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CMP-*N*-acetylneuraminic acid hydroxylase activity determines the wheat germ agglutinin-binding phenotype in two mutants of the lymphoma cell line MDAY-D2

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The dominant glycosylation mutants of MDAY-D2 mouse lymphoma cells, designated class 2 (D33W25 and D34W25) were selected for their resistance to the toxic effects of wheat germ agglutinin (WGA) and shown to express elevated levels of Neu5Gc. In accordance with this, the activity of CMP-Neu5Ac hydroxylase was found to be substantially higher in the mutant cells. The hydroxylase in the D33W25 mutant cells exhibited kinetic properties identical to those of the same enzyme from mouse liver. Growth rate experiments *in vivo* and *in vitro*, where the mutant cells grew more slowly at low cell densities in serum-free medium and also formed slower growing tumours in syngeneic mice, indicate that CMP-Neu5Ac hydroxylase expression may be associated with altered growth of the mutant cells.

Keywords: sialic acid; CMP-*N*-acetylneuraminic acid hydroxylase; wheat germ agglutinin; *N*-glycoloylneuraminic acid; glycosylation mutants

Abbreviations: WGA, wheat germ agglutinin; Neu5Ac, *N*-acetyl-β-D-neuraminic acid; Neu5Gc, *N*-glycoloyl-β-D-neuraminic acid; CMP-Neu5Ac, cytidine-5′-monophospho-*N*-acetylneuraminic acid; CMP-Neu5Gc, cytidine-5′-monophospho-*N*-glycoloylneuraminic acid; FACS, fluorescence-activated cell sorting; buffer A, triethylamine hydrogen carbonate, pH 7.6 (concentration given at appropriate points in the text); SFM, serum free medium; IMDM, Iscove's modified Dulbecco's medium. CMP-Neu5Ac hydroxylase, CMP-*N*-acetylneuraminate: NAD(P)H oxido-reductase (*N*-acetyl hydroxylating) (EC 1.14.99.18); CMP-sialate hydrolase (EC 3.1.4.40); sialic acid-pyruvate lyase (EC 4.1.3.3).

Glycosylation mutants of the highly metastatic murine lymphoreticular tumour cell line MDAY-D2 have previously been selected and partially characterized in an effort to determine whether specific classes of oligosaccharides contributed to tumor growth and metastasis [1–3]. Mutant cell lines were selected in toxic concentrations of plant lectins and then grouped into genetic complementation classes by somatic hybridization analysis. The class 2 mutants were selected for resistance to the toxic effects of WGA and the mutation was shown to be dominant in somatic cell hybrids made between mutant and MDAY-D2 cells. The observation that the mutant cells showed no change in sensitivity to lectins other than WGA suggested that sialic acid, a major cell surface ligand for WGA, may have been quantitatively or qualitatively altered in the mutant cells [4]. HPLC separation of sialic acid residues released from cellular glycoconjugates metabolically labelled with [^{3}H]glucosamine in the class 2 mutant D33W25 cells [1] revealed that the majority was in the form of Neu5Gc rather than the Neu5Ac found in the MDAY-D2 cells. The dominant character of the class 2 mutation suggested that a gene coding for CMP-Neu5Ac hydroxylase, the enzyme responsible for Neu5Gc biosynthesis [5, 6], may have been activated in the cells.

In the present study, we have addressed this question by further characterizing the interaction of WGA with the three cell lines and correlating this with their sialic acid composition and level of CMP-Neu5Ac hydroxylase activity. We have also characterized the hydroxylase in one of the mutant cell lines and compared it with the enzyme from other sources. Furthermore, a decrease in growth rate, possibly due to hydroxylase expression is reported.

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Materials and methods

Reagents

Unless stated otherwise, all reagents were of analytical grade from either Merck (Darmstadt, Germany), Sigma Chemicals (Deisenhofen, Germany) or Boehringer (Mannheim, Germany). CMP-[4,5,6,7,8,9-^{14}C]Neu5Ac (specific activity 272 μCi μmol^{-1}) was purchased from Amersham (Braunschweig, Germany). Unlabelled CMP-Neu5Ac was supplied by Sigma Chemicals (Deisenhofen, Germany). Free Neu5Ac and Neu5Gc were prepared as previously described [7, 8]. [6-^{3}H]Glucosamine (52.7 Ci mmol^{-1}) and [^{3}H]thymidine (25 Ci mmol^{-1}) were obtained from Amersham (Toronto, Canada). Sialic acid-pyruvate lyase (specific activity, 10 U(mg protein)$^{-1}$) was supplied by Sigma Chemicals. [6-^{3}H]-*N*-Acetylmannosamine was purchased from New England Nuclear (Dreieich, Germany). [^{3}H]-*N*-Glycoloylmannosamine was synthesized as previously described [9]. Ion exchange resins (Dowex 1-X8, 200–400 μm mesh and Dowex 50-X8, 50 μm mesh) were obtained from Bio-Rad (Munich, Germany).

