The biochemical consequences of α2,6(N) sialyltransferase induction by dexamethasone on sialoglycoprotein expression in the rat H411e hepatoma cell line

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Abstract Previous studies have demonstrated sialyltransferase (ST) enzyme activity to be induced in hepatic cells by corticosteroids. In this study, we used the H411e rat hepatoma cell line to further characterise this induction with particular reference to the subsequent changes in the pattern of sialoglycoprotein (SGP) expression. The induction of total ST activity by dexamethasone was concentration dependent with maximum induction occurring 12 h subsequent to drug addition. Western blot analysis demonstrated that the induction was associated with an increase in the expression of the α2,6(N) ST enzyme with no change in the expression levels of the α2,3(N) enzyme. While the induction resulted in an increase in the reaction velocity (V_{max}) of the enzyme for both the sugar donor (CMP-Neu5Ac) and the asialofetuin acceptor protein, there was no significant change in the enzyme affinity (K_m) for the substrates, suggestive of either an increase in the expression or efficiency of the existing enzyme(s) rather than an induction of novel ST enzymes. Lectin blot analysis of cellular glycoprotein expression demonstrated no change in the expression patterns of either α2,3 or α2,6-linked SGP following enzyme induction. These results suggest that the available acceptor sites for the terminal sialic acid group(s) may be fully occupied in the control cells and therefore there are no further sites onto which the sialic acid can be transferred following induction of ST enzyme activity. This may be due to the high basal enzyme levels in the control cells already exhausting endogenous acceptor sites.

Key words: Sialyltransferase; Hepatoma; Sialoglycoprotein; Lectin; Glucocorticoid

1. Introduction

Glycoproteins form a diverse group of complex macromolecules which are probably best defined as conjugated proteins which contain one or more heterosaccharides as their prosthetic group covalently bound to the polypeptide chain [1]. Glycosylation can be considered as one of the most common and diverse covalent modifications undertaken by newly synthesised proteins with this diversity lying in both the amino acids that are modified and the structures attached [2]. Sialyltransferases (ST) belong to the subclass of glycosyltransferases that catalyse the transfer of activated sialic acid (CMP-Neu5Ac) to terminal positions on the carbohydrate chains of sialoglycoproteins (SGP) and glycolipids (GL). The ST family comprises up to 19 enzymes all of which would be required to synthesise the sialyloligosaccharide sequences known to date, with each enzyme being distinguished on the basis of both its specificity for the sequence and the anomeric linkage formed between the sialic acid and the acceptor oligosaccharide [3,4]. It is worthy of note that, because of its size and negative charge, Neu5Ac is considered as one of the most important constituents of the carbohydrate moiety of many glycoconjugates [5].

ST expression has been shown to be induced in hepatic cells by the synthetic glucocorticoid dexamethasone (dex) both in vivo [6,7] and in vitro [8]. The observed increases in the ST mRNA levels are achieved through transcriptional enhancement [8] with increased stabilisation of the existing mRNA not appearing to play a role [8,9]. This induction appears to be relatively selective for the α2,6(N) ST enzyme (E.C. 2.4.99.1), which catalyses the transfer sialic acid onto the Gal residue of an N-linked oligosaccharide chain [10], and the induction also displays a cell-type specificity [11].

Considering that SGP and GL both play an important role in cell structure and function, conditions altering cellular ST activity and hence sialylation would be expected to have a profound influence on cell function [5]. While particular interest has been shown to date in the effects of dex on hepatic cell ST enzyme activity, there is little data available on the biochemical consequences of this induction. The aim of this study was, therefore, to gain a greater understanding of this induction of ST by dex using the H411e hepatoma cell line, as an in vitro model system, with particular interest in the subsequent expression of the resultant SGP species.

