a gift of Prof. S. Tsuji (Wako, Japan) [29]. The B104 cells were transfected using the calcium chloride technique as previously described [27]. Briefly, the cells were plated at 5×10^3 cells/cm^2 in a 90 mm petri dish. Twenty-four hours post plating, up to 50 μg of the ST3N or ST3N-III plasmids were added to the cells. For transient transfection, the cells were harvested 24–48 hours after the addition of the cDNA. For stable transfection with the ST3N cDNA clone, the cells were washed three times with serum-free DMEM and incubated in full medium containing geneticin (800 μg/ml) 24 hours after the addition of the plasmid. After 7–10 days, individual transfected colonies were evident and were picked up for sub-cloning. For biochemical analysis, the clones were cultured to confluency and harvested by scraping in phosphate-buffered saline (PBS) and the cell pellets stored at −20°C prior to use.

Sialyltransferase activity

Total ST activity in the cell lysates was determined as previously described using cytidine-5-monophosphate-4,5,6,7,8,9-^{14}C-N-acetylneuraminic acid (CMP-^{14}C-Neu5Ac, Radiochemical Centre, Amersham; specific activity 293 mCi/mmol) as the sialic acid donor and asialofetuin (ASF; Sigma) as an exogenous acceptor [30].

In vitro transcription and translation of the ST3N cDNA clones

In vitro transcription and translation reactions were carried out using components supplied by the TNT T7 Quick-coupled Transcription/Translation System (Promega). Briefly, 1 μg of DNA template was transcribed and translated with 40 μl of TNT T7 Quick Master mix and 2 μl (20 μCi) of [^{35}S] methionine (Translation grade: at 100 Ci/mmol). The reaction was carried out at 30°C for 90 min. The DNA templates used were either the pcDNA I or the pcDNA III plasmids, both of which encoded for the ST3N cDNA gene and contained the T7 promoter. Following completion of the reaction, 20 μl of the translation reaction was removed and the protein components separated by one-dimensional SDS-Polyacrylamide gel electrophoresis using a 10% running gel and a 5% stacking gel. The [^{35}S] methionine labelled proteins were visualised by fluorography using the ENHANCE solution (DuPont).

Protein analysis

The protein content of the harvested cells was determined using the Folin phenol reagent [31]. The individual polypeptide components of the samples were separated by discontinuous SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore) by electroblotting. Sialoglycoprotein expression was analysed by lectin affinity blot analysis (DIG glycan differentiation kit, Boehringer Mannheim) and the NCAM and PSA epitopes were determined by Western blot analysis using an anti-NCAM polyclonal antiserum and the 5A5 anti-PSA monoclonal antibody respectively. The blots were scanned using a Hewlett Packard 6100C flatbed scanner and densitometric analysis was carried out using the NIH image software.

Immunocytochemistry

Cells were plated on 18 mm glass coverslips at a density of 2.7×10^4 cells/cm^2. After washing with PBS, the cells were fixed for 10 min in 3.5% (w/v) paraformaldehyde in Hank's buffered salt solution (HBSS) and blocked for 10 min in 20 mM glycine in PBS. The cells were then permeabilised for 30 min in 0.1% (w/v) saponin in PBS prior to their incubation

in primary antibody in sap/PBS (1:100 dilution). After washing, the cells were incubated for 30 min in FITC-labelled anti-rabbit antibody (Sigma) (1:80 dilution) and visualised, after washing and mounting, by immunofluorescence microscopy using a Zeiss LSM510 Axioplan 2 laser scanning confocal microscope.

Semi-quantitative analysis of cellular mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from B104 cells using the TRIZOL reagent (Gibco BRL) which is a monophasic solution of phenol and guanidine isothiocyanate. The RNA concentration was determined by spectrophotometry. Specific primers, which flank a unique region of the ST3N cDNA, were used for the first strand cDNA synthesis and the subsequent PCR amplification steps. The ST3N cDNA sequence was obtained from the NCBI entrez database and the following primers were designed: primer-1: 5′-GAC ACT GCG CAT CAC CTA-3′ (Bases 609–626); primer-2: 5′-CAT GAG GCC ATT GTT GAA-3′ (Bases 882–864) and primer-3: 5′-CTC GTC ACA GCC GTG TAG-3′ (Bases 948–931).