Cell culture conditions

Cell lines: The origin and properties of the highly metastatic DBA/2 strain lymphoreticular tumour cell line, MDAY-D2, have been previously described [10]. The D33W25 and D34W25 cell lines were selected from MDAY-D2 without prior mutagen treatment in medium containing 25 μg ml^{-1} WGA. The two mutants are independent isolates which were selected in a single round of growth in WGA-containing medium [11].

Sensitivity to WGA: The sensitivity of tumour cells to the toxic effects of WGA was assayed by inoculating 2×10^4 cells into 96 well micro-test plates containing serial dilutions of WGA (Sigma). After 24 h, cells were pulsed with 2 μCi per well of [^{3}H]thymidine for 4 h, then harvested onto glass fibre discs using a Titertek harvester. The discs were washed, and bound radioactivity was quantified in a liquid scintillation counter.

Cell surface WGA binding: Tumour cells were washed with PBS and 2×10^6 cells ml^{-1} were incubated for 1 h with WGA at room temperature. Cells were washed twice with PBS and then incubated with a 1/3000 dilution of rabbit anti-WGA antiserum (Boehringer). The cells were then washed and incubated with a 1/3000 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma) at room temperature for 1 h. The cells were then fixed with 0.25% glutaraldehyde and analysed by FACS using a Coulter EPICS-C machine. Background was determined by omitting the anti-WGA antiserum and subtracted from the experimental values.

Tumour cell growth rates in vitro *and* in vivo: MDAY-D2 and its sublines were routinely propagated in minimal essential medium (Eagle's) supplemented with 5% foetal calf serum in a humidified, 5% CO_2 atmosphere. To measure growth rates in the presence of serum, the cells were harvested, washed twice with PBS and cultures were seeded at 10^4 cells ml^{-1}. Duplicate wells were counted on the following 5 days. For growth in SFM, the cells were grown for 48 h in fresh SFM which consisted of Iscove's modified Dulbecco's medium (IMDM), supplemented with 5 μg ml^{-1} human transferrin, 1 μg ml^{-1} bovine serum albumin and 1.2 μM ethanolamine. The cells were then harvested, washed twice in PBS and reseeded in fresh SFM at a density of 10^4 cells ml^{-1}. Cell number in duplicate cultures was determined on the following 5 days. Logarithmic transformations were performed upon the daily cell counts in order to obtain the number of cell doublings at each time point harvest. A plot of cell doublings versus time was produced by linear regression and slopes were compared by the Student *t*-test.

To compare tumour growth rates *in vivo*, 10^5 tumour cells were injected subcutaneously into syngeneic DBA/2J mice. The minimum and maximum tumour diameters were measured and used to estimate the tumour size index $(\sqrt{D_{min} \times D_{max}}/2)^2\pi$, at several time intervals after injection.

Thin-layer chromatography: Thin-layer chromatography was performed on 10 × 20 cm glass-backed HPTLC cellulose plates (Merck, Darmstadt, Germany) pre-run in the 20 cm direction in solvent system 1 (see below). Solvent system 1, butanol:propanol:0.1 M HCl, 1:2:1 by vol; solvent system 2, butanol:acetic acid:H_2O, 50:12:25 by vol; solvent system 3, 95% ethanol:1 M ammonium acetate, pH 7.3, 7:3 by vol. In radio-TLC analyses, the distribution of radioactivity was quantified using a Berthold 'Tracemaster' linear analyser with computerized integration and a Gaussian peak-fitting algorithm.

Metabolic labelling of cell-associated sialic acids

Tumour cells from each cell line at 2.5×10^6 ml^{-1} were incubated overnight in RPMI with 10% the normal glucose concentration (i.e., 200 mg l^{-1}), 10% dialysed foetal calf serum and 50 μCi ml^{-1} [6-^{3}H]glucosamine. The cells were washed three times in phosphate-buffered saline, suspended in 2 ml 0.1 M NaCl, 2 mM PMSF, 0.024 trypsin inhibitor units ml^{-1} of aprotinin (Sigma), then rapidly freeze-thawed three times to lyse the cells. The lysate was centrifuged at 120 000 × *g* for 1 h. The supernatant was stored frozen at −20°C and the pellet was extracted with chloroform:methanol:H_2O, 2:1:0.05 by vol, for 15 min at 20°C. The lipid and insoluble protein fractions were separated and dried. The supernatant, protein and lipid fractions were worked up and analysed for [^{3}H]sialic acid composition as described below.

