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Efficient biochemical engineering of cellular sialic acids using an unphysiological sialic acid precursor in cells lacking UDP-*N*-acetylglucosamine 2-epimerase

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Abstract Sialic acids comprise a family of terminal sugars essential for a variety of biological recognition systems. *N*-Propanoylmannosamine, an unphysiological sialic acid precursor, is taken up and metabolized by mammalian cells resulting in oligosaccharide-bound *N*-propanoylneuraminic acid. *N*-Propanoylmannosamine, applied to endogenously hyposialylated subclones of the myeloid leukemia HL60 and of the B-cell lymphoma BJA-B, both deficient in UDP-*N*-acetylglucosamine 2-epimerase, is efficiently metabolized to CMP-*N*-propanoylneuraminic acid resulting in up to 85% of glycoconjugate-associated sialic acids being unphysiological *N*-propanoylneuraminic acid. Thus, UDP-*N*-acetylglucosamine 2-epimerase-deficient cell lines provide an important experimental progress in engineering cells to display an almost homogeneous population of defined, structurally altered sialic acids. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Biochemical engineering; *N*-Propanoylmannosamine; Sialic acid; UDP-*N*-acetylglucosamine 2-epimerase

1. Introduction

Sialic acids are the most abundant terminal sugar moieties on the surface of eukaryotic cells. As a component of oligosaccharides on glycoconjugates, sialic acids are crucial for many biological processes, such as cellular adhesion [1], formation or masking of recognition determinants [2,3], and stabilization of the structure of glycoproteins [4]. Also, cell surface sialylation has been implicated in the tumorigenicity and metastatic behavior of malignant cells [5,6].

Sialic acid analogues have frequently been employed in in vitro systems to shed light on the role of sialic acids and specific side groups of these 9-carbon aminosugars for sialic acid-dependent cell–cell or pathogen–cell interactions [7,8]. More recently, through the development of unphysiological *N*-substituted D-mannosamine derivatives *N*-acyl side chain modifications of sialic acids could also be introduced into living cells (for review see Keppler et al. [9]). These synthetic D-mannosamines were shown to be taken up by cells, metabolized by the promiscuous sialic acid biosynthetic pathway, and expressed as *N*-acyl-modified sialic acids on the cell surface. A number of subsequent studies have demonstrated a crucial role of the *N*-acyl side chain in various biological processes, including the binding of several viruses to their cellular sialoglycoconjugate receptors [10,11], the proliferation of neuronal cells [12], the density-dependent inhibition of proliferation in human fibroblasts [13] and the binding of myelin-associated glycoprotein to neuronal cells [14].

Synthetic D-mannosamine derivatives likely compete with the physiological precursor *N*-acetylmannosamine (ManNAc) and its metabolic products for the sialic acid biosynthetic machinery, resulting in low to moderate rates of the expression of modified sialic acids on the cell surface. We therefore hypothesized that in the absence of this physiological competition the incorporation efficiency of structurally altered, *N*-acyl-modified sialic acids could be further increased. To test this we exposed two recently identified, endogenously hyposialylated hematopoietic cell lines to *N*-propanoylmannosamine (ManNProp). Due to a lack of UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase), the key enzyme of sialic acid biosynthesis, subclones of the myeloid leukemia HL60 (HL60-I) and the B-cell lymphoma BJA-B (BJA-B K20) cannot synthesize ManNAc and sialic acid de novo. These endogenously hyposialylated cells are only able to recruit a small amount of sialic acids from serum sialoglycoconjugates, probably through a salvage pathway [15]. Here we demonstrate that up to 85% of the total cellular sialic acids can be engineered to carry unphysiological *N*-propanoyl residues in these cell lines.

2. Materials and methods

2.1. Materials

ManNProp was chemically synthesized as previously described [16]. Fluorescein isothiocyanate (FITC)-conjugated *Limax flavus* agglutinin (LFA) was from EY Laboratories (San Mateo, CA, USA). All other chemicals were from Sigma (Deisenhofen, Germany).

