Biochemical engineering of the *N*-acyl side chain of sialic acid leads to increased calcium influx from intracellular compartments and promotes differentiation of HL60 cells

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Abstract **Sialylation of glycoconjugates is essential for mammalian cells. Sialic acid is synthesized in the cytosol from *N*-acetylmannosamine by several consecutive steps. Using *N*-propanoylmannosamine, a novel precursor of sialic acid, we are able to incorporate unnatural sialic acids with a prolonged *N*-acyl side chain (e.g., *N*-propanoylneuraminic acid) into glycoconjugates taking advance of the cellular sialylation machinery. Here, we report that unnatural sialylation of HL60-cells leads to an increased release of intracellular calcium after application of thapsigargin, an inhibitor of SERCA Ca^{2+}-ATPases. Furthermore, this increased intracellular calcium concentration leads to an increased adhesion to fibronectin. Finally, we observed an increase of the lectin galectin-3, a marker of monocytic differentiation of HL60-cells.**

Keywords: Cell adhesion; Sialic acid; Signal transduction; Calcium; Galectin-3

1. Introduction

Glycosylation represents the most common post-translational modification of proteins. Glycans of glycoproteins are synthesized in the Golgi apparatus by specific glycosyltransferases, which attach nucleotide-activated monosaccharides to specific residues of glycoproteins. One terminal monosaccharide of nearly all glycoproteins is sialic acid. The most frequent sialic acid in humans is *N*-acetylneuraminic acid (Neu5Ac). The physiological precursor of all sialic acids is *N*-acetyl D-mannosamine (ManNAc). It has been shown that synthetic *N*-acyl-modified D-mannosamines, such as *N*-propanoyl D-mannosamine (ManNProp), can be taken up by cells and efficiently metabolized to the respective *N*-acyl-modified sialic acid in vitro and in vivo [1–4]. These synthetic, unnatural sialic acid precursors are metabolized by the promiscuous sialic acid biosynthetic pathway in the cytosol and are incorporated into cell surface sialoglycoconjugates, where, depending on the cell type, they replace 10–85% of normal, physiological sialic acids (for review: [5]). This method of introducing novel, unphysiological sialic acids into cellular glycoconjugates has been termed biochemical engineering of the side chain of sialic acid (Fig. 1).

Application of ManNProp as sialic acid precursors to different biological systems has revealed important and unexpected functions of the *N*-acyl side chain of sialic acids, such as stimulation of neural cells [6–8].

Sialic acids are involved in the differentiation and maturation of lymphocytes [9,10]. Application of ManNProp leads to an incorporation of Neu5Prop into human T-cells. These biochemical engineered T-cells show increase in the peptidase-activity of CD26 [11], a costimulator of lymphocytes. In agreement with this, an incorporation of Neu5Prop into CD26 was observed [12]. Recently, we demonstrated that application of HL60-cells with ManNProp activates the β1-integrins (VLA4 or VLA5), resulting in an increased adhesion of HL60-cells to fibronectin [13].

Here, we report that application of ManNProp, e.g., incorporation of Neu5Prop into HL60-cells, leads to an increased release of intracellular calcium in the presence of thapsigargin, an inhibitor of SERCA Ca^{2+}-ATPases. Using cell adhesion assays, we demonstrate that the increased intracellular calcium concentration stimulates adhesion of HL60-cells to fibronectin. Finally, we measured an increase of galectin-3 expression on protein level, a marker of monocytic differentiation of HL60-cells.

2. Materials and methods

2.1. Cell culture and antibodies

HL60 cells were propagated as suspension cultures in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere containing 5% CO_2 at 37 °C. Stock solutions of ManNAc or ManNProp were prepared by dissolving the sugars in the medium. The final working concentration was 5 mM. Galectin-3 antibody was a gift of Dr. R. Probstmeier (Bonn).

2.2. Sialic acid precursors

The physiological precursor for sialic acid (ManNAc was purchased from Sigma). The synthetic derivative of sialic acid precursor ManNProp was synthesized as described in [14].

2.3. Sialic acid quantification

Sialic acid was quantified by the periodate/resorcinol method. In brief, lysates from 20×10^6 cells were oxidized in 250 μl with 5 μl of 0.4 M periodic acid at 37 °C for 90 min, followed by 15 min boiling in 500 μl of 6% resorcinol/2.5 mM $CuSO_4$/44% HCl. After cooling for a

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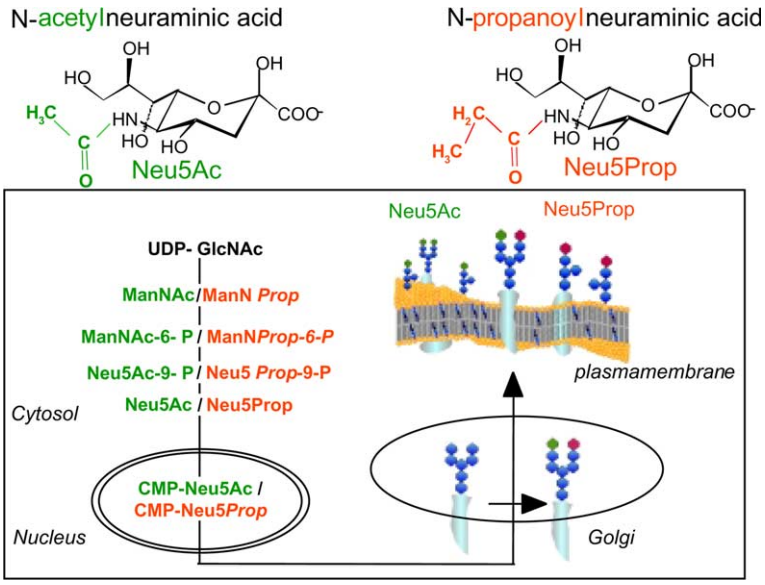