Soluble fraction: The supernatant fraction was applied to a 1 × 3 cm column of Dowex 1-X8 (200–400 mesh, hydrogen carbonate counterion) equilibrated in 20 mM triethylamine hydrogen carbonate buffer, pH 7.6 (buffer A) and washed with 20 ml of the same buffer. The wash fraction was

discarded and the column was eluted with 6 ml 0.5 M buffer A. Under these conditions, CMP-bound and free sialic acids were obtained [12]. The eluate was lyophilized and resuspended in 100 mM HEPES, pH 7.4 and acidified to pH 1.0 with 1 M HCl for 1 min at room temperature to hydrolyse the [^{3}H]sialic acids from the CMP nucleotides. After neutralizing with NaOH, the acid-treated eluates were diluted to 3 ml with water and applied to 1 × 3 cm columns of Dowex 1-X8 (200–400 mesh, formate counterion). These were washed with 20 ml water and eluted with 6 ml 1 M formic acid. The eluates were lyophilized, resuspended in 0.1 ml water and 20 µl aliquots were analysed by radio-TLC in system 1. All samples were co-chromatographed with authentic non-radioactive Neu5Ac and Neu5Gc which, after staining with the orcinol:Fe^{3+}:HCl reagent [13], allowed identification of radioactive zones. As a final check on the identity of the [^{3}H]sialic acids, a further 40 µl sample was subjected to 1 h incubation at 37°C with 0.8 U sialic acid-pyruvate lyase in a final volume of 0.12 ml 40 mM potassium phosphate buffer, pH 7.2. The reactions were stopped by addition of 2 volumes of ice-cold water and the released [^{3}H]-*N*-acylmannosamines were isolated free from their parent [^{3}H]sialic acids by passing each incubation mixture through 2 ml columns of Dowex 1-X8 (formate counterion) followed by 2 ml columns of Dowex 50-X8 (H^+ counterion). The tandem columns were washed with 20 ml water and the unbound material was lyophilized, resuspended in 100 µl water and subjected to radio-TLC analysis using solvent system 2. Radioactive *N*-acetylmannosamine and *N*-glycoloylmannosamine standards were chromatographed in adjacent lanes.

Protein and lipid fractions: The dried protein and lipid extracts were subjected to a mild acid hydrolysis in HCl (pH 1) for 1 h at 80°C. After dilution with 1.5 ml cold water, the hydrolysates were applied to 2 ml columns of Dowex 1-X8 (formate counterion). After washing the columns with 15 ml water, the [^{3}H]sialic acids were eluted with 6 ml 1 M formic acid. The lyophilized eluates were resuspended in 0.1 ml water and subjected to radio-TLC analysis (solvent system 1).

Determination of CMP-Neu5Ac hydroxylase activity in fractionated cells

Cell breakage and fractionation: Cell pellets, each of approximate volume 0.5–1.0 ml, were suspended into 5 vol ice-cold 50 mM HEPES:NaOH, pH 7.4, using a narrow pipette and were homogenized on ice using a Branson B-12 sonicator fitted with a microtip for 3 × 10 s allowing 10 s cooling between bursts at a power of about 65 W. The homogenates were centrifuged at 100 000 × *g* (Beckman Ti40 rotor) at 4°C for 70 min. The clear high-speed supernatants were carefully removed and the pellets were resuspended in the same starting volume of the HEPES: NaOH buffer by sonication and were centrifuged again under the same conditions as described above. The supernatants were discarded and the washed pellets were resuspended in 1 ml of the HEPES:NaOH buffer containing 1% Triton X-100. The high-speed supernatants were dialysed separately against 500 ml of the HEPES:NaOH over a period of 16 h at 4°C. The protein concentration in the pellets was about 16 mg ml^{-1} and in the supernatants around 7–13 mg ml^{-1}.

Determination of CMP-Neu5Ac hydroxylase activity: Unless stated otherwise, CMP-Neu5Ac hydroxylase assays were carried out in duplicate at 37°C using 10 µM CMP-[^{14}C]Neu5Ac substrate (50 nCi per incubation), 1 mM NADH and 0.5 mM $FeSO_4$ [6]. The details of individual experiments are indicated in the legends to the relevant figures. Reactions were quenched by addition of HCl to a final concentration of 140 mM, followed by a period of 10 min on ice and centrifugation at 2000 × *g* for 5 min. The formation of CMP-[^{14}C]Neu5Gc, measured as acid-released [^{14}C]Neu5Gc, was determined by quantitative radio-TLC [6].

As indicated in the results, the possible hydrolysis of CMP-sialic acid glycosides by CMP-sialic acid hydrolase was tested for in 60 min incubations under the same conditions described for the hydroxylase. Reactions were stopped by addition of ethanol to a final concentration of 70% by vol. After centrifugation, a 10 µl sample of the supernatant was analysed for [^{14}C]sialic acid and CMP-[^{14}C]sialic acid by quantitative radio-TLC in solvent system 3 [6].

