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Study of *O*-sialylation of glycoproteins in C6 glioma cells treated with retinoic acid

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When treated with retinoic acid *in vivo*, C6 glioma cells show an enhancement of CMP-Neu5Ac:Galβ 1-3 GalNAc-R α-2,3 sialyltransferase activity. A 300 kDa glycoprotein was detected by lectin affinoblotting in retinoic acid-treated C6 cells which stained weakly or not at all in control cells. Comparative studies with different lectins demonstrated that this glycoprotein contains α2,3 Neu5Ac Gal-GalNAc O-glycan moieties. Cultures in the presence of an inhibitor of O-glycan synthesis (*N*-acetylgalactosaminide α-*O*-benzyl) demonstrated that enhancement of staining of the 300 kDa glycoprotein was not due to the increase of the α2,3 sialyltransferase but to the *de novo* synthesis of the polypeptide chain of this glycoprotein.

Keywords: retinoic acid, C6 glioma cells, lectin-affinoblotting, α2,3 sialyltransferase

Abbreviations: RA, retinoic acid; Neu5Ac, *N*-acetylneuraminic acid; CMP-Neu5Ac, cytidine 5′ monophosphosialate; α2,3 ST, CMP-Neu5Ac:Galβ 1-3 GalNAc-R α-2,3 sialyltransferase; GalNAcα-*O*-benzyl, *N*-acetylgalactosaminide α-*O*-benzyl; Galβ1-3GalNAcα-*O*-benzyl:Galactosyl β1-3*N*-acetylgalactosaminide α-*O*-benzyl; TBS, Tris-HCl buffer 50 mM pH 7.5 containing NaCl 0.15 M and Tween 20 0.05%; B1 buffer, TBS containing $MgCl_2$ 1 mM, $MnCl_2$ 1 mM and $CaCl_2$ 1 mM.

Introduction

Retinoic acid (RA) and synthetic analogues of vitamin A have been known for a long time to affect the proliferation of many cultured cells, including embryonic carcinoma, melanoma, leukaemia, epithelial and neuroblastoma cells [1]. In addition, retinoids often induce cellular differentiation, accompanied by the suppression of cellular properties associated with the transformed phenotype, such as anchorage-independent growth [2–5]. Such properties of retinoids have been used for the prevention and treatment of various cancers [6, 7], especially acute promyelocytic leukaemia [8–11] and lung cancers [12]. Ability of retinoids to modulate cellular differentiation indicates that they act at the level of the nucleus [13] and two families of nuclear receptors have been described, the retinoic acid receptors (RARs) α, β and γ [14–16] and the retinoid X receptors (RXRs) [17, 18]. RXR receptors were originally classified as orphan receptors before 9-*cis* retinoic acid was discovered to bind and to activate this family of receptors [19–22]. These receptors are RA-inducible enhancer factors belonging to the super-family of steroid/thyroid nuclear receptors [23]. Receptors may reflect a heterogeneity of function and/or a tissue specific expression [24, 25]. Some pharmacological aspects of RA are directly related to RA receptors. In acute promyelocytic leukaemia (PML), a reciprocal chromosomal translocation in t(15;17) disrupts the *RAR*α gene leading to a chimeric PML/RARαmRNA [9, 10].

Translocation of RA from plasma membranes to nuclear receptors is postulated to be performed via binding to a cytoplasmic protein (cellular retinoic acid binding protein, CRABP-I) acting in cell differentiation [26] and neurogenesis [27, 28]. CRABP-I is an acidic protein of low molecular weight (14 700 Da) [29]. In

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brain, it is expressed in neurons but not in glia cells [30]. CRABP-I has the ability to alter RA metabolism [31, 32] and therefore may act by control of the intracellular RA concentration rather than by transfer of RA. Using molecular cloning of cDNA, Giguere *et al.* [33] identified a second CRABP isoform (CRABP II) [34], the expression of which was positively regulated by RA. Induction of CRABP II is mediated by RAR-RXR heterodimers bound to a Far Upstream Retinoic Acid-Responsive Element (RARE) [35]. In addition, another retinoid-binding protein with a lower molecular weight (4000 Da) has also been described [36].

With regards to glycoprotein biosynthesis, pioneer work of Lotan *et al.* [37] has demonstrated in HeLa cells changes in surface glycoproteins induced by RA. These authors have also demonstrated specific changes in cell surface glycoconjugates of S91 murine melanoma RA-treated cells [38] and stimulation of sialyltransferase activity [39]. More recently, Lotan *et al.* [40] have correlated the growth inhibition of two murine melanoma cell lines to the enhancement of gp160 and to an asialofetuin sialyltransferase activity. Enhancement of fucosyl and galactosyltransferase activities were also observed [41]. In the F9 cell line, RA treatment led to increased α2,3 galactosyltransferase activity [42] and to increased fucosylation of high molecular weight glycoproteins [43].

In previous studies, we have demonstrated that a sialyltransferase was activated when cultured C6 glioma cells were treated with RA [44] and that there exists a correlation between increased sialyltransferase activity and the enhancement of cell adhesion. In the present work, we have studied the enhancement of sialyltransferase activity in relation to changes in the composition of glycoproteins in RA-treated C6 glioma cells.

Materials and methods

MATERIALS

All reagents were of analytical grade. Fetuin (grade III), GalNAcα-*O*-benzyl, Galβ1-3GalNAcα-*O*-benzyl, all-*trans* retinoic acid, neuraminidase from *Vibrio cholerae* (EC 3.2.1.18) and biotin-labelled *Helix pomatia* agglutinin were from Sigma; digoxigenin-labelled lectins, "DIG Glycan Differentiation Kit" and related reagents were from Boehringer-Mannheim. Digoxigenin-labelled lectins were: ACA from *Amaranthus caudatus*; ConA from *Canavalia ensiformis* (jack bean); DSA from *Datura stramonium*; GNA from *Galanthus nivalis*; MAA from *Maackia amurensis*; PNA from *Arachis hypogea* (peanut); SNA from *Sambucus nigra* (eider). CMP-*N*-[^{14}C]-acetylneuraminic acid (CMP-[^{14}C]-Neu5Ac, specific activity 300 Ci mol^{-1}; 11.1 GBq mmol^{-1}) and [^{3}H] retinoic acid (specific activity 49.3 Ci mmol^{-1}; 1.8 TBq mmol^{-1}) were from Dupont de Nemours (NEN). Protogold kit (Stabilized Colloidal Gold solution for the sensitive staining of protein blots) was from BioCell (British BioCell International). SDS-PAGE was performed using a Mini Protean II Dual Slab Cell from Bio-Rad and transfer made with a Mini Trans Blot Cell (Bio-Rad).