2. Materials and methods

2.1. Cell culture

H411e rat hepatoma cells (American Type Culture Collection) were cultured routinely in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), 1% (v/v) penicillin and 1% (v/v) streptomycin as previously described [12]. Cells were split (passaged) by trypsinisation and re-seeded at a density of 7.5×10^5 cells per 75 cm^2 flask. The cells were harvested by scraping in phosphate buffered saline solution (PBS), centrifuged for 10 min at 3000 rpm and resuspended in water by sonication. The protein content of the samples was quantified using the Folin phenol reagent [13]. For analysis of released SGP, the cells were incubated in serum-free medium for 2 h to remove serum-derived glycoproteins and then incubated in fresh serum-free medium for 18 h. At this stage, there was no evidence of cell death or the dislodgement of cells from the culture substrate. The medium was then collected, centrifuged at $15000\times g$ for 10 min to remove any cell debris and stored at −20°C until use. All of the biochemical assays were carried out at least in duplicate on at least three sets of individually harvested cells of increasing passage number.

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2.2. Sialyltransferase assay

Total sialyltransferase activity was determined as previously described [14] 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), which did not contain any detectable residual sialic acid, as an exogenous acceptor. Under the assay conditions used, there was minimal transfer of sialic acid to endogenous protein acceptors as has previously been reported [15].

2.3. Determination of enzyme kinetic parameters

In order to determine the kinetic parameters of the enzyme, ST activity was assayed using varying concentrations of ASF and CMP-Neu5Ac. The kinetic parameters, K_m and V_{max}, were calculated using the Enzfit programme for enzyme kinetics [16].

2.4. Sialoglycoprotein analysis

Glycoconjugate expression was determined by lectin blot analysis as previously described [17]. Briefly, the individual peptides of 50μg protein samples were separated by discontinuous SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore) by electroblotting. Glycoproteins containing sialic acid attached to Gal residues in α2,3-linkages and to Gal/GalNAc residues in α2,6-linkages were detected using the MAA and SNA lectins, respectively, and visualised using the DIG Glycan Differentiation Kit (Boehringer Mannheim).

2.5. Sialyltransferase enzyme expression

The expression of the α2,3(N) and α2,6(N) ST enzymes were determined using antisera raised against the individual enzymes. The antiserum to rat α2,6(*N*) ST has been described previously [18]; a new rabbit polyclonal antiserum has been raised to a fusion protein consisting of β-galactosidase fused with full length Jurkat cell-derived α2,3(N) ST and expressed in *E. coli*. [19]. The fusion protein was solubilized from inclusion bodies as described previously [20], separated on SDS-PAGE and injected into a rabbit. The resulting antiserum was found to be monospecific to a soluble form of the enzyme expressed in Sf9 cells using the baculovirus expression system (kindly provided by Dr. M. Streiff, Novartis); characterization included ELISA, immunofluorescence of recombinant antigen expressed in COS cells and immunoblotting (Burger et al., submitted for publication). In all tissue cultured cells investigated by immunofluorescence the antiserum produced a typical Golgi staining pattern. The antiserum proved to be crossreactive with rat and mouse enzyme. The protein components of the H411e cell pellets were separated by SDS-PAGE and transferred to PVDF membranes as described and the binding of the ST antisera was detected using the ECL detection system (Amersham). The autoradiographs were scanned using a Glyco FACE imaging system and the immunoreactive bands were quantified by densitometric analysis using the NIH Image software package. The staining intensities of the blots were proportional to the protein loaded within the range employed in the assay (data not shown) and the variability between samples was less than 5% (see Fig. 3).