Results and discussion

Tumour cell growth rates in vivo *and* in vitro

The growth rates of the three tumour cell lines in medium containing foetal calf serum were identical, with doubling times of 12–13 h for each line (data not shown). In serum-free medium (SFM), however, the log phase growth rates differed. The rates of division of MDAY-D2 and D34W25 cells were about equal while the growth rate of D33W25 cells was significantly lower. The growth curves of the two mutant cell lines exhibited an initial lag lasting about two days, after which logarithmic cell growth began (Fig. 1). For cultures initiated at 2500 cells ml^{-1}, the doubling times in log phase growth were 14.8 h, 15.0 h and 25.9 h, for MDAY-D2, D34W25 and D33W25, respectively. Comparable results were obtained when cultures were initiated at 5000 cells ml^{-1}, D34W25 and D33W25 again showing an initial lag in growth followed by log phase growth with doubling times of 15.5 h, 14.5 h and 21.3 h, for MDAY-D2, D34W25 and D33W25, respectively.

The slower growth rates of the mutant D33W25 in the serum-free medium suggest that the effect of a growth factor may be suppressed in these cells. Indeed, the growth rate of MDAY-D2 cell lines in SFM has been shown recently

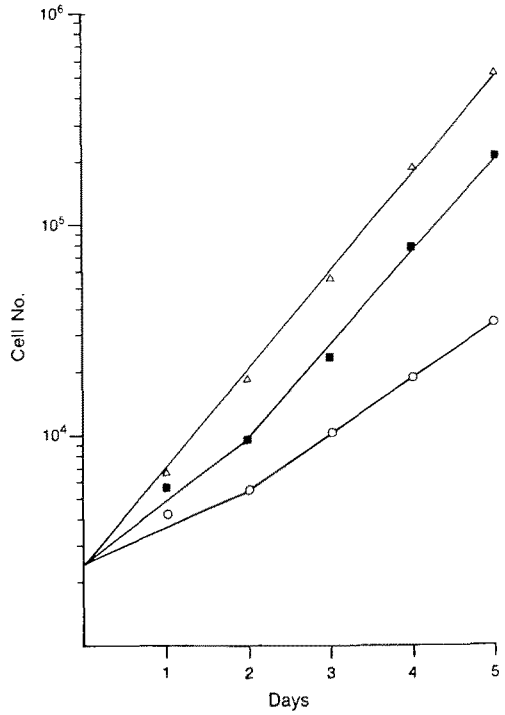

Figure 1. Log phase growth of MDAY-D2, D33W25 and D34W25 cell lines in serum-free medium. Cultures were initiated with 2500 tumour cells per ml medium on day 0 and duplicate cultures were counted daily for 5 days. The growth rates of MDAY-D2 (△), D33W25 (○) and D34W25 (■) cell lines determined by regression of the cell counts measured on days 3, 4 and 5 were 14.8, 25.9 and 15.0 h, respectively.

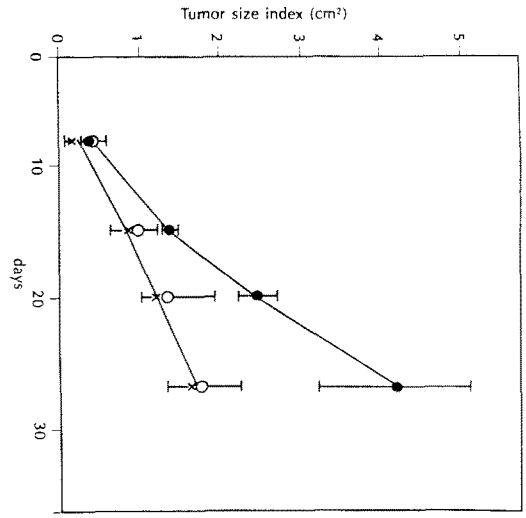

Figure 2. Solid tumour growth of MDAY-D2, D33W25 and D34W25 in DBA/J mice. Tumour cells (10^5 per mouse) MDAY-D2 (●), D33W25 (×) and D34W25 (○), were injected subcutaneously and the tumour size index was determined as described in the Materials and methods section. Mean tumour size indices +/− SD (5 mice per group) have been plotted.

to be accelerated by secretion of an autocrine growth factor [14]. It is therefore possible that the expression of Neu5Gc in the glycoconjugates of this mutant cell line may reduce responsiveness of the cells to autocrine stimulation. The fact that these cells grew slowest at the lower seeding density (i.e., 2500 cells compared to 5000 per ml), while no density-dependent effects were observed in the presence of serum, is consistent with this idea. Alternatively, the mutant cells may produce less autocrine growth stimulating factors. These possibilities will be the subject of future investigations.

In several other experimental tumour models, the ability of tumour cell lines to proliferate in the absence of growth factors in culture has been correlated with enhanced tumour growth when the cells are injected into mice [15, 16]. The reduced growth rate of tumours in mice injected with mutant tumour cells (Fig. 2) suggests that, for these cell lines, a similar relationship may exist between growth in serum free medium and tumour growth rates in mice. Recent studies on glycosylation mutants of MDAY-D2 as well as swainsonine-treated tumour cells suggest that loss or truncation of complex N-linked oligosaccharides results in reduced utilization of autocrine growth factors *in vitro* and reduced tumour growth when cells are injected into mice [14, 17]. The present results suggest that changes in expression of Neu5Gc may also influence tumour cell growth rates in growth factor deprived environments.