CELL CULTURE

C6 glioma cells were routinely cultured as previously described [45] in Costar 75 cm^2 flasks at 37 °C in an atmosphere of 95% O_2 and 5% CO_2. The medium was Dulbecco's modified Eagle minimal essential medium (DMEM, Flow, Labsystems, France) containing 200 U ml^{-1} of penicillin and 200 μg ml^{-1} of streptomycin (GIBCO BRL, Life Technologies, France) and supplemented with inactivated fetal calf serum (FCS, Seromed, France). Cultures at 80–100% confluency were incubated in the dark for various periods of time with 10 μM of RA in DMEM medium. Before the cells were collected, they were washed in the culture flasks with a Tris saline buffer (10 mM Tris-HCl, 8.2 g l^{-1} NaCl, pH 7.2), scraped out with a rubber policeman, suspended in the same buffer and centrifuged for 10 min at 300 × **g**. Viability of the cells was routinely checked by Trypan blue exclusion. SKN-SH SY5Y cells were cultured as C6 glioma cells but in RPMI medium (GIBCO). Enzymatic assays were performed on the pellets suspended with a Potter-Elvejhem homogenizer in 2 ml of 50 mM Tris-HCl, pH 7.2 ('cell homogenate').

PREPARATION OF SIALYLTRANSFERASE ACCEPTORS

Mucin from ovine submaxillary glands, asialofetuin and ovine asialomucin were prepared as previously reported [46]. Galactosylation of ovine asialomucin with microsomes from porcine submaxillary gland was performed according to Van den Eijnden *et al.* [47] and Baubichon-Cortay *et al.* [46].

Alkaline $NaBH_4$ degradation of asialofetuin was performed in 0.1 M NaOH containing 0.8 M $NaBH_4$ for 68 h at 37 °C, as described by Spiro and Bhoyroo [48]. The reduced oligosaccharides were separated by gel filtration on a column (1.4 cm × 152 cm) of Bio-Gel P-4 (200–400 mesh) in 0.1 M pyridine acetate pH 5.0.

Asialofetuin (30 mg) was incubated for 60 min in 2 ml of 50 mM Tris-HCl, pH 8.4, 25 mM EDTA, 0.2% SDS, 0.5% mercaptoethanol, Triton X100 was added to 0.1% and deglycosylation was performed with 5 U of N-glycanase F (N-glycopeptidase F from *Flavobacterium meningosepticum*, EC 3.2.2.18) for 8 h at 37 °C followed by the addition of 5 U of N-glycanase and overnight incubation. Products were chromatographed on a Bio-Gel P100 column in 50 mM ammonium carbonate pH 7.0, and detergents removed on a column of Bio-Beads SM2 (Bio-Rad) in 10 mM sodium phosphate.

SIALYLTRANSFERASE (EC 2.4.99.4) ASSAYS

CMP-Neu5Ac:Galβ1-3 GalNAc-R α-2,3 sialyltransferase was assayed as described by Broquet *et al.* [49]. The standard incubation medium contained in a total volume of 250 μl:200 μl of Tris buffer containing cell homogenate, 400 μg of asialofetuin, 0.5% Triton X100, 5 mM $MnCl_2$, 40 mM Mes, pH 6.0, and CMP[^{14}C]Neu5Ac (50 nCi). The incubation was performed at 20 °C for 120 min, and the reaction was stopped with 2 ml of a mixture of trichloroacetic acid (10% w/v) and phosphotungstic acid (5% w/v). The precipitate was filtered on GF-B Whatman filters and radioactivity determined. Assays were performed in triplicate. Other assays were performed as above but with 400 μg of one of the following acceptors: native fetuin, asialomucin from ovine submaxillary glands and asialofetuin treated with N-glycanase F.

When Galβ1-3GalNAcα-*O*-benzyl or GalNAcα-*O*-benzyl at 0.05–0.2 M were used as acceptors, the incubation was stopped by adding 1 volume of ethanol. Samples were centrifuged at 3000 × **g** for 5 min and supernatants were processed by descending paper chromatography in ethyl acetate:pyridine:water (10:4:3 by volume) [50, 51]. As a control, some experiments were performed using 1 mU of purified porcine liver Galβ1-3GalNAcα2,3sialyltransferase (Boehringer) in place of C6 cell homogenate.

LECTIN AFFINOBLOTTING

Western blotting with digoxigenin-labelled lectins

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 4–15% gradient separating gel and a 4% stacking gel. Gradient gels were prepared either with Mini-Protean II Multi-Casting Chamber (Bio-Rad) or obtained from Bio-Rad. Prestained molecular weight markers (Bio-Rad) were loaded in adjacent lanes. Proteins were transferred to nitrocellulose (Schleicher and Schuell) and stained with Ponceau S to control the uniformity of transfer. After transfer, the nitrocellulose membranes were blocked with 0.5 g of casein in 100 ml of TBS (Tris-HCl buffer 50 mM pH 7.5 containing NaCl 0.15 M and Tween 20 0.05%) for 30 min, washed twice with TBS for 10 min, and with TBS containing $MgCl_2$ 1 mM, $MnCl_2$ 1 mM and $CaCl_2$ 1 mM ('B1 buffer') for 10 min. The nitrocellulose membranes were incubated for 1 h with digoxigenin-labelled lectins. Digoxigenin-labelled lectin amounts were for 10 ml of B1 buffer: 10 μg for DSA, GNA and SNA; 30 μg for ACA; 40 μg for Con A; 100 μg for PNA. Detection was performed as described by the manufacturer with an antidigoxigenin antibody conjugated with alkaline phosphatase. After transfer some nitrocellulose membranes were processed overnight at 37 °C with 1 U ml^{-1} of neuraminidase in Na acetate, pH 5.5, 4 mM $CaCl_2$ and washed with TBS before lectin affinoblotting.

Helix pomatia affino-blotting

Biotin-labelled *Helix pomatia* agglutinin (3 μg ml^{-1} B1 buffer) and avidin-peroxidase at 0.2 μg ml^{-1} were used for staining. Peroxidase reaction was performed with diaminobenzidine (6 mg in 10 ml of 50 mM Tris-HCl, pH 7.6) and 10 μl of H_2O_2 30%.