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Abbreviations: HPAEC, high performance anion exchange chromatography; FITC, fluorescein isothiocyanate; LFA, *Limax flavus* agglutinin; ManNAc, *N*-acetylmannosamine; ManNProp, *N*-propanoylmannosamine; NeuAc, *N*-acetylneuraminic acid; NeuProp, *N*-propanoylneuraminic acid; UDP-GlcNAc 2-epimerase, UDP-*N*-acetylglucosamine 2-epimerase; VVA, *Vicia villosa* agglutinin

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2.2. Cell culture and sugar treatment

The hyposialylated subclones HL60-I, spontaneously derived from wild-type HL60 cells [15], BJA-B K20, produced by limited dilution of BJA-B cells [17], and two normally sialylated reference cell lines, HL60-II and BJA-B K88 were propagated as suspension cultures in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in a humidified 5% CO_2, 37°C atmosphere. Stock solutions of ManNAc or ManNProp were prepared by dissolving the sugars in medium and subsequent filter sterilization. Cells were seeded at 3×10^5/ml, sugars were added to concentrations of 10 mM, if not indicated otherwise, and cells were cultured in the presence or absence of the sugars for the indicated time periods.

2.3. Quantification of cellular CMP-sialic acids

CMP-sialic acids were prepared and determined by the method of Fritsch et al. [18]. In brief, cells ($1–2\times10^7$) were sonicated in 2 ml of 50 mM ammonium hydrogencarbonate, pH 8.0, and the lysate was centrifuged at $100\,000\times g$ for 30 min. Protein was precipitated by adjusting the supernatant to 70% ethanol. The soluble fraction was applied to a DEAE-Sephacel column. The column was washed with water, sialic acids were quantitatively washed out with 20 mM ammonium acetate, CMP-sialic acids were eluted with 70 mM ammonium acetate. CMP-sialic acids were analyzed by Dionex high performance anion exchange chromatography (HPAEC) using a CarboPac PA-100 column. CMP-sialic acid content was given as nmol/mg of protein. For determination of relative amounts of CMP-*N*-acetylneuraminic acid (CMP-NeuAc) and CMP-*N*-propanoylneuraminic acid (CMP-NeuProp) in cellular CMP-sialic acid fractions, sialic acids were released for 1 h with 2 M acetic acid at 80°C [19] and *N*-acylneuraminic acids were analyzed by HPLC as described below.

2.4. Quantification of membrane-bound sialic acids and N-acylneuraminic acids

PBS-washed cells (1×10^7) were lysed by hypotonic shock in 1 ml water (15 min, 4°C). The crude membrane fraction was pelleted by centrifugation at $10\,000\times g$ for 15 min. The pellet was washed twice with water and lyophilized. Content of membrane glycoconjugate-bound sialic acid was determined by hydrolyzing the pellet for 1 h with 2 M acetic acid at 80°C [19]. Sialic acids were quantified by the thiobarbituric acid method [20] and HPLC analysis, as described below. Similar results were obtained by both methods.

NeuAc and NeuProp were quantified by HPLC analysis. pH values of the acetic acid hydrolysates were adjusted to 4 and further purification was carried out on a cation exchanger (AG-50W-X12, H^+ form; Bio-Rad, München, Germany). *N*-acetylneuraminic acids were fluorescence-labeled according to a method of Hara et al. [21] and analyzed on a reversed-phase C18 column as described [10].

2.5. Flow cytometry analysis

Cells (2×10^6) were washed twice in cold PBS and subsequently incubated in 100 μl PBS containing FITC-labeled lectins, either 20 μg/ml LFA or 50 μg/ml *Vicia villosa* agglutinin (VVA), for 45 min on ice in the dark. After washing with PBS cells were resuspended in 500 μl PBS and analyzed by flow cytometry on a Beckton Dickinson FACScan using Cellquest II software.