The first strand cDNA synthesis from the mRNA was carried out using the Superscript II system (GIBCO BRL) using 2 μg of total RNA from cells and primer-3. The resulting cDNA was stored at 4°C and subsequently served as a template for amplification by PCR, which was first denatured for 5 min at 95°C. The PCR reaction was carried out using the Ampli-Taq DNA Polymerase system (Perkin Elmer) and primers 1 and 2. The PCR reaction was incubated for 2 min at 80°C, which is the hot start step, and was permitted to continue for 35 cycles (95°C for 20 sec, 60°C for 45 sec and 70°C for 45 sec). A final extension step was also carried out (70°C for 7 min). The PCR products were separated by agarose gel electrophoresis and visualised by staining with ethidium bromide. The gel was scanned using a Glyco FACE imager and the band density analysed using the NIH image software.

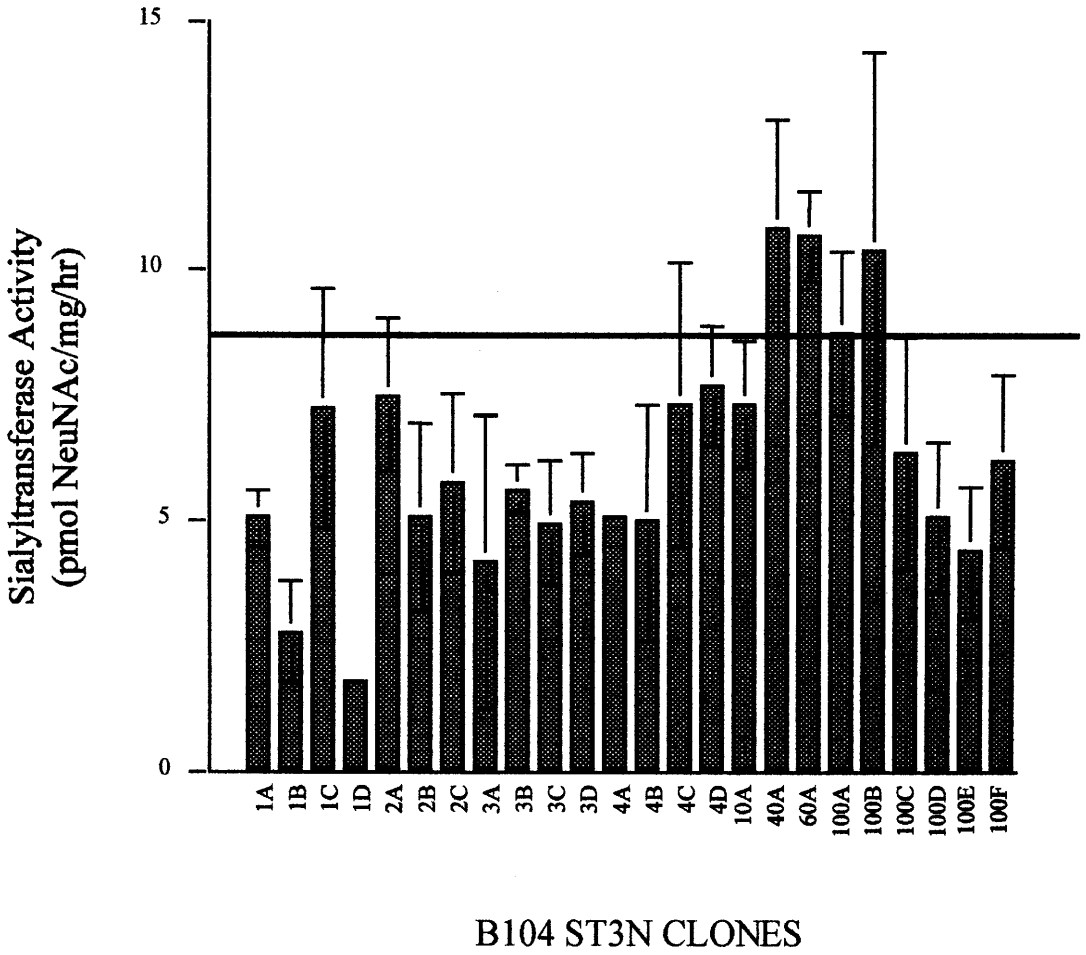

Figure 1. Total ST activities of B104 rat neuroblastoma cells transfected with cDNA coding for the ST3N enzyme. The horizontal line represents the activity of the control (untransfected) cells.