WGA binding and toxicity on mutant and wild type tumour cell lines

The WGA sensitivity of the parental MDAY-D2 cells and the mutants D33W25 and D34W25 are shown in Fig. 3(a). From these results, it is evident that at WGA concentrations up to 13 μg ml^{-1} the growth of neither mutant cell line was significantly inhibited, while the rate of division of MDAY-D2 cells was dramatically reduced. At higher concentrations of WGA, however, the D34W25 cells exhibited considerable sensitivity to this lectin, while the D33W25 cells still remained largely unaffected. Since the binding of lectins to cell surface glycoconjugates appears to be responsible for their toxic action, decreased WGA binding to the class 2 mutants may provide an explanation for their resistance to WGA.

The interaction between WGA and the various cell lines was further investigated in experiments using FACS analysis. The results in Fig. 3(b) clearly show that the extent of WGA-binding exhibited by each cell type followed the order MDAY-D2 > D34W25 > D33W25, suggesting that the sensitivity of a cell to WGA correlates with its WGA binding capacity.

Determination of sialic acid composition of each cell line by metabolic labelling

Previous investigations suggest that the reduced binding of WGA by D33W25 cells may be the result of incorporation

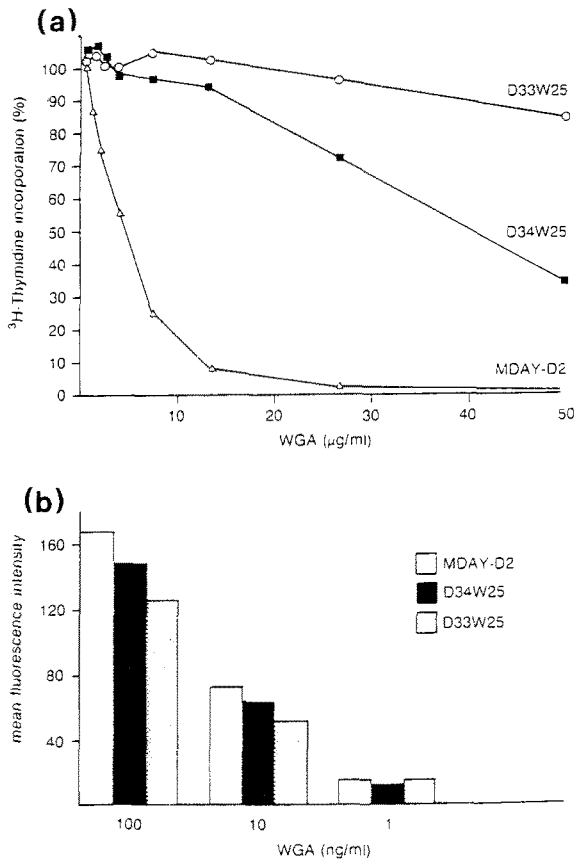

Figure 3. (a) Sensitivity of MDAY-D2, D33W25 and D34W25 cell lines to the toxic effects of WGA. Tumour cells were grown in the presence of increasing concentrations of WGA as indicated on the abscissa for 24 h, then pulsed labelled for 4 h with [^{3}H]thymidine as described in the Materials and methods section. (b) FACS analysis of WGA binding to the tumour cells. Cells were reacted with WGA at 100 ng ml^{-1}, then treated with rabbit anti-WGA antiserum followed by FITC-coated goat anti-rabbit IgG and analysed by FACS as described in the Materials and methods section. The mean fluorescence intensity of the cells is plotted for each cell type +/− SD.

of the sialic acid Neu5Gc in place of Neu5Ac [1]. Metabolic labelling experiments were performed therefore to examine this possibility in both class 2 mutants described here.

Since extracts of [^{3}H]sialic acids from the protein and lipid fractions were relatively free of other radioactive compounds, their analyses proved to be fairly straightforward. By contrast, the soluble fractions contained a large number of tritiated compounds, even after fractionation on two anion-exchange columns (Dowex 1-X8) under different conditions (see the Materials and methods section). For this reason, the relative amounts of [^{3}H]Neu5Ac and [^{3}H]Neu5Gc were quantified as their neutral radioactive *N*-acylmannosamines after a specific enzymatic cleavage using sialic acid-pyruvate lyase and subsequent fractionation by ion exchange chromatography.

Table 1. Sialic acid composition in fractionated cells metabolically labelled with [^{3}H]glucosamine[a].

Fraction	*Cell type*		
	MDAY-D2	D33W25	D34W25
Protein[1]	16	84	89
Lipid[1]	9	71	84
Soluble[2]	20	81	77

[a] Numbers represent the relative composition of [^{3}H]Neu5Gc in each fraction, expressed as a percentage of the total [^{3}H]sialic acid ([^{3}H]Neu5Gc and [^{3}H]Neu5Ac). Analyses were carried out by radio-TLC of (1) intact [^{3}H]sialic acids or (2) [^{3}H]-*N*-acylmannosamines generated by the action of sialic acid-pyruvate lyase on partially purified [^{3}H]sialic acids.