Staining of protein blots

Staining of proteins was performed with Protogold Colloidal Gold Solution (BioCell) which gives intense dark red stain by hydrophobic and ionic interactions with proteins. Protogold solution was used as indicated by the manufacturer.

Protein assay

Total protein content was determined using the bicinchoninic acid protein reagent (Sigma) [52].

Results and discussion

Cultured C6 glioma cells were treated with 10 μM all-*trans* retinoic acid from 30 min to 96 h. After 24 h or more, the cells showed typical morphological changes [53] and an increased sialyltransferase activity [44]. Under these conditions, the profileration rate related either to cell number or protein content was not significantly affected.

Retinoic acid treatment of C6 glioma cells leads to increased CMP-Neu5Ac:Galβ1-3 GalNAc-R α-2,3 sialyltransferase (α2,3 ST)

In C6 glioma cells, under our assay conditions, the only sialyltransferase detected was the α2,3 ST transferring Neu5Ac to O-linked Gal-GalNAc [45, 49, 54]. The increase of sialyltransferase activity in RA-treated cells may be due to: (i) the emergence of an α2,6 sialyltransferase acting on O-glycans not previously detected in untreated cells; (ii) an increase of the α2,3 ST acting on O-glycans; or (iii) a sialyltransferase acting on N-glycans.

To check the first hypothesis, we have performed sialyltransferase assays as described [55] with native fetuin as acceptors because fetuin contains two *O*-glycan chains which can be sialylated in α2,6 linkage to Gal NAc. However, no α2,6 sialyltransferase activity was detected in RA-treated cells. An additional control was performed to check possible sialylation of endogenous glycoprotein using digoxigenin-labelled SNA lectin after Western blotting. However, both RA-treated and control C6 cells showed negative staining with SNA indicating the absence of the Neu5Ac α2,6 linkage.

To check the second hypothesis, we used glycoprotein acceptors lacking N-glycan chains. For this purpose,

asialofetuin was treated with N-glycanase and the effectiveness of the treatment was checked by dot blot of the product with digoxigenin-labelled DSA (*Datura stramonium*) specific for Gal β(1-4) GlcNAc disaccharides. We also used ovine asialomucin, which possesses only O-glycan residues, previously galactosylated as described [46]. As shown in Fig. 1, removal of N-glycans from asialofetuin had no effect on sialyltransferase activation by RA. In addition, activation of sialyltransferase with RA was also obtained with galactosylated asialomucin, but to a lower extent due to the lower effectiveness of the acceptor. As a control, the products of sialyltransferase assays obtained with control and RA-treated cells were checked as follows: β-elimination was performed on [^{14}C]Neu5Ac labelled asialofetuin and the oligosaccharides obtained were chromatographed on Bio-Gel P-4. As shown in Fig. 2, enhancement of sialylation in treated cells corresponded only to enhancement of O-glycan sialylation of Gal-GalNAc residues. No previously undetected N-glycan sialylation appeared. It is concluded that the increase of sialyltransferase activity following RA treatment results only from activation of the α2,3 ST previously detected in untreated cells.

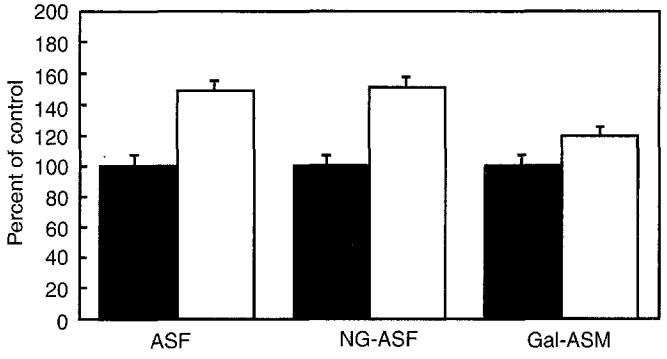

Figure 1. Study of sialylation specificity with different exogenous acceptors. C6 glioma cells were treated or not treated with 10 μM retinoic acid for 24 h then collected and sialyltransferase activity assayed with the following acceptors:asialofetuin (ASF), N-glycanase-treated asialofetuin (NG-ASF) and galactosylated asialomucin (Gal-ASM) prepared as described in Materials and methods. Specific activities (calculated for 1 mg of protein) were related to specific activity of control (without RA). ■, control cells, □, RA-treated cells.

The increase of α2,3 ST is not related to activation of a cellular retinoic acid binding protein (CRABP)

RA is known to act on protein biosynthesis after binding to nuclear receptors, generally after translocation attached to a cytoplasmic protein (CRABP). However, we were unable to demonstrate the existence of CRABP in C6 cells. Effectiveness of the method used was checked with the SKN SH SY5Y neuroblastoma cell line which has a CRABP activity. As shown in Fig. 3, this cell line possesses a protein which binds RA. As some papers assert that RA binding to retinoic acid receptors can

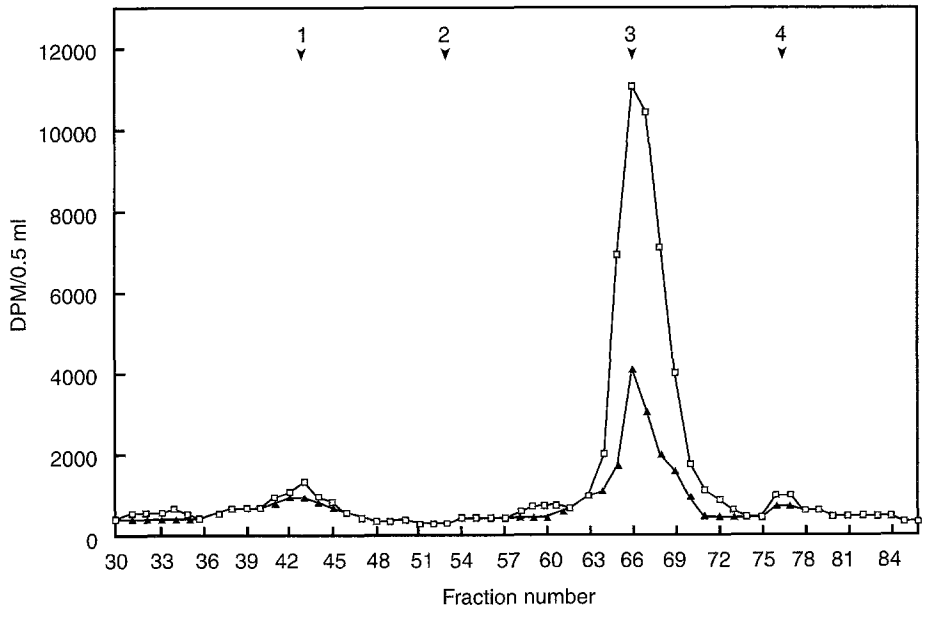

Figure 2. Gel filtration of alkaline borohydride-treated [^{14}C] sialylated asialofetuin on Bio-Gel chromatography. The alkaline borohydride-treated samples were placed on a column (152 × 1.4 cm) of Bio-Gel P4 (200–400 mesh), equilibrated with 0.1 M pyridine-acetate buffer, pH 5.0 and eluted with the same buffer. Fractions of 2 ml were collected and assayed for radioactivity. ▲, control cells, □, RA-treated cells; arrow 1, N-glycans; arrow 2, Disialo O-glycans from fetuin; arrow 3, Monosialo O-glycans from fetuin; arrow 4, CMP-Neu5Ac.