Results

In general, there was a decrease in total cellular sialyltransferase (ST) activity in the B104 rat neuroblastoma clones transfected with the ST3N cDNA, although there was some variability in the enzyme activities in individual clones (Fig. 1). In order to confirm that this effect was not clonal, cells were transiently transfected separately with two individual ST3N cDNA clones obtained from different sources. In both cases, there was an inverse dose-response relationship with a decrease in total ST activity corresponding to an increase in ST3N cDNA transfected into the cells (Fig. 2). The ability of the clones to generate a full-length protein was confirmed by an *in vitro* transcription and translation reaction that generated a protein of 35 kDa, which is the predicted size of the enzyme protein (Fig. 3). The inverse relationship between the expression of the ST3N enzyme and total ST activity was confirmed by semi-quantitative PCR analysis of ST3N mRNA levels with an increase in ST3N mRNA levels in those cell lines with decreased enzyme activity (Fig. 4). This relationship was highly significant ($p = 0.009$) as determined by linear regression analysis. There was, however, no change in the expression level of the ST6N enzyme within the cell (data not shown).

The expression pattern of the ST3N enzyme protein was examined in the control and transfected cells by immunocytochemical analysis. In the control (untransfected) B104 cells, there was significant nuclear and perinuclear staining with homogenous staining throughout this region (Fig. 5a). In the ST3N transfects, however, the enzyme appeared to be redistributed with the staining being of a more punctate nature and this located in discrete perinuclear regions (Fig. 5b). The antibody to ST3N used in the study was not suitable for Western blot analysis so it was not possible to quantitate the total amount of enzyme protein expressed in the cells.

There was a slight increase in the level of expression of α2,3-linked sialoglycoproteins in the clonal cell lines, as detected by MAA lectin staining, with this increase being inversely related to the total cellular ST activity (Fig. 6). This increase was, however, in good agreement with the change