3. Results and discussion

3.1. Quantification of membrane-bound sialic acids

To test whether hyposialylated cells can metabolize and incorporate unphysiological, *N*-acyl-modified mannosamines more efficiently, we cultivated HL60-I and BJA-B K20, and two normally sialylated reference cell lines, HL60-II and BJA-B K88 [15,17], in the presence of ManNProp. In a first experiment hyposialylated HL60-I and BJA-B K20 cells were cultivated in the presence of 10 mM ManNAc or ManNProp for 3 days and subsequently, the membrane-bound sialic acid content was quantified by the thiobarbituric acid method (Table 1). This assay detects both *N*-acetyl- and *N*-propanoylneuraminic acids [20].

In HL60-I and BJA-B K20 cells ManNAc and ManNProp treatment increased sialylation, approximately 5- and 3-fold, respectively (Table 1). *N*-Acylmannosamine-treated K20 cells even reached the sialylation status of normally sialylated K88 cells. This suggests that the physiological and unphysiological sialic acid precursor had been metabolized to the respective *N*-acylneuraminic acids at a similar rate and these had then been incorporated into cell surface glycoconjugates. The overall sialic acid content of UDP-GlcNAc 2-epimerase-competent HL60-II and BJA-B K88 cells was unaffected by either D-mannosamine derivative probably reflecting a preexisting saturation of all acceptor sites for sialic acid in these cells.

Next, we analyzed cell surface sialylation in ManNAc- and ManNProp-treated cells by flow cytometry using two fluorochrome-labeled sialic acid-sensitive lectins. In agreement with the results described above, both ManNAc- and ManNProp-treated HL60-I cells showed a drastically increased binding of the sialic acid-specific lectin LFA (Fig. 1), indicative of an increased cell surface sialylation. Interestingly, the increase in LFA binding in ManNProp-treated cells was about 2-fold less than that of ManNAc-treated cells. As shown in Table 1 this is not due to a lower cell surface sialylation in ManNProp-treated cells. Most likely it reflects a reduced binding affinity of LFA to *N*-propanoyl-modified sialic acids. This is supported by data obtained for the second lectin, VVA, which recognizes penultimate carbohydrate structures that can be masked by sialic acids [17]. VVA showed an equivalent or even slightly higher reduction in ManNProp-treated com-

Table 1
CMP-sialic acids and membrane-bound sialic acids in HL60 and BJA-B subclones with or without sugar treatment

Cells	Sialic acid content (nmol/10^7 cells)	CMP-sialic acid content (nmol/10^7 cells)
HL60-I	0.2 ± 0.1	< 0.02
HL60-I+ManNAc	1.2 ± 0.4	0.24 ± 0.04
HL60-I+ManNProp	1.0 ± 0.4	0.26 ± 0.06
HL60-II	2.5 ± 0.4	0.28 ± 0.06
HL60-II+ManNAc	2.8 ± 0.8	1.04 ± 0.12
HL60-II+ManNProp	2.8 ± 0.7	0.26 ± 0.08
BJA-B K20	1.0 ± 0.3	< 0.02
BJA-B K20+ManNAc	2.9 ± 0.4	1.06 ± 0.14
BJA-B K20+ManNProp	3.1 ± 0.4	0.78 ± 0.16
BJA-B K88	3.1 ± 0.5	0.34 ± 0.08
BJA-B K88+ManNAc	3.3 ± 0.6	2.50 ± 0.04
BJA-B K88+ManNProp	3.0 ± 0.4	1.12 ± 0.06

Cells were incubated with 10 mM ManNAc or ManNProp for 3 days. CMP-sialic acid content was determined by HPAEC analysis, sialic acid content was determined by the thiobarbituric acid method. Values are means ± S.D. of four (sialic acid content) and three (CMP-sialic acid content) values, respectively.