The results presented in Table 1 show that, in the wild type cells, MDAY-D2, [^{3}H]glucosamine was incorporated mainly into Neu5Ac. In the lipid- and protein-associated fractions, Neu5Ac made up 91% and 84%, respectively, of total sialic acid. In contrast, the [^{3}H]sialic acids of the mutant cells, D33W25 and D34W25, consisted of approximately 80% Neu5Gc. A closer examination of the results in Table 1 reveals that the protein fractions contained a slightly larger proportion of Neu5Gc than the lipid fractions, though the general tendency indicated a close correlation between the relative ratios of Neu5Gc and Neu5Ac bound to lipid and protein of one particular cell type.

The sialic acid compositions of the glycoconjugates from the cell lines tested therefore correlate with the WGA binding specificity, since this lectin binds to glycoconjugates which are sialylated with Neu5Ac, but poorly to those terminated with Neu5Gc [18, 19].

Interestingly, the pattern of sialic acid types in the soluble fractions closely mirrored the sialic acid composition in the protein or lipid fractions of the corresponding cell lines (Table 1). Since the soluble fractions contained free or CMP-bound [^{3}H]sialic acids and no glycoconjugate-bound sialic acid (see the Materials and methods section), these results tie in well with the suggestion that the relative amounts of CMP-Neu5Gc and CMP-Neu5Ac produced by a cell determine the Neu5Gc/Neu5Ac ratio in the resulting sialoglycoconjugates [20, 21].

Activity of the CMP-Neu5Ac hydroxylase in supernatant and particulate fractions of the cell lines

The results of a time-course experiment using dialysed high-speed supernatants from all three cell lines are shown in Fig. 4. The hydroxylase activity was readily detected in supernatants of both D33W25 and D34W25 cell lines, turnover being linear with time up to approximately 1 h. The specific activities calculated from these curves were: D33W25, 9.0 (SD 0.49) pmol min^{-1} (mg protein)$^{-1}$; and D34W25, 2.2 (SD 0.1) pmol min^{-1} (mg protein)$^{-1}$. In the

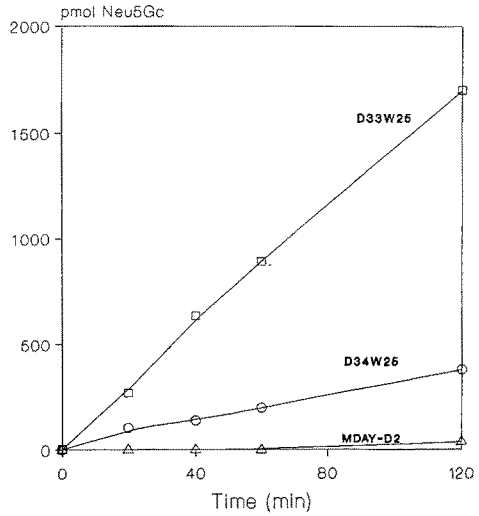

Figure 4. Time course of CMP-Neu5Ac hydroxylation by high-speed supernatants of MDAY-D2, D33W25 and D34W25 cell lines. Dialysed high-speed supernatants of MDAY-D2, D33W25 and D34W25 cells containing 1.32, 1.65 and 1.49 mg protein, respectively, were incubated in duplicate at 37°C in the presence of 10 μM CMP-[^{14}C]Neu5Ac, 1 mM NADH and 0.5 mM $FeSO_4$ in a final volume of 0.24 ml. After the time intervals indicated, 30 μl aliquots were removed and tested for [^{14}C]Neu5Gc as described in the Materials and methods section.

MDAY-D2 supernatants, no activity was detectable in two preparations, though a trace of activity (approximately 0.14 pmol min^{-1} (mg protein)$^{-1}$) was detectable in a third one.

No CMP-Neu5Ac hydroxylase was detectable in the membrane fractions of either MDAY-D2 or D34W25 cells. The particulate fraction of the D33W25 cells did, however, contain 11% of the total hydroxylase activity, though at a much lower specific activity (3.2 pmol min^{-1} (mg protein)$^{-1}$) than in the supernatant. Very little CMP-sialic acid hydrolase activity was detected in the particulate fractions, its possible interference in the hydroxylase assays was thus excluded. Since the homogenization of cultured cells is generally difficult [22], the activity detected in the pellet fraction may have resulted from incomplete cell breakage. It must additionally be noted that the hydroxylase tests on particulate fractions were performed in the presence of Triton X-100 which can activate this enzyme (see [6] and later in these results). The activity in the membrane fractions may thus have been overestimated in comparison with the soluble enzyme. The CMP-Neu5Ac hydroxylase in D33W25 cells is therefore a soluble enzyme, as was reported for the hydroxylase in mouse liver [6], pig submandibular glands [5, 23] and rat intestine [21].