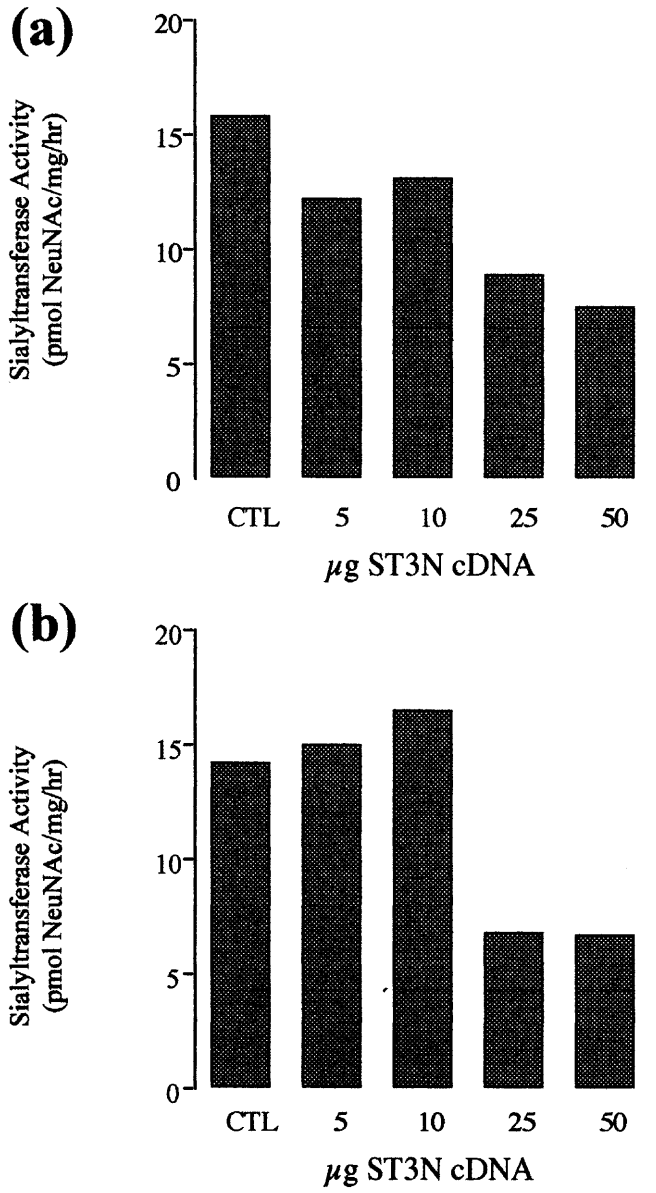

Figure 2. Total cellular ST activity following the transient transfection of B104 rat neuroblastoma cells with increasing concentrations of (a) ST3N and (b) ST3N III cDNA clones.

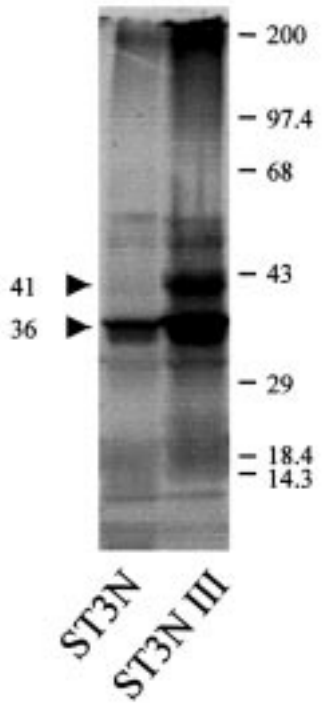

Figure 3. *In vitro* transcription and translation of the ST3N and ST3N III cDNA clones. The proteins were labelled with [^{35}S] methionine and visualised by fluorography. The migration of the molecular weight markers is indicated.