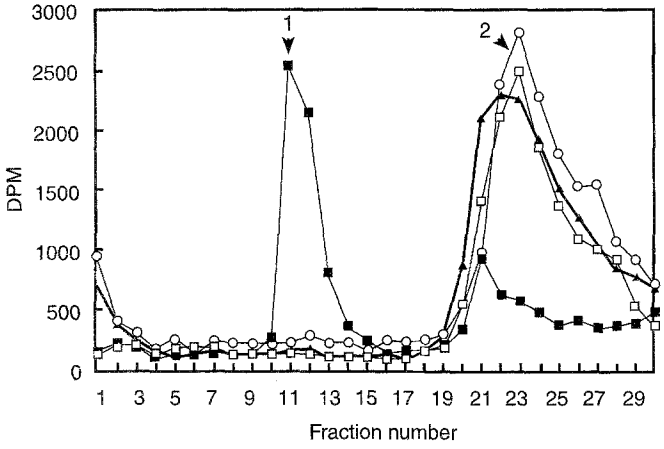

Figure 3. Search for a cellular retinoic acid-binding protein in C6 glioma and neuroblastoma SKN SH SY5Y cells. The supernatant (500 μg of protein) obtained from centrifugation for 30 min at 100 000 × **g** of a cellular homogenate was incubated overnight at 4 °C with tritiated RA (3.7 kBq, 2 pmol) and then chromatographed on a Bio-Gel P30 column (35 × 1 cm). Elution was performed with 50 mM Tris-HCl, 0.2 M NaCl, pH 7.5. ▲, C6 glioma cells + [^{3}H] RA; ○, C6 glioma cells cultured with RA + [^{3}H] RA; ■, SKN SH SY5Y cells + [^{3}H] RA; □, SKN SH SY5Y cells + [^{3}H] RA + unlabelled RA in excess (200 pmol); arrow 1, CRABP; arrow 2, free RA.

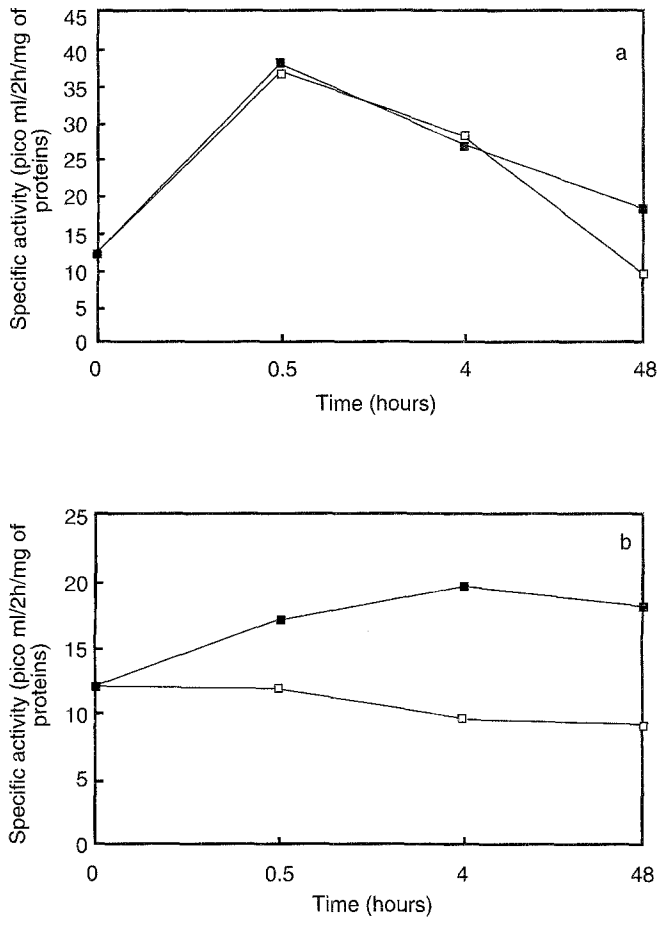

Figure 4. Effect of treatment time with retinoic acid upon α2,3 ST activity. (a) C6 glioma cells were cultured for 30 min, 4 h or 48 h without (□) or with (■) 10 μM RA. Cells were collected and sialyltransferase assayed with asialofetuin as acceptor. Specific activities were expressed as picomol per mg of proteins for time of incubation (2 h). (b) As in Fig. 4a, but after time indicated medium was changed and the cultures continued up to 48 h without RA.

induce CRABP [56], we checked if a CRABP activity appeared in RA-treated C6 cells, which could possibly explain the effects observed. As shown in Fig. 3, no binding protein was detected in RA-treated cells. As Sani *et al.* [36] have described a smaller cytosolic RA-binding protein (4000 Da), we used Sephadex G15 instead of Biogel P30 in our isolation protocol. However no binding protein of small molecular weight was found whether or not the C6 cells were treated (data not shown).

The increase in α2,3 ST corresponds to a de novo synthesis

Immediately after subculture (time from 30 min to 4 h), we observed an increase in α2,3ST activity in control C6 glioma cells and the same increase in RA-treated cells (Fig. 4a). The activation of α2,3 ST due to RA appeared only after 24 h or more of culture in medium with RA [44]. When C6 glioma cells were treated for a short time (30 min to 4 h) with RA, the increase in α2,3 ST activity was also observed but only if culture was extended to 24 h or more in medium without RA (Fig. 4b). This delay in enhancement indicates that the RA effect is due to a *de novo* biosynthesis of the α2,3 ST polypeptidic chain.