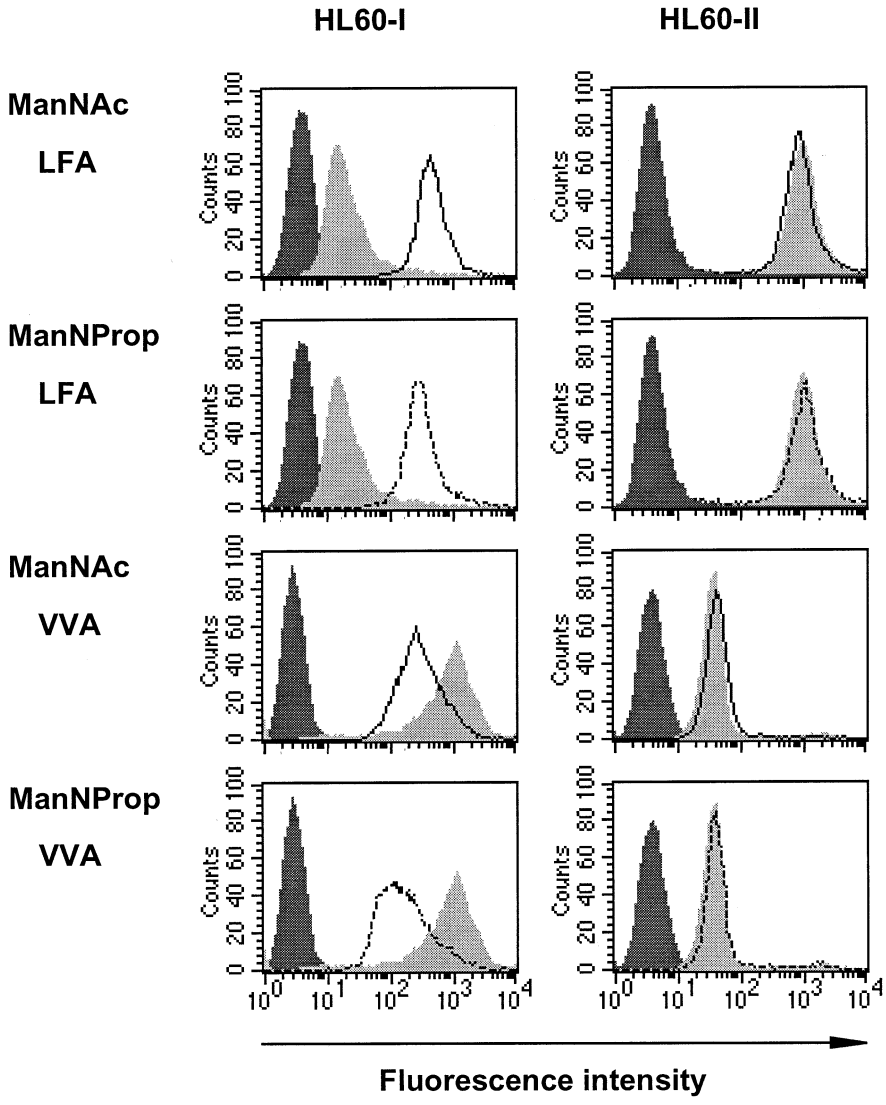

Fig. 1. Detection of cell surface sialic acids by flow cytometry in HL60 subclones with or without sugar treatment. Histograms illustrate the expression of sialic acids (LFA) or unmasked penultimate *N*-acetylgalactosamine residues (VVA) on the cell surface of hyposialylated HL60-I and normally sialylated HL60-II cells as determined by flow cytometry. Cells were treated with ManNAc or ManNProp for 3 days. The histograms for untreated cells (light gray) and cells without lectin staining (dark gray) are shown as references.

pared to ManNAc-treated HL60-I cells (Fig. 1). Furthermore, a model of the sialic acid binding site of LFA has been previously proposed, in which an important role of the *N*-acetyl group for the interaction with this lectin was put forward [22], suggesting that an aliphatic elongation of *N*-acyl side chain could interfere with lectin binding. In HL60-II cells no significant changes in LFA or VVA binding were observed (Fig. 2). This is consistent with the unchanged total sialic acid content of these cells after sugar treatment and with the low percentage of structurally altered sialic acids following ManNProp treatment (Fig. 3).