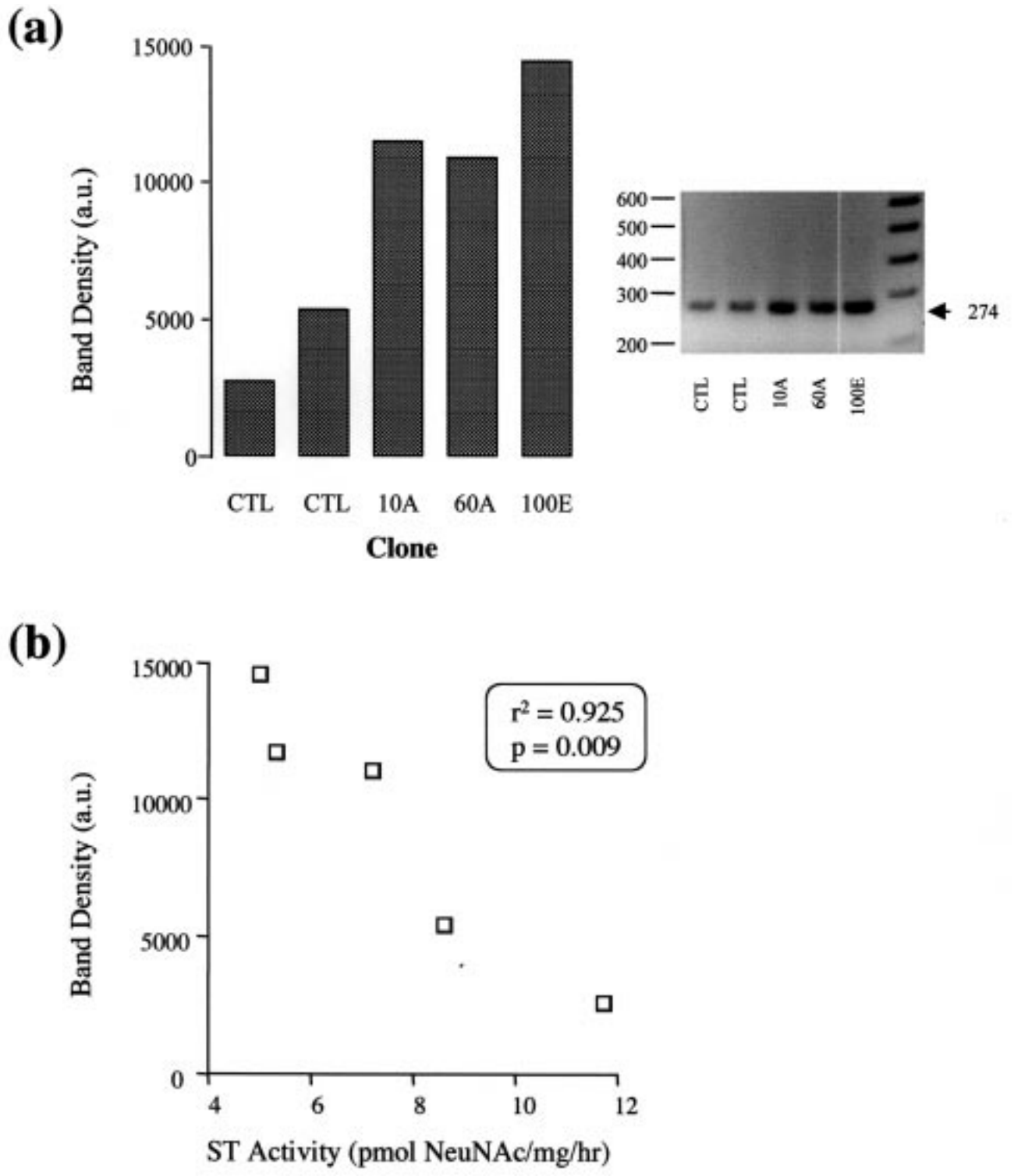

Figure 4. (a) Semi-quantitative PCR analysis of ST3N mRNA levels in control and transfected B104 clones. (b) Inverse relationship between ST3N mRNA levels and total cellular ST activity. $r^2 = 0.925$, $p = 0.009$.

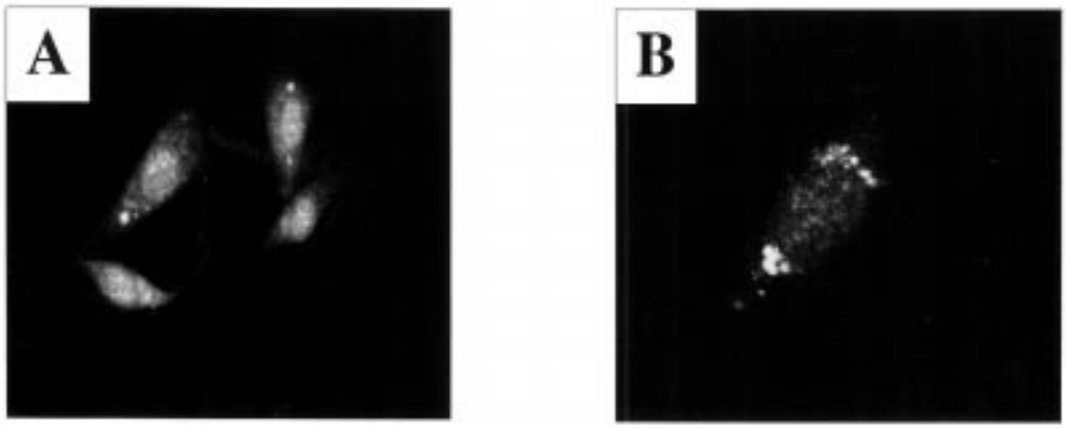

Figure 5. Immunocytochemical analysis of (A) control and (B) ST3N-transfected B104 rat neuroblastoma cells using an anti-ST3N antiserum.

observed in ST3N mRNA levels (Fig. 4). There was no change, however, in the expression pattern of α2,6 sialoglycoproteins as determined by SNA lectin blot analysis. These changes in the MAA lectin blot suggest that the general glycosylation machinery is still intact in the transfected cells.

The expression of the polysialic acid (PSA) oligosaccharide chain of the neural cell adhesion molecule (NCAM) was investigated. Previous studies have shown that both the ST3N and ST6N enzymes, which catalyse the transfer of the core sialic acid residue of the PSA chain, may play a vital role in controlling PSA expression [21,25]. The level of expression of the PSA epitope in the transfected cells was in good agreement with the expression of α2,3 sialoglycoproteins and with ST3N enzyme levels (Fig. 7a). This was, however, inversely proportional to total ST activity, although there were no changes in the level of expression of the NCAM protein backbone in the transfected cells (Fig. 7b).