We then checked if the RA activation of α2,3 ST was due to enhancement of biosynthesis of all the cellular proteins of C6 glioma cells or to a specific effect on α2,3 sialyltransferase. C6 cells were cultured with 0.5 μg ml^{-1} of cycloheximide or RA or both, and processed as above. Protein biosynthesis was measured both by the total amount of protein after 24 h culture and by [^{3}H] leucine incorporation. As shown in Fig. 5, cycloheximide inhibited protein biosynthesis at levels up to 50% of control, whether the C6 glioma cells were also cultured with RA or not. In contrast, while the total activity of α2,3 ST in cycloheximide treated cells was lowered by the same amount as total protein, the ratio between the activities in RA treated cells and control cells was the same (×2) in cells treated or not treated with cycloheximide. Cycloheximide therefore inhibited α2,3 ST activity to the same extent as protein synthesis but could not inhibit the specific enhancement of α2,3 ST obtained with RA treatment. These results indicate that RA induced the biosynthesis of α2,3 ST.

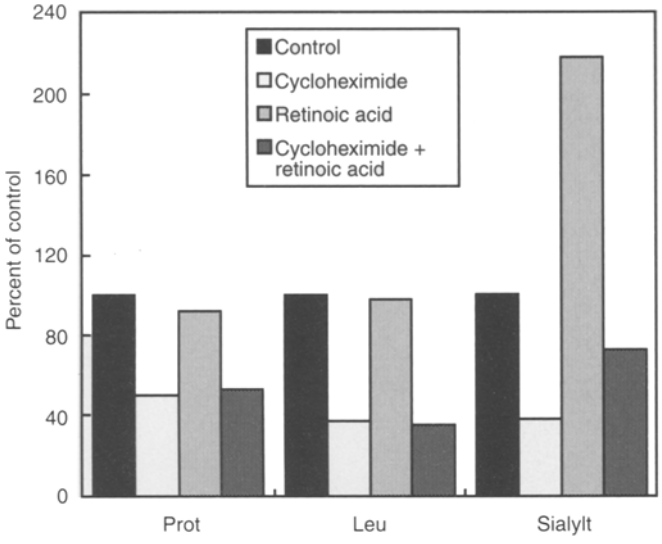

Figure 5. Effect of cycloheximide on protein biosynthesis and α2,3 ST in C6 glioma cells treated or not treated with RA. C6 cells were treated for 24 h with 10 μM RA or 0.5 $\mu g\,ml^{-1}$ cycloheximide or both. Total proteins (prot) were measured with bicinchoninic acid assay, [^{3}H] leucine incorporation (Leu) by a pulse for 30 min with 10 μl [^{3}H] leucine (180 $kBq\,ml^{-1}$) and α2,3 ST (sialylt) related to mg of proteins. All the results were then related to C6 control cell values taken as equal to 100.

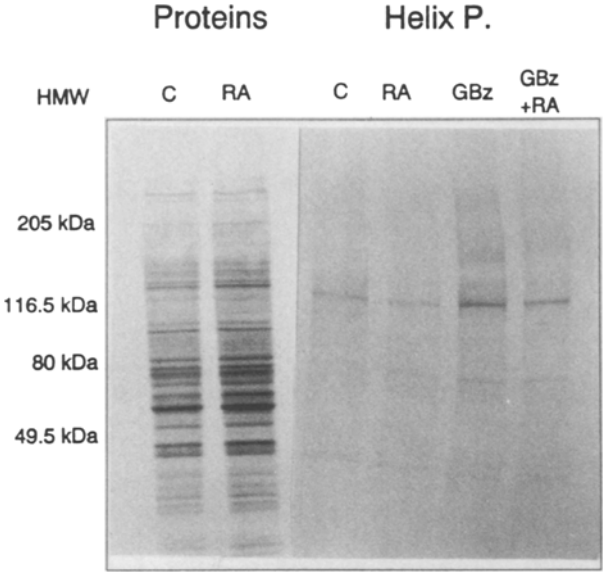

Figure 6. Protein and *Helix promatia* agglutinin staining. Cells were cultured for 72 h with 10 μM RA (RA), or 2.5 mM GalNAcα-*O*-benzyl (GBz) or both (RA + GBz) and collected. C was Control. SDS-PAGE was performed using 4–15% gradient gels. Prestained molecular weight markers from Bio-Rad were run in adjacent lanes. After electrotransfer, proteins were stained with the Protogold solution and GalNAc residues by affinoblotting with biotin-labelled *Helix promatia*. In this and subsequent figures, when indicated, molecular weight markers were myosin (205 kDa), β-galactosidase (116.5 kDa), bovine serum albumin (80 kDa) and ovalbumin (49.5 kDa) for high range prestained standards (HMW); and β-galactosidase (116.5 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa) and soybean tryspin inhibitor (27.5 kDa) for low range prestained standards (LMW).

Study of glycoprotein pattern of control and RA-treated C6 glioma cells

Since RA induces the specific synthesis of α2,3 ST acitivity, we determined whether the glycoprotein pattern of C6 glioma cells was affected by RA-treatment. C6 glioma glycoproteins were analysed by Western blotting with digoxigenin-labelled lectins. Since O-glycan biosynthesis may be implicated, experiments were also performed with *N*-acetylgalactosaminide α-*O*-benzyl (GalNAcα-*O*-benzyl) which has been reported to be an inhibitor of the biosynthesis of O-glycans [57, 58].

As shown in Fig. 6 (Protogold staining), the protein pattern was not significantly affected by RA-treatment. As demonstrated by GNA and ConA affinoblotting (Fig. 7), no evident change was detected, respectively, in high-mannose and lactosamine type N-glycans.

With SNA, specific for α2,6Neu5Ac, no glycoprotein band was detected either in control or RA-treated C6 cells (data not shown) and this result may be correlated with our previous data which demonstrated that α2,6 sialyltransferase activity was not present in C6 glioma cells.

With PNA, specific for Galβ1,3GalNAc, no glycoprotein band was detected in C6 cells, RA-treated or not, indicating that there were no significant amounts of O-glycan-type glycoproteins with free terminal Gal in C6 cells (Fig. 8).

With MAA, specific for α2,3 Neu5Ac either on N- or O-glycans, major glycoprotein bands were detected at levels corresponding to about 75, 120 and 170 kDa. A band is also visible at 220 kDa which was lowered after RA-treatment. In contrast, in RA-treated cells, one high molecular mass band (about 300 kDa) appeared, which was stained faintly in control cells (Fig. 8).