3.2. Determination of CMP-sialic acids

In order to gain further insight into the metabolism of externally applied *N*-acylmannosamines in both UDP-GlcNAc 2-epimerase-deficient and -competent cells, we investigated the intracellular CMP-sialic acid pool. In human cells CMP-activated sialic acids are normally found as CMP-NeuAc, since no formation of CMP-*N*-glycolylneuraminic acid takes place due to a defect in the respective hydroxylase [23,24]. Other sialic acid modifications, including *O*-acetylation and *O*-methylation, occur after transfer of NeuAc to oligosaccharide chains on glycoconjugates [3]. In untreated BJA-B K20 cells a CMP-NeuAc peak could not be unambiguously defined (Fig. 2A) and their CMP-NeuAc concentration was accordingly set to be lower than 0.02 nmol/10^7 cells, which is the limit of detection of this assay [18]. After treatment of BJA-B K20 cells with ManNAc the chromatogram displayed a peak (Fig. 2B) with an identical elution time and elution profile as commercially available CMP-NeuAc and as CMP-NeuAc detected in BJA-B K88 cells (data not shown).

Treatment of BJA-B K20 cells with ManNProp resulted in a distinct elution peak with a slightly shorter elution time (0.4 min) compared to CMP-NeuAc (Fig. 2C). This is consistent with the small structural difference between CMP-NeuAc and CMP-NeuProp, the latter representing the dominant species of CMP-sialic acid expected to be synthesized in ManNProp-treated K20 cells. However, the only minor difference in elu-

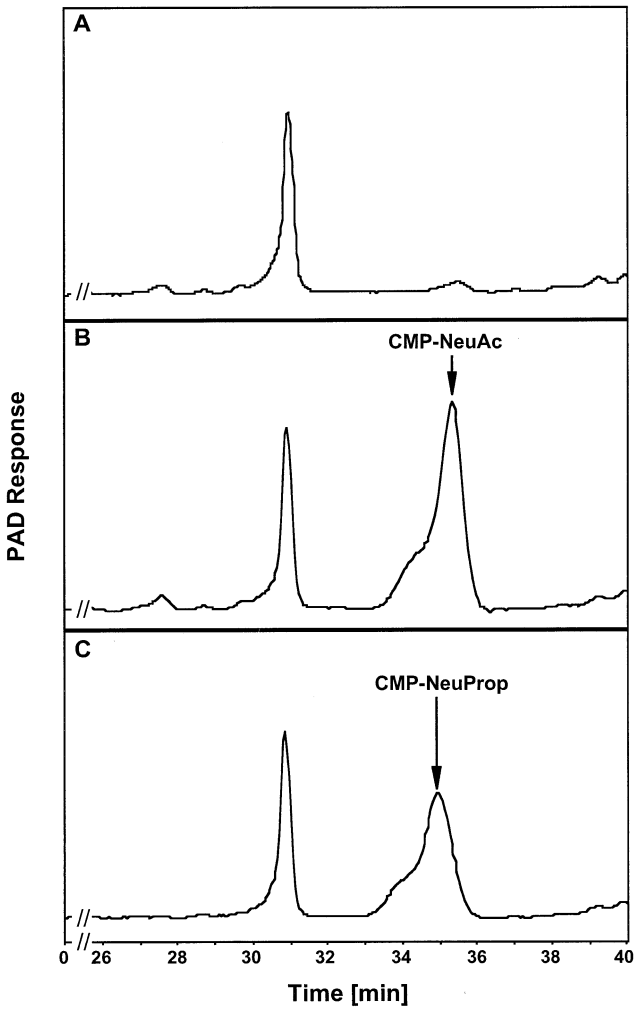

Fig. 2. Detection of cellular CMP-sialic acids after treatment with *N*-acylmannosamines. The CMP-sialic acid fraction was isolated from (A) untreated, (B) ManNAc-treated, or (C) ManNProp-treated BJA-B K20 cells (10 mM, 3 days) and analyzed by HPAEC as described in Section 2. At about 31 min a constant, sample-derived but unidentified peak appeared, which served as internal standard.