3. Results and discussion

There was a dose-related induction of total cellular ST activity in the H411e cells by dex, with maximum induction being reached at [10^{-7} M] (Fig. 1a). A lag period of approximately 3 h after drug addition was observed prior to enzyme induction, with the plateau being reached at 12 h after treatment with dex (Fig. 1b). The dose-response relationship between dex and cellular ST activity were in good agreement with those previously reported for hepatic cells [8,9] and indicated that the H411e cells have a significant capacity for ST

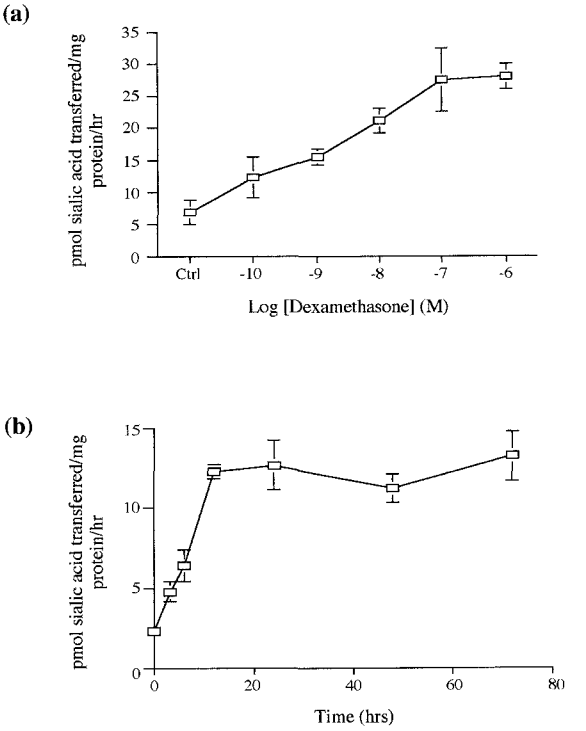

Fig. 1. (a) Dose-response effect of dex on the ST activity of H411e cells treated with drug for 24 h prior to harvesting. (b) Time course of ST induction by dex (10^{-6} M). The cells were plated at 10^4 cells/cm^2 and treated at time 0 (24 h post plating) with dex. The cells were harvested at the indicated time points and the cellular ST activity determined. Values represent mean ± S.E.M. ($n = 3$).

enzyme induction subsequent to drug addition. This is an important observation as agents including cytokines and glucocorticoids act synergistically in vivo to raise the levels of many glycoproteins during the acute phase response [6]. The presence of this reserve capacity for induction would suggest that the H411e cells appear to be a good model for in vivo hepatocyte function where dex induction of ST activity has also been observed [7]. The lag period of approximately 3 h between the addition of drug and the induction effect was in agreement with previous reports that dex causes a transcriptional enhancement of ST mRNA [8]. This was supported by the observation that the activity of the enzyme in the dex treated cells remained significantly above control levels for at least 24 h after the withdrawal of the drug and the direct addition of dex to the enzyme assay had no direct effect on the activity (data not shown).

The kinetics of enzyme induction were then determined by independently varying the concentrations of the CMP-Neu5Ac sialic acid donor and the asialofetuin acceptor. For the control cells, when the concentrations of CMP-Neu5Ac were varied, the kinetic parameters were calculated as $K_m = 0.068 \pm 0.039$ mM and $V_{max} = 3.35 \pm 0.414$ pmol/mg per hour following Lineweaver-Burk analysis (Fig. 2a). Upon treatment with dexamethasone, the K_m was calculated as