Discussion

The expression and subcellular distribution of individual glycosyltransferase enzymes within the cell determines the glycosylation states of proteins as they pass through the ER and Golgi following translation. As the protein glycosylation state may have a particular effect on both the function as well as the processing of the protein, an upset in the general glycosylation process may therefore have widespread implications for the general glycoprotein biosynthetic pathway [1,32]. This study reports an upset in the activities of total cellular STN activity following transfection with cDNAs cloning for the ST3N enzyme with this decrease being inversely related to the level of mRNA expression levels (Fig. 4). While the decrease in enzyme activity is modest, it is in fact the absence of an increase in activity that is the most striking feature of the cells. Previous studies in both our lab and in others have all reported an increase in ST activity following the overexpression of a specific STN enzyme [21,33]. Therefore, it could be postulated that overexpression of ST3N in the B104 cell line may interfere with the general sialylation.

Neuronally-derived cells have previously been reported to express high levels of the ST3N enzyme [4]. Previous studies in this lab have demonstrated that transfection of B104 rat or SHSY-5Y human neuroblastoma cell lines with the ST6N enzyme resulted in a 40-fold increase in enzyme activity [21]. However, the low basal levels of the ST6N enzyme in the cells may account for this increase in enzyme expression and activity. As the B104 cells express a high basal level of ST3N, a further increase in expression levels may actually act to upset the homeostasis of protein expression within the *trans*-Golgi and TGN with a potential disruption in the subcellular localisation of the enzyme (Fig. 5). As the correct location of the enzyme within the Golgi and TGN determines its function, an upset in this localisation may have a particular influence on the cellular protein glycosylation state. In fact, previous studies have demonstrated that treatment of cells with GalNAc-α-*O*-benzyl, which inhibits sialylation, resulted in an upset in the intracellular protein transport in HT-29 human colon carcinoma cell line [34]. Furthermore, the overexpression of ST6N in glioma cells, which results in an abnormal sialylation of membrane-bound integrin proteins, may be associated with an altered positioning of the enzyme in the oligosaccharide biosynthetic pathway [35].

There was an increase in ST3N enzyme mRNA levels in the transfected cell lines, as determined by semi-quantitative PCR analysis, with an inverse relationship between mRNA levels and total enzyme activity. Similar results have been reported from studies with α1,3-galactosyltransferase where an increase in enzyme mRNA levels, following treatment of cells with inflammatory cytokines, resulted in a decrease in enzyme specific activity. This could be attributed to a decrease in the stability of the protein or the induction of different isoforms of the enzyme with an altered functionality [36]. The subcellular localisation of the enzyme has a particular influence on its functional capacity [37] and the glycosylation process is