tion times between the CMP-activated *N*-acetyl and *N*-propanoylneuraminic acids prevented a clear differentiation of the two compounds in mixed populations, in particular in cell lines with an intact de novo sialic acid biosynthesis. Consequently, the HPAEC analysis was only used for quantification of the total CMP-sialic acid pool.

In both hyposialylated cell lines CMP-sialic acid pools became detectable only after treatment with *N*-acylmannosamines (Table 1). Interestingly, also the CMP-sialic acid pool of ManNAc-treated HL60-II and BJA-B K88 cells, and of ManNProp-treated K88 cells, increased significantly. This is in agreement with results of Fritsch et al. [18] and indicates a bypass of the tightly regulated feedback function of UDP-GlcNAc 2-epimerase [25] by supplying the sialic acid biosynthetic pathway with the epimerase reaction product ManNAc, or its unphysiological *N*-propanoyl analogue. Notably, both HL60-II and BJA-B K88 cells showed a preference for the physiological ManNAc, as indicated by a higher CMP-sialic acid concentration in cells treated with ManNAc or ManNProp at equimolar concentrations. Despite the well documented general promiscuity of the sialic acid biosynthetic machinery towards *N*-acyl modifications [9], these results imply either a reduced uptake or a less efficient metabolism of the *N*-propanoyl analogue to the respective CMP-sialic acid in these cells.

3.3. Relative amounts of N-propanoyl-modified membrane-bound sialic acids and CMP-sialic acids

For an unambiguous discrimination of NeuAc and NeuProp we used HPLC analysis on a reversed-phase column [10]. In a first experiment the relative amount of glycosidically-linked NeuProp on the cell surface of ManNProp-treated cells was investigated over the course of 4 days (Fig. 3). ManNProp treatment at 10 mM for only one day resulted in the expression of significant levels of NeuProp on the cell surface of HL60 and BJA-B cells. Maximal incorporation of the unphysiological precursor was reached after about 3 days. Treatment of cells at 5 mM resulted in a very similar kinetic, but the overall incorporation levels were between 12% (HL60-I) and 40% (HL60-II) lower than those at 10 mM (data not shown).

The kinetics are consistent with data from Ferwerda et al. [26], which showed a maximal incorporation of [^{3}H]ManNAc into sialic acids of rat brain glycoconjugates after about 2 days. Since flux of these compounds through the sialic acid biosynthetic pathway is thought only to take a few hours [26], these much longer intervals for cells to reach maximal incorporation most likely reflect the relatively slow turnover of sialic acids on preexisting cellular glycoconjugates. The average half-life of sialic acid on liver cell surface glycoproteins is in the range of 25–30 h [27]. Consequently, up to 3 days are required to reach maximal replacement of NeuAc by NeuProp in normally sialylated cells.

The ratios of relative concentrations of physiological and unphysiological CMP-sialic acids and membrane-bound sialic acids in HL60-I and HL60-II cells were almost identical (Fig. 4). In contrast, both BJA-B subclones showed a lower relative percentage of *N*-propanoyl-modified compounds in the sialic acid fraction as compared to the CMP-sialic acid fraction. These findings suggest that in HL60 cells the Golgi CMP-sialic acid transporter as well as the most active sialyltransfer-

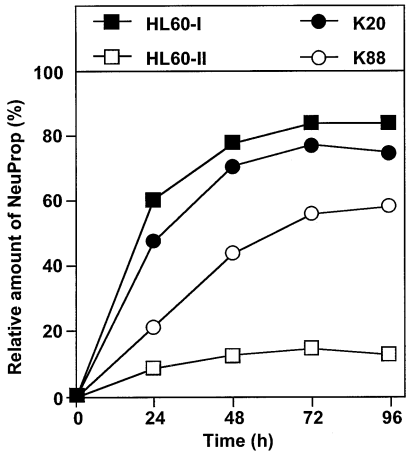

Fig. 3. Kinetic of ManNProp incorporation into membrane-bound sialic acids. Cells were treated with ManNProp (10 mM) and, at the indicated time points, the relative concentration of NeuProp in membrane-bound sialic acids was determined by HPLC analysis. Values are means of two independent experiments.