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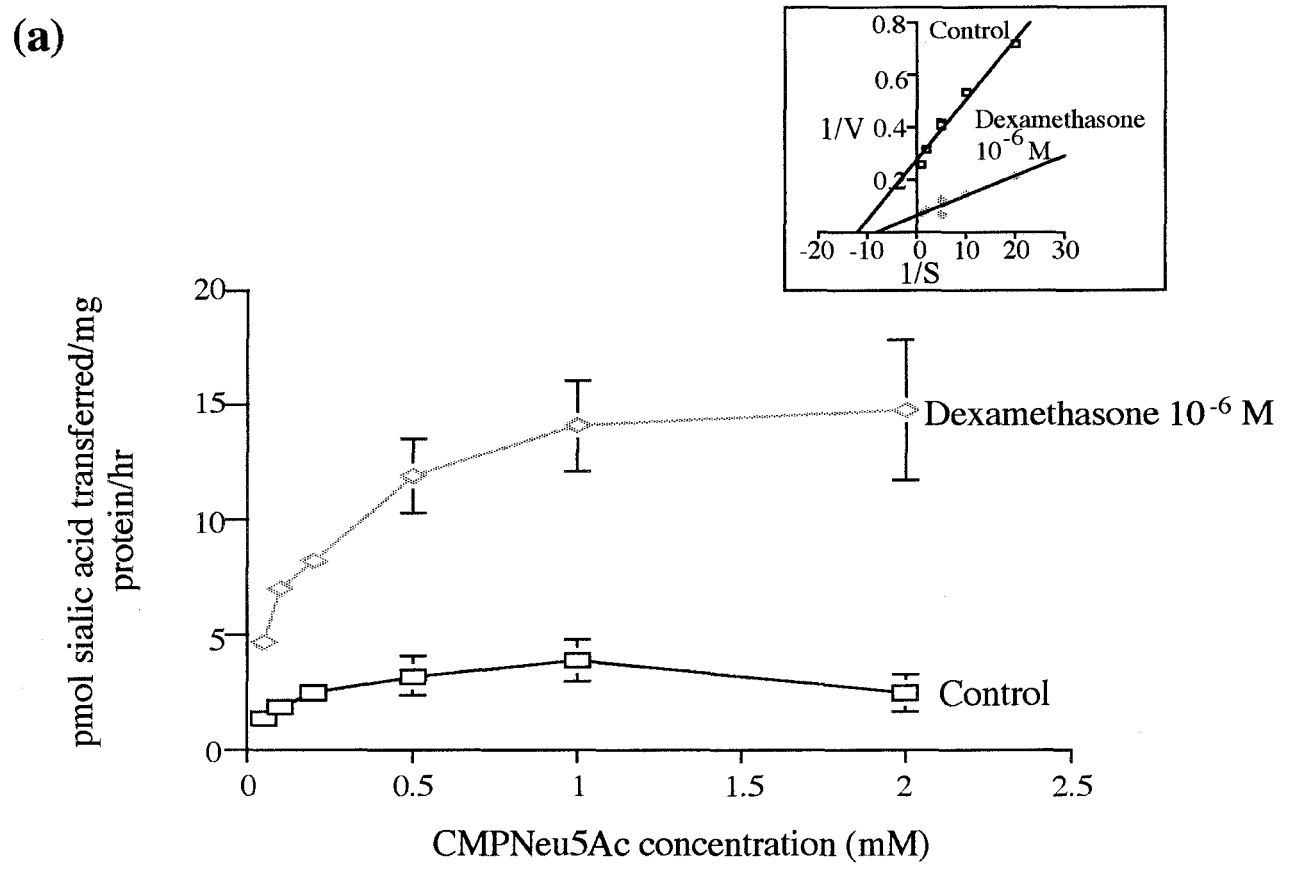
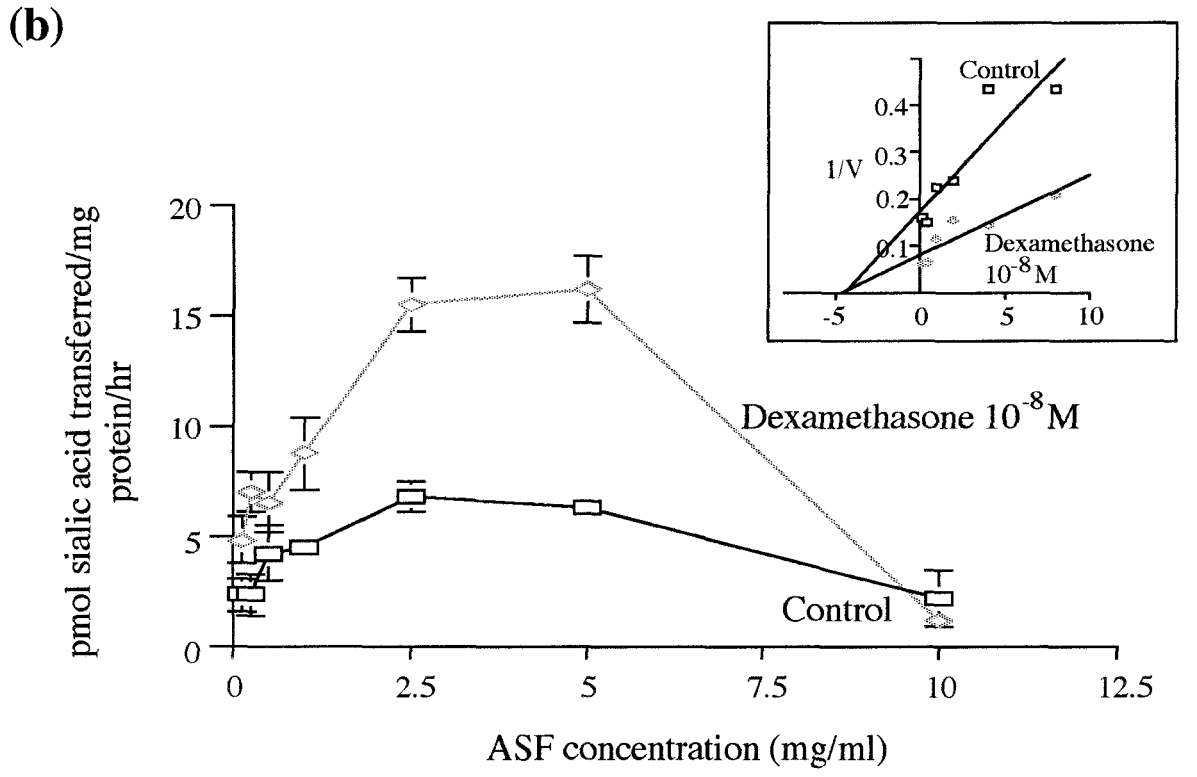
Fig. 2. The effect of dex treatment on the ST enzyme kinetics. Cells were cultured as described and assayed for ST activity using (a) increasing concentrations of CMP-Neu5Ac [0.05 mM] to [2 mM] in the presence of 1 mg ASF and (b) increasing concentrations of ASF [0.125 mg/ml] to [10 mg/ml] in the presence of 1 mM CMP-Neu5Ac. The values represent mean ± S.E.M. ($n = 3$) and the kinetic parameters were calculated by Lineweaver-Burk analysis.


