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The role of protein phosphorylation in α2,6(*N*)-sialyltransferase activity

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Abstract

Sialoglycoproteins play a key role in both brain development and neuronal plasticity with their sialylation state being controlled by the sialyltransferase (STN) family of enzymes. In this study, we have determined the role of specific kinase enzymes in the expression and catalytic activity of the α2,6 STN (ST6N) isozyme. The catalytic activity was moderately decreased following the inhibition of GSK3β with LiCl. However, there was a significant increase in catalytic activity following activation of protein kinase C (PKC) by phorbol ester. There was no change in the expression levels of the enzyme protein following any of the treatments. The changes in enzyme catalytic activity were also mirrored by the expression of both protein-bound sialic acid and the polysialic acid oligosaccharide group attached to the neural cell adhesion molecule, NCAM. These results provide further evidence for the role of second messenger-associated kinase enzymes in the modulation of the cell glycosylation potential.

Keywords: Sialyltransferase; Kinase; Glycoprotein; NCAM; Polysialic acid

The transfer of sialic acid onto nascent N-linked oligosaccharide chains is catalysed by the members of the sialyltransferase (STN) family of enzymes [1]. The available evidence suggests that the control of STN expression and activity is multifactorial and, indeed, there is an increasing body of evidence to suggest an interaction between second messenger system activation and enzyme catalytic activity [2,3]. Certain members of the STN family have been reported to be phosphorylated, with the phosphorylation state playing a key role in determining the enzyme catalytic activity. The ganglioside ST enzymes CMP-NeuAc:LacCer α2,3ST (ST-I), CMP-NeuAc:GM3 α2,8ST (ST-II), and CMP-NeuAc:GM1 α2,3ST (ST-IV) can be directly phosphorylated following the addition of purified protein kinase C (PKC) to an ST-enriched Triton extract derived from rat brain microsomes [4,5]. Similar results were obtained following the treatment of NG108-15 cells, a neuroblastoma × glioma hybrid cell line, with agents that altered kinase or phosphatase activities [5].

The nerve cell adhesion molecule, NCAM, plays a pivotal role both during brain development in addition to modifying neurochemical events associated with synaptic plasticity in the adult. The polysialic acid (PSA) oligosaccharide chain, that contains up to 80 negatively charged sialic acid residues joined in α2,8 linkages, is attached to the core protein (to generate PSA-NCAM) coincident with a requirement for a decrease in the strength of cell–cell interactions [6]. While PSA is normally attached to the penultimate galactose residue of the core sugar in an α2,3 linkage [7], we have previously reported that it can also be attached in an α2,6 linkage in cells overexpressing the ST6N enzyme [8]. The factors controlling the transient expression of PSA-NCAM associated with plastic events in the adult brain remain to be clarified although the activation of second messenger systems to alter the protein phosphorylation state is likely to play a key role [9]. The aim of this study was to investigate the role of second messenger system activation in the expression and activities of the ST6N enzyme using a human neuroblastoma cell line that primarily expresses this form of the enzyme in parallel with PSA-NCAM [8].

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Methods

The 6B100 subclone of the SH-SY5Y human neuroblastoma cell line was cultured as previously described in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) foetal calf serum, penicillin (2 U/ml), and streptomycin (0.25 mg/ml) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 [8]. They were seeded at a density of 2.2×10^6 cells per 75 cm^2 tissue culture flasks (Greiner, Germany) and routinely harvested by scraping into phosphate-buffered saline (PBS) upon reaching approximately 80% confluency [8]. The cells were treated with LiCl (10 μM) for 18 h or phorbol ester (100 μM) for 2 or 4 h prior to harvesting. The protein content of the cell preparations was determined colorimetrically [10] and they were stored in aliquots at −20 °C until use.