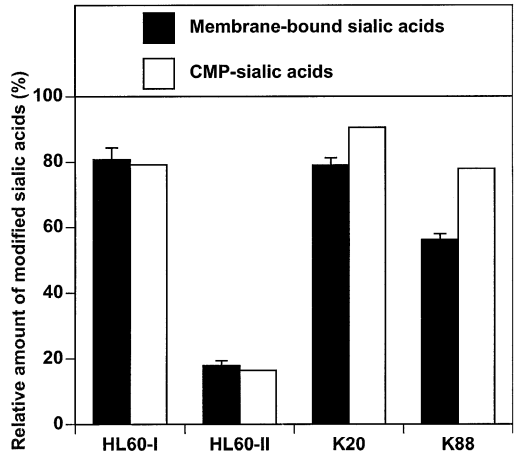

Fig. 4. Relative amounts of *N*-propanoyl-modified membrane-bound sialic acids and CMP-sialic acids in HL60 and BJA-B subclones after treatment with ManNProp. Cells were treated with ManNProp (10 mM) for 3 days. CMP-sialic acids and membrane-bound sialic acids were purified, sialic acids were released by acetic acid hydrolysis, and the percentage of NeuProp in total sialic acids was determined by reversed-phase HPLC analysis. Values for membrane-bound sialic acids represent the arithmetic means ± S.D. of four independent determinations. Values for CMP-sialic acids are arithmetic means of two independent determinations where relative differences between determinations were less than 5%.

ases utilize CMP-NeuProp and CMP-NeuAc with comparable efficiency. The drop in the relative representation of the *N*-propanoyl metabolite in the sialic acid fraction in BJA-B cells could, for example, be explained by a lower affinity of one or several of the most prominent sialyltransferases in these B-cells.

The most efficient NeuProp incorporation was found in HL60-I and BJA-B K20 cells, both of which are UDP-GlcNAc 2-epimerase-deficient (Fig. 3). In contrast, HL60-II cells, which display a high UDP-GlcNAc 2-epimerase activity (50 ± 11 μU/mg of protein [15]), showed a relative NeuProp incorporation of only 15% after 3 days of ManNProp treatment. In BJA-B K88 cells with a moderate 2-epimerase activity (18 ± 2 μU/mg of protein [15]) 56% of total sialic acids were structurally modified. These results show an inverse correlation between the incorporation of unphysiological *N*-acylmannosamines into sialic acids and the activity of UDP-GlcNAc 2-epimerase. Different levels of UDP-GlcNAc 2-epimerase activity probably translate into different amounts of intracellular ManNAc being synthesized, which compete directly with ManNProp for advancing in the biosynthetic pathway. This is underlined by results from Yarema et al. [28], showing that in Jurkat cells with a hyperactive UDP-GlcNAc 2-epimerase of the sialuria type almost no modified sialic acids were incorporated after treatment with modified mannosamine precursors due to an extremely high production level of competing ManNAc.

The very effective incorporation of NeuProp in the hyposialylated subclones resulted in up to 85% modified sialic acids in cell surface sialoglycoconjugates. A similar efficiency has only been described for peracetylated mannosamine precursors (up to 80% [14]). Therefore, the combination of synthetic *N*-acylmannosamine treatment with UDP-GlcNAc 2-epimerase-deficient cells can be used for the engineering of cells with almost homogeneous populations of modified sialic acids, which should further improve the studies of sialic acid-dependent ligand–receptor interactions.

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