These results thus establish a clear inverse correlation between both WGA binding and sensitivity of the three cell lines and the levels of CMP-Neu5Ac hydroxylase activity. Although the relative amounts of glycoconjugate-bound Neu5Gc generally fit in with this pattern, the MDAY-D2 cells appeared to possess a disproportionately low hydroxylase level in comparison to the low, though readily detectable, quantities of Neu5Gc. Furthermore, the D34W25 cells expressed roughly the same amount of Neu5Gc as the D33W25 cells despite the significantly lower hydroxylase activity and stronger WGA binding exhibited by the former cells. Evidently, other factors, such as the rate of glycoconjugate turnover or possibly the extent of sialylation, may be involved.

Characterization of the soluble CMP-Neu5Ac hydroxylase from the D33W25 cell line

Although CMP-Neu5Ac hydroxylase activity has been demonstrated in several tissues [5, 6, 21] and one cell line [23], only the mouse liver and pig submandibular gland enzymes have been examined with regard to their kinetics and cofactor requirements [5, 6]. It was therefore of interest to compare these enzymes with the hydroxylase in one of the mutant cell lines described in this report.

Cofactor requirements: The effects of various cofactors on enzyme activity are shown in Fig. 5. In the absence of added cofactors, the activity was very low in dialysed supernatants. Activity could be stimulated by the addition of both Fe^{2+} and NADH separately, a very marked stimulation being obtained when both cofactors were added together. With NADPH and Fe^{2+}, the enzyme behaved essentially the same, although this pyridine nucleotide did not support activity as effectively as NADH.

Significantly, the CMP-Neu5Ac hydroxylase activity in non-dialysed supernatants exhibited a considerably lower dependency on exogenously added cofactors. Thus, using a non-dialysed high-speed supernatant, the addition of

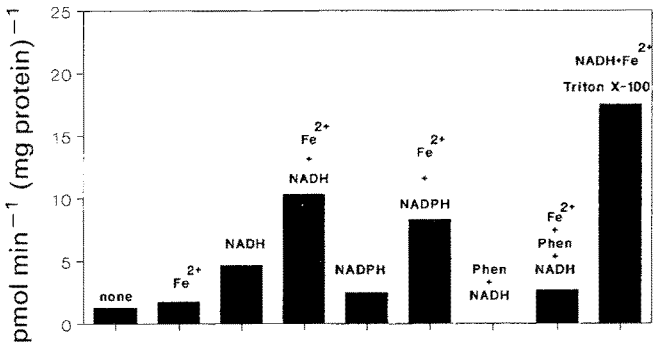

Figure 5. Influence of various substances and cofactors on the activity of CMP-Neu5Ac hydroxylase from D33W25 cells. Duplicate incubations were performed over a period of 50 min at 37°C in a final volume of 60 μl, containing dialysed high-speed supernatant from D33W25 cells (655 μg protein), 10 μM CMP-[^{14}C]Neu5Ac and substances (indicated above the respective bars of the histogram) at the following concentrations: NADH, 1 mM; NADPH, 1 mM; $FeSO_4$, 0.5 mM; *o*-phenanthroline, 1 mM; Triton X-100, 1% by weight.

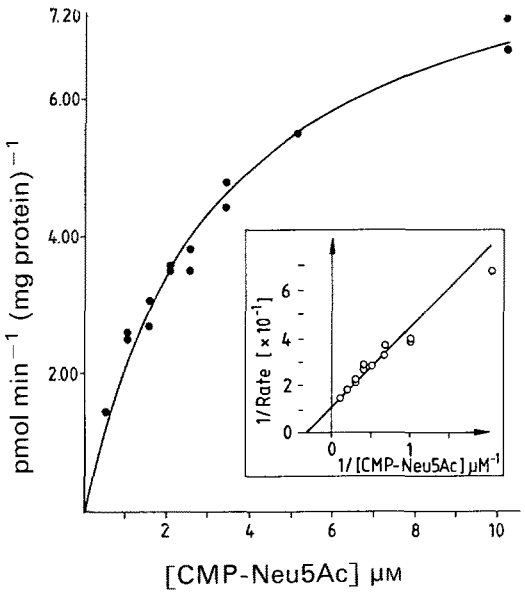

Figure 6. Effect of CMP-Neu5Ac concentration on activity of the hydroxylase. Dialysed high-speed supernatant from D33W25 cells (420 μg protein) was incubated in a final volume of 60 μl at 37°C in the presence of 1 mM NADH, 0.5 mM $FeSO_4$ and the indicated concentrations of CMP-[^{14}C]Neu5Ac. Incubation times were varied according to CMP-[^{14}C]Neu5Ac concentration in order to ensure linear kinetics: 10 μM, 40 min; 5 μM, 20 min; 3.3, 2.5 and 2 μM, 15 min; 1.5 μM, 12 min).