(a)
0.8
0.6
1/V 0.4
0.2
Control
Dexamethasone
10-6 M
-20 -10 0 10 20 30
1/S
pmol sialic acid transferred/mg protein/hr
20
15
10
5
0
0 0.5 1 1.5 2 2.5
Dexamethasone 10-6 M
Control
CMPNeu5Ac concentration (mM)


(b)
Control
0.4
1/V 0.3
0.2
0.1
Dexamethasone
10-8 M
-5 0 5 10
pmol sialic acid transferred/mg protein/hr
20
15
10
5
0
0 2.5 5 7.5 10 12.5
Dexamethasone 10-8 M
Control
ASF concentration (mg/ml)

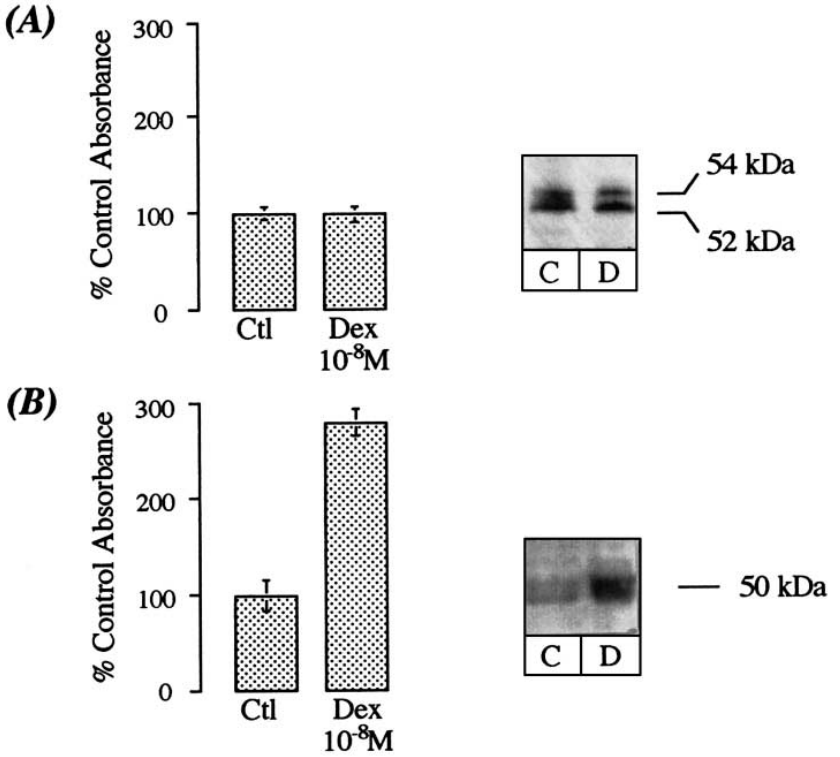


Fig. 3. The expression of the (A) α2,3 and (B) α2,6 ST enzymes in the H411e cells following treatment with dex [10^{-8} M] for 24 h as determined by Western blot analysis. Graphs represent mean ± S.E.M. ($n = 3$) of densitometric values.

0.144 ± 0.022 mM (mean ± standard deviation) and the V_{max} as 15.5 ± 0.65 pmol/mg per hour. The increase in the latter parameter was calculated as being significant upon analysis using the Enzfit programme. A constant concentration of CMP-Neu5Ac (1.0 mM), which was significantly in excess of the K_m value so as not to act as a limiting factor, was then used to determine the kinetic parameters of the ASF acceptor (Fig. 2b). A saturation effect was observed for ASF at concentrations above 5 mg/ml and the substrate was shown to act as an inhibitor of ST activity at a concentration of 10 mg/ml (Fig. 2b). The kinetic parameters for ASF, as determined again by Lineweaver-Burk analysis, were $K_m = 7.79 \pm 2.32 \times 10^{-2}$ mg/ml and $V_{max} = 0.70 \pm 0.58$ pmol/mg per hour for the untreated cells, and $K_m = 13.2 \pm 5.51 \times 10^{-2}$ mg/ml and $V_{max} = 18.10 \pm 2.43$ pmol/mg per hour, following administration of dex [10^{-8} M]. Statistical analysis demonstrated that V_{max} was the only parameter significantly altered following dex administration. The change in the enzyme V_{max} for the sialic acid donor and the protein acceptor would suggest that the observed increase in ST activity was due to the induction of the levels of the enzyme species. It is unlikely that there was an induction of a novel ST species as the affinity for ASF was shown to be unaffected by dex induction of the enzyme.

The contribution of individual ST enzymes to the increase in enzyme activity was then determined using enzyme-specific antisera (Fig. 3). The antiserum directed against the α2,6(N) ST labelled a protein band of 50 kDa which is in good agree-