ACA binds to the same residues as PNA, but Gal may or may not be substituted with α2,3Neu5Ac. ACA detected the high molecular weight (300 kDa) glycoprotein in RA-treated cells but stained this band very slightly in control cells (Fig. 8). These results are in accordance with the hypothesis that the 300 kDa glycoprotein is O-glycosylated and that these glycans chains are α2,3Neu5Ac substituted.

An additional control was performed by direct processing of the blots with neuraminidase before staining with ACA and PNA. As shown in Fig. 9, bands previously stained with ACA but not with PNA were now clearly stained with PNA indicating that the 300 kDa band from

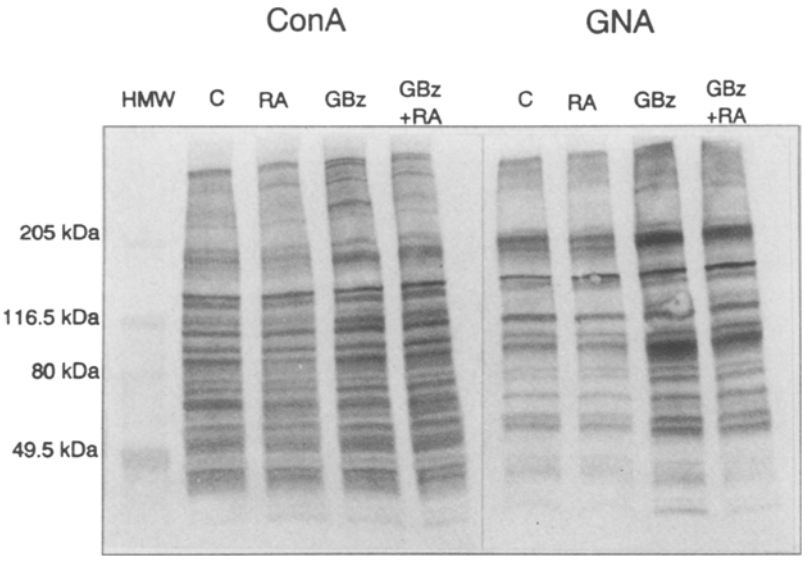

Figure 7. ConA and GNA affinoblottings. Affinoblottings were performed with digoxigenin-labelled ConA and GNA (as indicated in Materials and methods) on cells processed as indicated in Fig. 6.

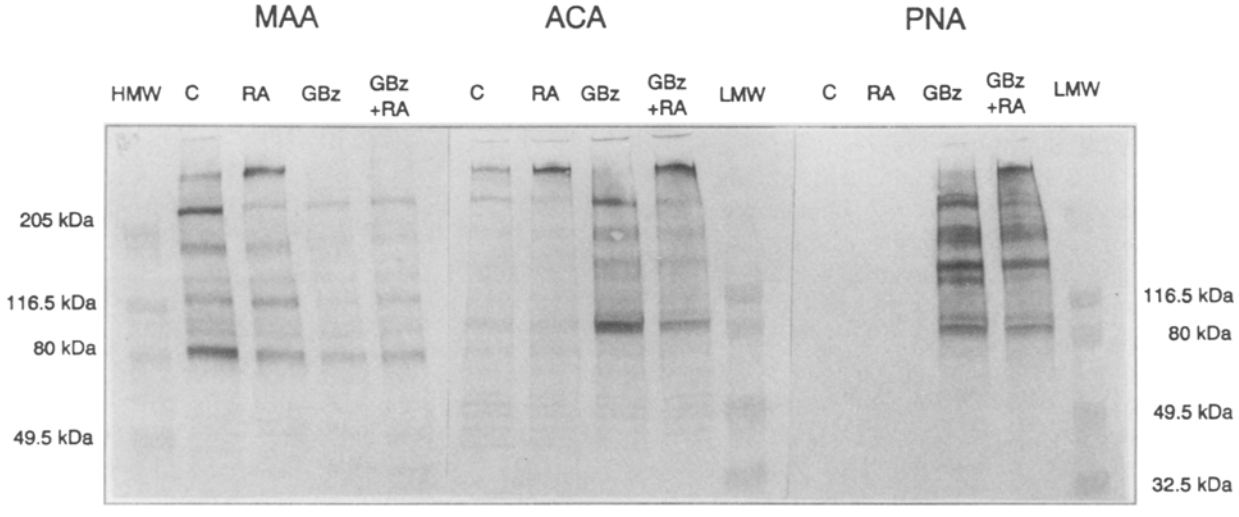

Figure 8. MAA, ACA and PNA affinoblottings. Affinoblottings were performed with digoxigenin-labelled MAA, ACA and PNA on cells processed as indicated in Fig. 6.

RA-treated cells is a glycoprotein with O-glycan chains having the structure Neu5Ac α2,3 Galβ1,3 GalNAc.

Effect of GalNAcα-O-benzyl on glycoprotein pattern of control and RA-treated C6 glioma cells

Simultaneously with the previous experiments, we performed SDS-PAGE and affinoblotting on C6 glioma cells treated with GalNAc-α-*O*-benzyl and with GalNAc-α-*O*-benzyl *plus* RA. For this purpose, the C6 cells were cultured either with 10 μM RA or 2.5 mM GalNAcα-*O*-benzyl or both. Afterwards, cells were collected and washed, and lectin affinoblotting was carried out.

As expected, treatment with GalNAcα-*O*-benzyl showed no modification of N-glycans, whether or not the C6 cells were treated with RA, as demonstrated with GNA and ConA (Fig. 7).