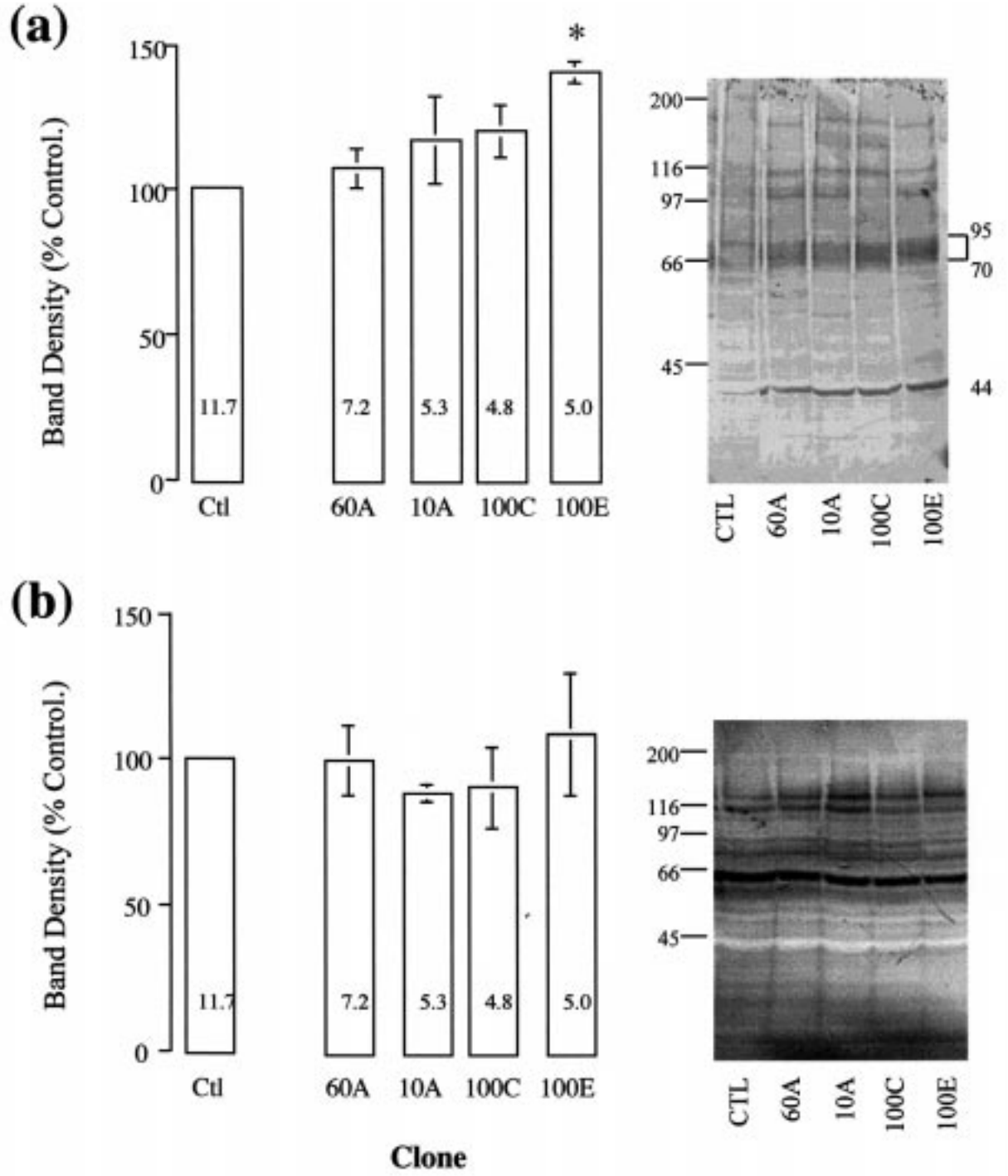

Figure 6. (a) MAA and (b) SNA lectin blot analysis of 50 μg samples of control and ST3N-transfected B104 cells. The migration of molecular weight standards (200, 116, 97, 66 & 45 kDa) is indicated. Control cells are in the left-hand lanes with clones of decreasing enzyme activity from left to right (enzyme activities of the individual clones are indicated within the bars). The intensity of the total staining in each lane was analysed by densitometric analysis. * indicated $p < 0.05$ vs. control.

dependent on the correct localisation of the individual glycosyltransferase enzymes. The overexpression of ST3N in the B104 cells resulted in an alteration in the immunocytochemical staining pattern of the enzyme within the cell and this may represent an altered distribution within the Golgi apparatus (Fig. 5). As the individual components of the Golgi are likely to have only a limited capacity for protein expression, a significant increase in the cellular levels of