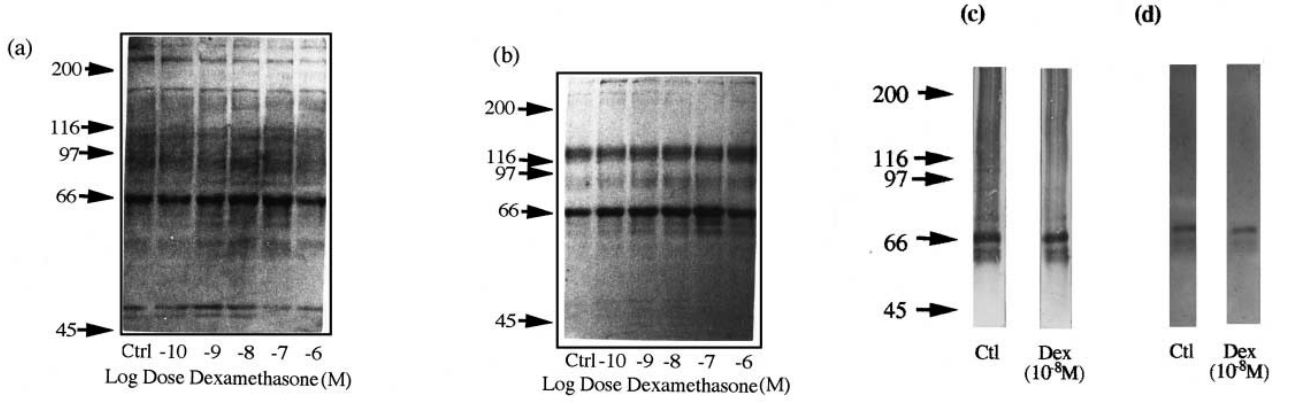


Fig. 4. The effect of dex treatment on the expression of cellular (a) α2,6- and (b) α2,3-linked SGPs and the release of secreted (c) α2,6- and (b) α2,3-linked SGPs from the H411e cell line. The migration of the molecular weight standards (in kDa) are indicated. The standards used were myosin (200 kDa), β galactosidase (116 kDa), phosphorylase B (97 kDa), albumin (66 kDa) and ovalbumin (45 kDa).

ment with previous reports [18]. The increase in the expression levels of this protein band closely mirrored the increase in total cellular ST levels following dex induction. The antiserum directed against the α2,3(N) ST labelled a protein doublet with a major band at 52 kDa and a minor band at 54 kDa. There was no change in the density of either band following dex treatment. While these results are in good agreement with previous reports of α2,6(N) ST induction in hepatoma cells by corticosteroids [10], this is the first confirmation that there is no induction of the α2,3(N) ST by dexamethasone. Furthermore, these results support the hypothesis of an induction of an endogenous enzyme species, as suggested by the enzyme kinetic parameters.

Having characterised the induction of ST by dex in the H411e cells, the expression of both cellular (Fig. 4a,b) and secreted (Fig. 4c,d) α2,6 and α2,3 SGPs were analysed using the SNA and MAA lectins which detect sialic acid linked to Gal/GalNAc and Gal residues in α2,6 and α2,3 linkages, respectively. In all cases, a variety of stained bands were detected indicating the expression of a large number of SGP species of diverse molecular size. However, while the induction of α2,6ST activity by dex did not alter the general pattern of lectin staining of either the cellular or secreted SGP species, there was a dose-related increase in both SNA and MAA labelling of a 66-kDa cellular protein (Fig. 4a,b). Because this effect was observed for both lectins, despite dex inducing an increase in the α2,6 ST enzyme alone, it is likely that this effect was due to a dex-induction of the protein backbone thus providing an increase in the substratum available for sialic acid transfer.

Therefore, although there was a significant increase in ST activity, primarily associated with the α2,6(N) ST enzyme, there were no general changes in the SGP expression pattern as determined by lectin blot analysis apart from that due to an increase in the levels of an individual protein backbone. As hepatoma cells have a very high endogenous ST activity [7], it could be proposed that the majority of the available penultimate Gal acceptor residues are already occupied under normal cellular conditions and thus, there would be no free acceptor sugar sites onto which to transfer the sialic acid following the dex-induced increase in ST activity. The fact that changes in neural SGP levels have been detected in vivo following induction of cellular ST activity [21] may be explained by the fact that the basal neural enzyme activity is considerably lower than hepatic tissue and therefore the penultimate Gal residues are not saturated with endogenous sialic acid [7]. Furthermore, we must consider that the H411e cell line has a tumor base and as metastatic cells have been reported to have an increase in cell surface sialic acid [22], this may also contribute to the saturation of the acceptor sugar residues. The lack of effect of increased enzyme activity on SGP expression may also be due to changes in the rate of turnover of SGP within the cell with a change in protein metabolic rate possibly masking the increase in sialyltransferase activity.