Sialyltransferase catalytic activity was determined as previously described using CMP-[^{14}C]-NeuNAc (Amersham, UK) as the sialic acid donor and asialofetuin (Sigma, UK) as the protein acceptor [11]. The individual polypeptide components of the samples were separated by discontinuous SDS–PAGE and transferred to PVDF membranes (Millipore) by electroblotting. The sugar content of the protein bands was analysed by lectin affinity blot analysis (DIG glycan differentiation kit, Boehringer–Mannheim) using the *Sambucus nigra* (SNA) lectin that labels the terminal N-linked Galα(2,6)NeuNAc disaccharide. The expression of the NCAM and ST6N proteins was determined by Western blot analysis using anti-ST6N (kindly provided by Prof. Eric Berger, Zurich) and an anti-NCAM antibody (Santa Cruz). The antibody binding was detected using a HRP-labelled second antibody (Scottish Antibody Production Unit) and visualised using the ECL detection reagent (Amersham) [8].

Results and discussion

A number of previous studies have proposed that post-translational modifications play a key role in the control of the catalytic activity of ganglioside-associated ST isozymes. However, the majority of these studies have been carried out using isolated enzyme preparations rather than whole cells and so the physiological relevance of the findings remains unclear [12].

The 6B100 cell line is a stably transfected subclone of the SH-SY5Y human neuroblastoma cell line that expresses high levels of the ST6N enzyme and this accounts for at least 95% of the total STN catalytic activity [8]. Stimulation of protein kinase C in the control cells with phorbol ester (PDBu) resulted in a 1.5-fold increase in ST6N catalytic activity following 2 h treatment. There was a trend for this to decrease towards basal levels again at the 4-h time point. Inhibition of glycogen synthase kinase 3β (GSK-3β) following treatment with LiCl for 18 h resulted in a 50% decrease in ST6N catalytic activity. The changes in ST6N catalytic activities were not associated with any alterations in the enzyme protein expression levels as determined by Western blot analysis (Fig. 1B).

The expression of protein-associated α2,6-linked sialic acid residues labelled by the SNA lectin mirrored well the changes in enzyme activity in the cells, with a significant increase in staining intensity after both at 2 and 4 h treatment with PDBu (Fig. 2A). The greater expression at 4 h, when compared with the maximal stimulation of STN catalytic activity observed after 2 h, probably reflects a time lag between a change in enzyme activity and the subsequent de novo glycoprotein biosynthesis.

The cellular expression of PSA-NCAM following the different treatment strategies was also employed as an indicator of the cellular sialylation potential. The 6B100 clone, because of its high ST6N catalytic activity, expresses PSA-NCAM and can serve as a model system with which to investigate the effect of pharmacological agents on PSA expression [8]. Treatment with LiCl, which significantly decreased ST6N activity, attenuated PSA expression, as detected by a decrease in the molecular size of the NCAM protein banding pattern (Fig. 2B). PDBu treatment had no effect on PSA-NCAM expression as the N-linked oligosaccharide acceptor sites on the protein are likely to be fully occupied

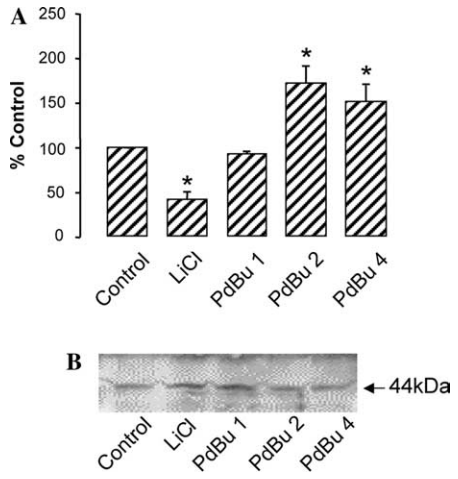

Fig. 1. (A) Total sialyltransferase (STN) activity and (B) ST6N protein expression in SH-SY5Y human neuroblastoma cells (6B100 clone) overexpressing ST6N following treatment with LiCl (10 μM for 18 h) of PDBu (100 μM for the time periods indicated). The values represent means ± SEM ($n = 3$) and * indicates $p < 0.01$ vs. control (ANOVA followed by Bonferroni's post hoc comparison test).