The MAA blot (Fig. 8) showed, after GalNAcα-*O*-benzyl treatment, the same pattern as in control and RA-

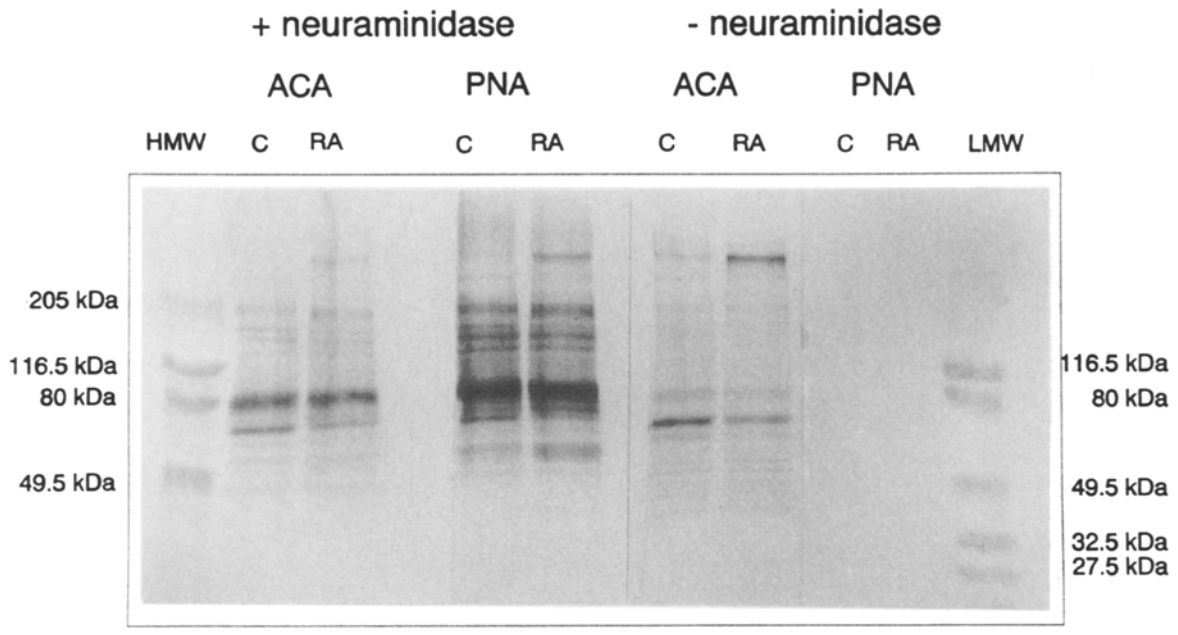

Figure 9. Effect of neuraminidase on affinoblots. After electrophoresis and before affinoblotting with ACA and PNA as in Fig. 8, one half of the blot was incubated with neuraminidase as indicated in Materials and Methods.

treated cells (with less intense staining) except that the 300 kDa band was no longer stained. On the PNA blot, a lot of bands appeared, which were not present in control cells. The ACA blot of GalNAcα-*O*-benzyl-treated cells (Fig. 8) showed the same pattern as the PNA blots as expected, since ACA is able to bind both to Neu5Acα2,3Galβ1,3GalNAc and Galβ1,3GalNAc. This indicates that GalNAcα-*O*-benzyl inhibits not by competition with substrates of *N*-acetyl-galactosaminyltransferase as suggested by Kuan [57], but by another mechanism leading to the accumulation of Gal-GalNAc. Similar results have previously been obtained by Huang *et al.* [59] in human colon cancer cells. These authors claimed that Galβ1,3-GalNAcα-*O*-benzyl generated *in situ* inhibits the elongation of Gal-GalNAc chains more efficiently than GalNAcα-*O*-benzyl due to inhibition of sialylation. However no direct evidence was provided. Huet *et al.* [51] demonstrated that in HT-29 cells selected by adaptation to methotrexate, GalNAcα-*O*-benzyl was metabolized to Galβ1,3GalNAcα-*O*-benzyl.

Using asialofetuin as substrate, Galβ1,3-GalNAc-α-*O*-benzyl is a powerful competitive inhibitor of the sialyltransferase activity of C6 glioma cells, while GalNAcα-*O*-benzyl is not (Fig. 10A); the same effect is observed in RA-treated cells (Fig. 10B). GalNAcα-*O*-benzyl-treated cells were assayed without exogenous acceptor, giving a product which was chromatographically similar to the one obtained by incubation of purified α2,3 sialyltransferase with Galβ1,3-GalNAcα-*O*-benzyl (Fig. 11). Homogenate from C6 cells treated with GalNAcα-*O*-benzyl and heat-inactivated was used as a substrate for α2,3 ST either from C6 control cells or

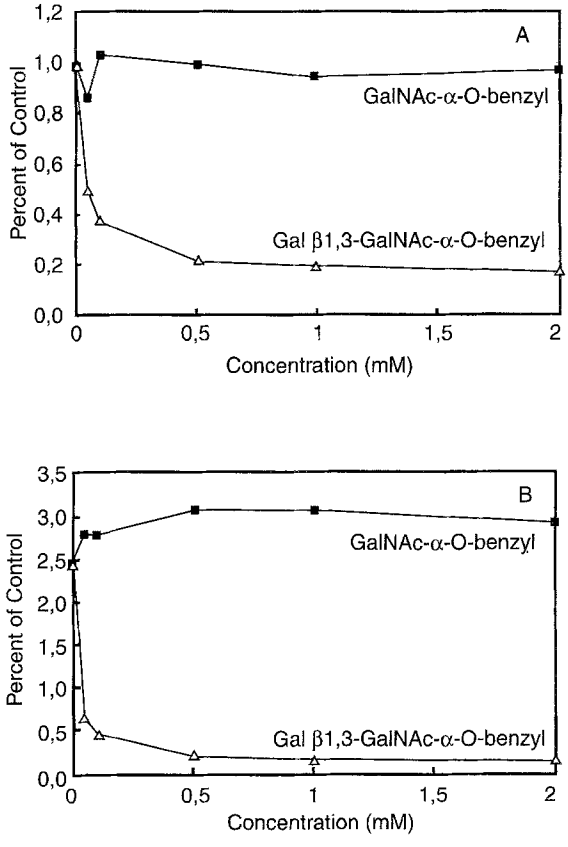

Figure 10. Effect of GalNAcα-*O*-benzyl and Galβ1-3GalNAcα-*O*-benzyl on the transfer of Neu5Ac to asialofetuin. GalNAcα-*O*-benzyl or Galβl-3GalNAcα-*O*-benzyl was added to the incubation medium of the α2,3 ST assay at the concentration indicated in the abscissa. Assays were performed both on homogenates from C6 control (panel A) or RA-treated (panel B) cells.