While the dex-induced increase in cellular α2,6(N) expression in hepatoma cells does not appear to alter the sialylation state of cellular or secreted SGP, subtle changes in protein sialylation, which were undetectable by the lectin blot analysis, cannot be ruled out. Furthermore, previous studies have demonstrated that dex treatment of H411e hepatoma cells also results in an increase in secreted enzyme [18] although it did not prove possible to assay secreted enzyme levels using this in vitro system. While the function of the soluble form of ST has not been clarified, it will be interesting to investigate the functional consequences of an increase in serum ST following dex induction of hepatic ST.

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References

[1] Bahl, O.P. (1992) in: Glycoconjugates: Composition, Structure and Function (Allen, H.J. and Kisailus, E.C., Eds.), pp. 1–12, Marcel Dekker, New York.
[2] Lis, H. and Sharon, N. (1993) Eur. J. Biochem. 218, 1–27.
[3] Harduin-Lepers, A., Recchi, M.A. and Delannoy, P. (1995) Glycobiology 5, 741–758.
[4] Weinstein, J., Lee, E.U., McEntee, K., Lai, P.-H. and Paulson, J.C. (1987) J. Biol. Chem. 262, 17735–17743.
[5] Montreuil, J. (1980) Adv. Carbohydr. Chem. Biochem. 37, 157–223.
[6] Kaplan, H.A., Woloski, B.M.R.N.J., Hellman, M. and Jamieson, J.C. (1983) J. Biol. Chem. 258, 11505–11509.
[7] Coughlan, C.M., Seckl, J.R., Fox, D.J., Unsworth, R. and Breen, K.C. (1996) Glycobiology 6, 15–22.
[8] Wang, X., O'Hanlon, T.P. and Lau, J.T.Y. (1989) J. Biol. Chem. 264, 1854–1859.
[9] Wang, X.C., Smith, T.J. and Lau, J.T.Y. (1990) J. Biol. Chem. 265, 17849–17853.
[10] Bosshart, H. and Berger, E.G. (1992) Eur. J. Biochem. 208, 341–349.
[11] Vandamme, V., Pierce, A., Verbert, A. and Dellannoy, P. (1993) Eur. J. Biochem. 211, 135–140.
[12] Breen, K.C. and Ronayne, E.M. (1994) Neuroreport 5, 970–972.
[13] Lowry, G.H., Rosenbrough, N.L., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Breen, K.C. and Regan, C.M. (1986) J. Neurochem. 47, 1176–1180.
[15] Breen, K.C. and Regan, C.M. (1986) J. Neurochem. 47, 1176–1180.
[16] Lyles, G.A. and Chalmers, J. (1995) Biochem. Pharmacol. 49, 416–419.
[17] Gillian, A.M. and Breen, K.C. (1995) in: Research Advances in Alzheimer's Disease and Related Disorders (Iqbal, K. et al., Eds.), pp. 429–436, Wiley, New York.
[18] Bosshart, H. and Berger, E.G. (1992) Eur. J. Biochem. 208, 341–349.
[19] Burger, P., Kleene, R. and Berger, E.G. (1996) Experientia 52, A4.
[20] Wattzele, G., Bachofner, R. and Berger, E.G. (1991) J. Cell. Biochem. 52, 275–288.
[21] Coughlan, C.M., Seckl, J.M. and Breen, K.C. (1996) Cell. Mol. Neurobiol. 16, 431–436.
[22] Hebert, E. and Monsigny, M. (1993) Biol. Cell 79, 97–109.