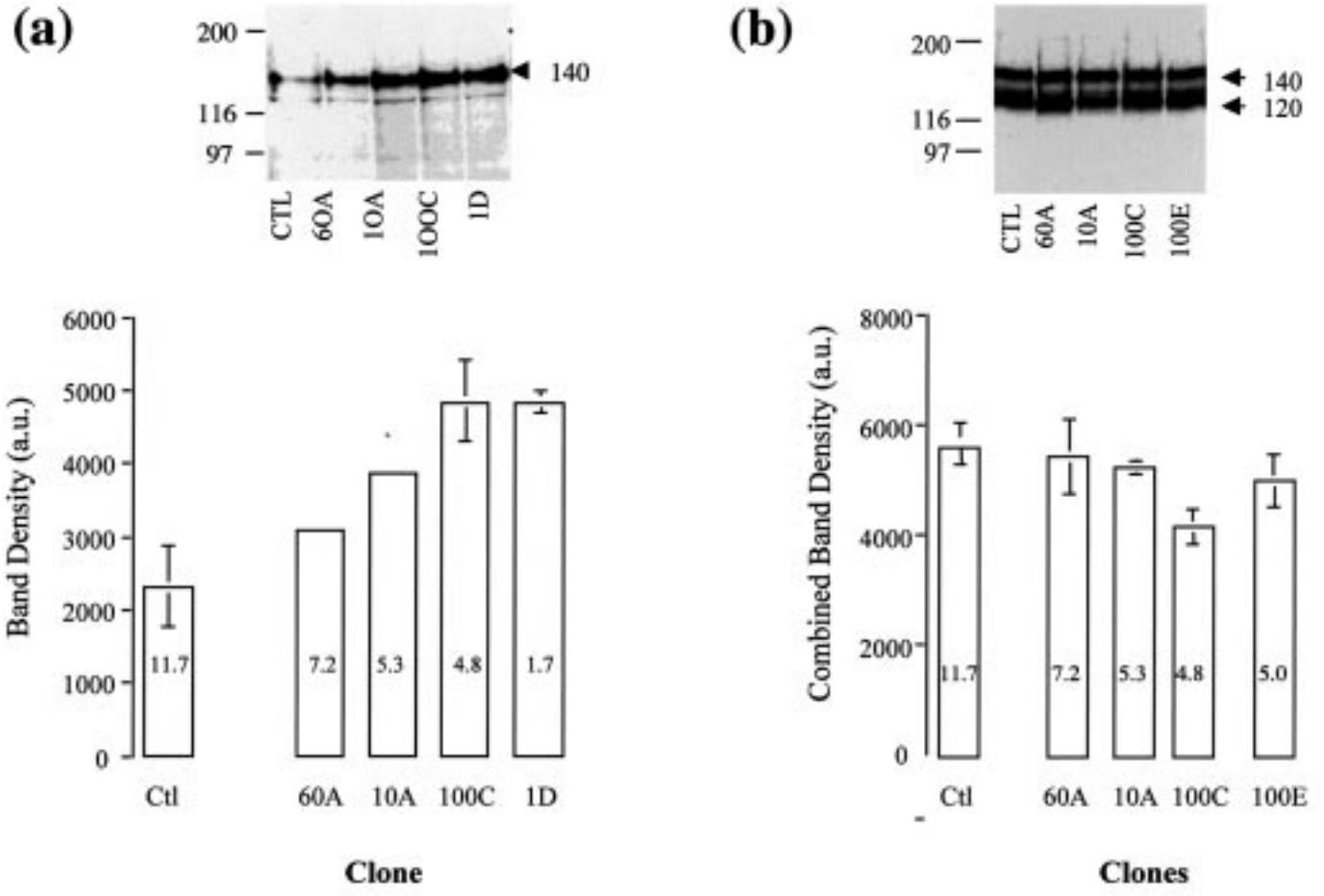

Figure 7. Western blot analysis of (a) PSA and (b) NCAM in 50 μg samples of control and ST3N-transfected B104 cells. The molecular weights of the protein bands in kDa are indicated. Control cells are in the left-hand lanes with clones of decreasing enzyme activity from left to right (enzyme activities of the individual clones are indicated within the bars). The intensity of the total staining in each lane was analysed by densitometric analysis.

ST3N may well result in an upset in the localisation of this enzyme, or indeed of others in the N-linked oligosaccharide pathway.

Although there was a decrease in the total ST activity in the transfected cells, there was a moderate increase in the level of lectin staining of the α2,3 sialoglycoproteins in addition to an up-regulation in the expression of the PSA epitope. While these changes parallel the increase in ST3N expression in the cells, they would appear to be contradictory to the overall trend of a decrease in total cellular ST activity. It must be remembered, however, that the total ST activity, as determined using asialofetuin as an acceptor, includes the activities of a number of ST enzymes which catalyse the transfer of sialic acid in both N- and O-linkages. Therefore, the alteration in ST3N expression and subcellular distribution may preferentially modulate the activities of other selected ST enzymes with a consequent decrease in total ST enzyme activity whilst maintaining or even increasing slightly the ST3N potential.

This study suggests that there is a complex relationship between individual sialyltransferases at the level of the Golgi and changes in the expression levels of an individual enzyme may have a significant influence on the overall sialylation potential of the cell.

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