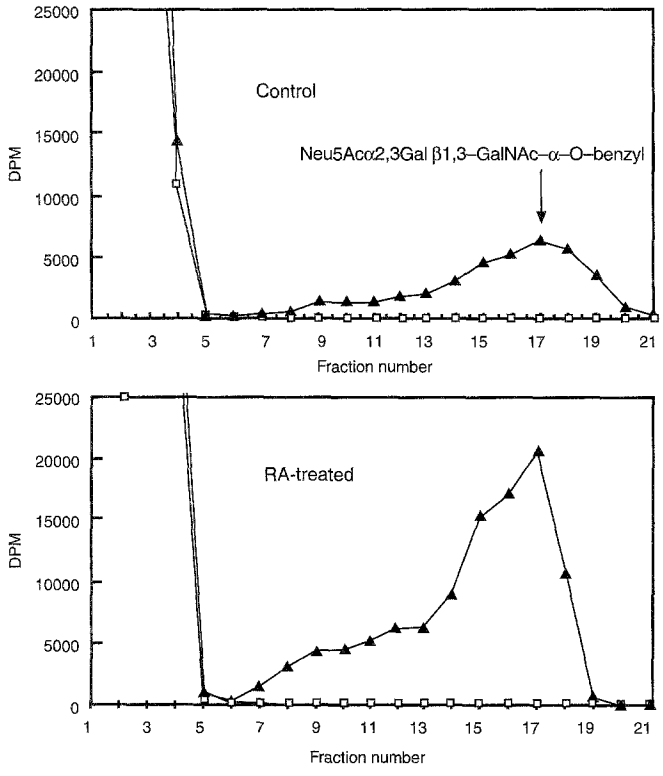

Figure 11. Detection of Galβ1-3GalNAcα-*O*-benzyl in homogenates of C6 glioma cells treated with 2.5 mM GalNAcα-*O*-benzyl. Panel A: homogenates of control C6 cell cultured with (▲) or without (□) GalNAcα-*O*-benzyl were incubated under standard conditions of α2,3 ST assay but without exogenous acceptors and sialylated products were detected as described in Materials and methods. The arrow corresponds to the position of Neu5Acα2-3Galβ1-3GalNAcα-*O*-benzyl obtained by the sialylation of Galβ1-3GalNAcα-*O*-benzyl with porcine liver α-2,3 ST. Panel B: Same as panel A but with C6 glioma cells treated with 10 μM RA.

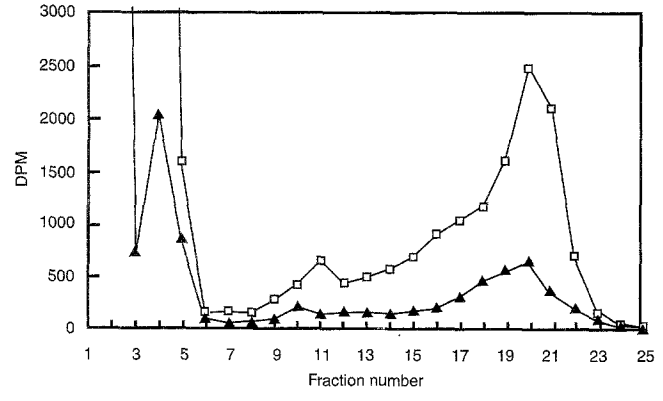

Figure 12. Detection of Galβ1-3GalNAcα-*O*-benzyl in inactivated homogenates of C6 glioma cells treated with 2.5 mM Gal NAcα-*O*-benzyl. Homogenates of C6 glioma cells cultured with GalNAcα-*O*-benzyl were denatured by heating and used as a substrate source in the α2,3 ST assay. Homogenate of control cells (□) or porcine liver α2,3 ST (▲) were used as the enzyme source.

from porcine liver; the same products were formed (Fig. 12). These results are consistent with the ones from Heut *et al.* [51] and we concluded that C6 cells are able to synthesize Galβ1,3GalNAcα-*O*-benzyl from GalNAcα-*O*-benzyl. Therefore, the PNA blotting patterns fit well with inhibition of the sialylation of *O*-glycoproteins by Galβ1,3GalNAc-α-*O*-benzyl. Equal amounts of protein led to a lower staining with ACA than with PNA (Fig. 8), but this did not reflect different amounts of Galβ1,3GalNAcαO-glycans as similar results were observed after treatment with neuraminidase (Fig. 9). The 300 kDa glycoprotein labelled in RA-treated C6 cells was labelled after RA *plus* GalNAcα-*O*-benzyl treatment on ACA blots but also on PNA blots, which demonstrated that lack of sialylation was due to competition with Galβ1,3-GalNAcα-*O*-benzyl generated *in situ* as mentioned above. Low MAA-staining of the 220 kDa band after RA-treatment might suggest that the corresponding glycoprotein is the precursor of the 300 kDa glycoprotein, however this is very unlikely as inhibition of sialylation by Galβ1,3-GalNAcα-*O*-benzyl led to the same Rf for the band (Fig. 8).

Helix pomatia (specific for GalNAc termini) affinoblotting patterns showed no differences after retinoic acid treatment. GalNAcα-*O*-benzyl treatment led to staining of a 60 kDa band (Fig. 6), while no band was stained in the 300 kDa range whatever the treatment. This agrees with our conclusions that the main structure obtained by GalNAcα-*O*-benzyl treatment is Gal-GalNAc and not GalNAc O-glycans. Furthermore, as we were unable to detect an hypothetical GalNAc O-glycan precursor of the 300 kDa glycoprotein, we may assume that this structure does not exist at a significant level in control C6 glioma cells.

Conclusion

RA-treatment of C6 glioma cells led both to increases of CMP-Neu5Ac:Galβ1-3 GalNAc-Rα-2,3sialyltransferase activity and the amount of a high molecular weight (300 kDa) glycoprotein. Affinoblotting led us to conclude that this 300 kDa glycoprotein contains O-glycans with the main structure α2,3Neu5Ac-Gal-GalNAc, suggesting a direct correlation between the two events. Such a correlation has previously been described by Gillepsie *et al.* [60] in developing thymocytes. Since PNA staining of the 300 kDa glycoprotein was not detected in control C6 glioma cells, it is concluded that RA-treatment did not lead to specific sialylation of a 300 kDa precursor glycoprotein. Effectiveness of PNA staining of Gal-GalNAc O-glycans was clearly documented both after the removal of Neu5Ac by processing of blots with neuraminidase and after inhibition of sialylation by Galβ1,3-GalNAcα-*O*-benzyl generated *in situ* from Gal-NAcα-*O*-benzyl. As negative staining was also obtained

with *Helix pomatia* agglutinin, we postulate that the entire O-glycan chain(s) is absent in control C6 glioma cells. As every glycosyltransferase is present in these control cells as demonstrated from lectin affinity blotting, we conclude that the polypeptide chain of the 300 kDa glycoprotein is also lacking in untreated cells and that control of the 300 kDa glycoprotein synthesis is at the expression level, and not due to increased α2,3 ST activity. The role of the 300 kDa glycoprotein induced by RA is presently under study.

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