NADH and $FeSO_4$ to 1 mM and 0.5 mM, respectively, gave rise to only a 27% increase in activity over an already significant hydroxylase turnover. These observations suggest that significant amounts of endogenous cofactors are present in the 120 000 × *g* supernatant.

In the absence of exogenously-added Fe^{2+}, NADH-supported activity was virtually abolished by the Fe^{2+} chelator *o*-phenanthroline (Fig. 5). This inhibition could be partially overcome by the addition of excess $FeSO_4$. A similar influence of *o*-phenanthroline both on the mouse liver hydroxylase (L. Shaw, unpublished results) and on the formation of Neu5Gc from free Neu5Ac in crude pig submandibular gland homogenates [24] has also been observed.

The CMP-Neu5Ac hydroxylase from these cells is therefore dependent on a reduced pyridine nucleotide and ferrous ions for its activity and, as such, closely resembles the enzyme in mouse liver and pig submandibular glands.

The non-ionic detergent, Triton X-100, at a concentration of 1% (w/v) was able to activate the enzyme by a factor of about 1.7-fold (Fig. 5). Previous investigations on the CMP-Neu5Ac hydroxylase from mouse liver [6] revealed a similar effect. The significance of this observation is unknown, as the enzyme from both sources is not associated with any membrane fraction after homogenization.

Apparent K_M for CMP-Neu5Ac: A kinetic analysis on the hydroxylase activity with respect to CMP-Neu5Ac concentration, under conditions where turnover was independent of NADH or $FeSO_4$ concentration, revealed that the apparent K_M for the sugar nucleotide substrate was 3.05 μM (SD 0.27 μM) (Fig. 6). This apparent high affinity for the sugar nucleotide substrate is in good agreement with kinetic studies on the enzyme from mouse liver and pig submandibular gland where K_M was 1.3 μM and 2.5 μM, respectively [6, 23].

Thus, with respect to solubility, cofactor requirements and substrate affinity, the hydroxylase extracted from the D33W25 cells is essentially identical to the CMP-Neu5Ac hydroxylase described in mouse liver and pig submandibular gland.

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References

1. Dennis JW (1986) *Cancer Res* **46**:4594–600.
2. Dennis JW, Laferté S, Waghorn C, Breitman ML, Kerbel RS (1987) *Science* **236**:582–5.
3. Dennis JW, Laferté S, Yagel S, Breitman M (1989) *Cancer Cells* **1**:87–92.
4. Peters BP, Ebisu S, Goldstein IJ, Flashner M (1979) *Biochemistry* **18**:5505–11.
5. Shaw L, Schauer R (1988) *Biol Chem Hoppe-Seyler* **369**:477–86.
6. Shaw L, Schauer R (1989) *Biochem J* **263**:355–63.
7. Nöhle U, Shukla AK, Schröder C, Reuter G, Schauer R, Kamerling JP, Vliegenthart JFG (1985) *Eur J Biochem* **152**:459–63.
8. Schauer R (1982) *Adv Carbohydr Chem Biochem* **40**:131–234.
9. Nöhle U, Beau J-M, Schauer R (1982) *Eur J Biochem* **126**:543–8.
10. Kerbel RS, Florian MS, Man S, Dennis JW, McKenzie I (1980) *J Natl Cancer Inst* **64**:1221–30.
11. Lagarde AE, Donaghue TP, Kerbel RS, Siminovitch L (1984) *Somatic Cell Molec Genet* **10**:503–19.
12. Beau J-M, Schauer R, Haverkamp J, Kamerling JP, Dorland L, Vliegenthart JFG (1984) *Eur J Biochem* **140**:203–8.
13. Schauer R (1987) *Methods Enzymol* **138**:132–61.
14. van der Elst I, Dennis JW (1991) *Exp Cell Res* **192**:612–21.
15. Rodeck U, Herlyn M, Menssen HD, Furlanetto RW, Koprowski H (1987) *Int J Cancer* **40**:687–90.
16. Chadwick DE, Lagarde AE (1988) *J Natl Cancer Inst* **80**:318–25.
17. Dennis JW, Kosh K, Yousefi S, van der Elst I (1990) *Cancer Res* **50**:1867–72.
18. Monsigny M, Roche A-C, Sene C, Maget-Dana R, Delmotte F (1980) *Eur J Biochem* **104**:147–53.

19. Maget-Dana R, Veh RW, Sander M, Roche A-C, Schauer R, Monsigny M (1981) *Eur J Biochem* **114**:11–6.
20. Lepers A, Shaw L, Schneckenburger P, Cacan R, Verbert A, Schauer R (1990) *Eur J Biochem* **193**:715–23.
21. Bouhours JF, Bouhours D (1989) *J Biol Chem* **264**:16992–99.
22. Howell KE, Devaney E, Gruenburg J (1989) *Trends Biochem Sci* **14**:44–7.
23. Muchmore EA, Milewski M, Varki A, Diaz S (1989) *J Biol Chem* **264**:20216–23.
24. Schauer R (1978) *Methods Enzymol* **50**:374–86.