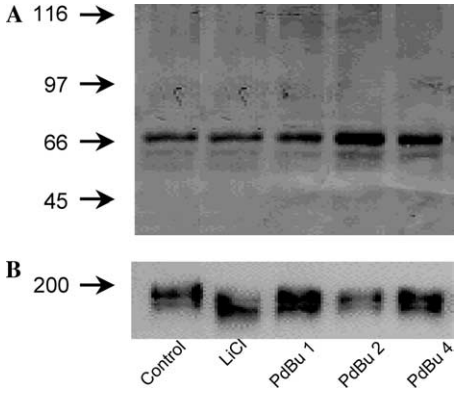

Fig. 2. (A) α2,6 Sialoglycoprotein and (B) NCAM expression in SH-SY5Y human neuroblastoma cells (6B100 clone) following treatment with LiCl (10 μM for 18 h) of PDBu (100 μM for the time periods indicated).

in the untreated cells and a further PDBu-associated increase in ST6N activity would be unlikely to further increase the level of PSA expression, as monitored by the molecular size of the protein. A similar saturable expression of PSA has been demonstrated in clones transfected with increasing levels of the ST6N cDNA and parallel catalytic activities [8].

Previous studies have proposed that the role of post-translational modifications may be ST enzyme-specific. Stimulation of PKC in neuro-2A mouse neuroblastoma cells, which express primarily the ST3N isozyme, resulted in a decrease in PSA expression. However, the time scale involved in those experiments suggests that the changes observed may be associated with alterations in the PKC enzyme expression levels rather than its catalytic activity [13]. In the present study, the time points investigated (up to 4 h with PDBu) are unlikely to alter enzyme expression but rather modify its catalytic activity [14]. Indeed, at the 4-h treatment point, there was evidence for an attenuation in the response, probably associated with a desensitisation of the enzyme to PDBu-stimulated induction and a parallel down-regulation in its catalytic activity. Studies on *O*-glycan sialylation by ST6O in C6 rat glioma cells reported that dephosphorylation by alkaline phosphatase in addition to phosphatase inhibition both decreased enzyme catalytic activity, suggesting that even small changes in enzyme phosphorylation may influence the enzyme function. Interestingly, that study reported that PKC activation had no effect on enzyme catalytic activity and this may reflect differential cellular and enzyme susceptibilities to changes in the cellular phosphorylation potential [15].

Additionally, there is in vivo evidence to support the potential role of PKCδ in the attenuation of PSA expression, potentially mediated by changes in the expression and/or activities of the polysialyltransferase enzymes that catalyse PSA expression [16]. However, in vivo studies have the additional complexity associated with the myriad of interacting biochemical pathways. Therefore, in vitro studies could be considered to exhibit greater potential for the dissection of individual biochemical pathways at a cellular level.

While the available evidence suggests the existence of differing mechanisms for the control of individual ST enzyme activities, potential disparities may also exist between the long- and short-term effects of second messenger system activation on STN enzyme activity. There is evidence for an increase in ST6N catalytic activity, but not expression levels (Fig. 1A) following relatively rapid changes in protein phosphorylation (up to 4 h) that may be associated with certain plastic events within the CNS such as long-term potentiation that display a similar time course [17]. However, in the longer term (over a period of days that would correspond temporally with plastic changes associated with memory consolidation), there is a decrease in STN catalytic activity associated with a phorbol ester-stimulated decrease in PKCδ expression levels [16]. Therefore, the in vitro results obtained in this study do not actually conflict with the previous reports due to the different time scales (and therefore biochemical mechanisms) involved. Indeed, the longer-term effects are likely to be associated with a number of additional factors which may include, for example, auto- and *trans*-glycosylation of individual glycosyltransferase enzymes [18].

The apparent conflict of results in the study of ST phosphorylation may also be explained by the different experimental approaches taken to address the question. The different experimental tools (e.g., the targeting of either phosphatase or kinase enzymes) may also account for some of the anomalous findings that have been reported. While there is strong evidence for a role of phosphorylation in the control of ST catalytic activity, the actual physiological role of the kinase and/or phosphatase enzymes remains unclear. The ST6N enzyme, for example, has been demonstrated to be phosphorylated in the stem and catalytic domains of the enzyme that lie within the lumen of the Golgi, possibly mediated by a member of the casein kinase family of enzymes [19]. However, the enzymes investigated in the current study are not located within the Golgi. This does not exclude, however, a biochemical cascade system whereby alterations in PKC or GSK3β activities may ultimately influence the activity of a kinase or phosphatase enzyme within the lumen of the *cis-medial* Golgi to modify the phosphorylation state of one or more of the ST enzymes contained within.

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