Fig. 1. Schematic representation of the incorporation of unnatural sialic acids into glycoproteins and structure of *N*-propanoylneuraminic acid. Note that sialic acids are activated in the nucleus to the respective CMP-activated sialic acid. Activated sialic acids are then transported across the cytosol into the golgi compartment, where specific sialiyltransferases are located.

few minutes, 500 μl tertbutyl alcohol was added, the samples were vortexed and centrifuged for 5 min to precipitate cell debris. Immediately after spinning, the supernatants were poured into OD cuvettes and read at 630 nm. Sialic acid concentrations were calculated by comparison with a standard curve (usually in a range of 0–250 μM sialic acid) and expressed in nanomoles per mg protein.

2.4. Preparation of cell extracts

Cell pellets were solubilized at 4 °C for 1 h in buffer containing 150 mM NaCl, 50 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton and protease inhibitor cocktail (Sigma) at pH 7.4. Cell extracts were centrifuged at 13 000 rpm for 30 min and supernatants were collected.

Protein concentration was determined in 96-well ELISA plates using 200 μl bicinchonic acid protein reagent (Pierce) and a 50 μl sample. Plates were evaluated in a 96-well ELISA reader (Spectra) at 570 nm.

2.5. Immunoblotting

Samples (150 μg/lane) were separated on SDS-polyacrylamide gels (BioRad) and transferred to nitrocellulose filters. Equal loading was controlled by actin staining (data not shown). The blots were blocked with 4% fat-free dried milk powder in PBS, incubated with the respective primary antibodies, washed with PBS and incubated with the appropriate secondary antibodies conjugated with peroxidase. After washing, proteins were detected by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions, and visualized by exposing the blots to a Fuji imager system (LAS) for time periods between 10 and 120 s.

2.6. Cell adhesion assay

For adhesion assays, 96-well plates were coated with fibronectin (10 μg/ml) for 16 h at 4 °C. Unspecific binding was blocked with 1% BSA for 4 h at 4 °C. Cells were incubated with serum-free medium for 2 h before the experiment. 3×10^4 cells in 100 μl were plated per well. After 1 h of incubation at 37 °C, unattached cells were rinsed away and attached cells were quantified using a cell adhesion quantification kit (Amplificon) according to the manufacturer's instructions.

2.7. Calcium measurements

Cells were cultivated for 48 h in the absence or presence of 10 mM ManNAc or ManNProp, respectively. 5×10^5 ml^{-1} cells were incubated with 1 μl Fluo-3 reagent (Molecular Probes) for 30 min at 37 °C. Cells were washed in indicator-free RPMI-medium (Biochrome) and incubated again for 30 min at 37 °C. Fluorescence was analyzed on a FACS-Vantage (Becton–Dickinson). The non-ratiometric Ca^{2+} indicator, fluo-3, was used to provide information about changes in relative calcium concentrations. Fluo-3 was excited at 488 nm with an argon laser and the fluorescence was measured at an emission wavelength above 510 nm. Acquisition of the fluorescence data and image analysis were performed using CellQuest (Becton–Dickinson) and standard PC evaluation software.

Maximal fluorescence intensity was calculated after application of 1 μg/ml ionomycin (Sigma), background fluorescence was calculated in the presence of 5 mM EGTA. Thapsigargin (Sigma) was used at 100 nM.

3. Results and discussion

3.1. ManNProp does not increase total sialylation in HL60-cells

HL60-cells have been demonstrated to metabolize ManNProp to Neu5Prop and express this on their cell surface [15]. In a first set of experiments, we quantified the total sialic acids of HL60-cells cultured in the absence and presence of ManNAc (the physiological precursor of sialic acid) or ManNProp, respectively. As shown in Fig. 2, neither addition of ManNAc nor addition of ManNProp could increase the total amount of sialic acid in HL60-cells. In all cases, we measured approximately 30 nmol sialic acid per mg cellular protein (Fig. 2).

This is of special interest, because HL60-cells metabolize both ManNAc and ManNProp to the corresponding sialic acid. After application of ManNProp, 20% of Neu5Ac is replaced by Neu5Prop (data not shown and [15]). The fact that the total concentration of sialic acids did not increase after application of ManNAc or ManNProp suggests a cellular regulation and upper limit of the total amount of sialic acids.

3.2. ManNProp modulates the release of Ca^{2+} from intracellular stores

Recently, we could demonstrate that application of ManNProp leads to an increased adhesion of HL60-cells to fibronectin [13]. This increased adhesion is mediated by

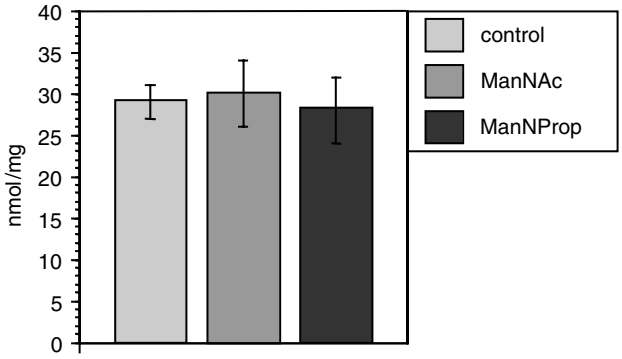

Fig. 2. Quantification of total sialic acid. HL60-cells were cultured in the absence or presence of 5 mM ManNAc or ManNProp for 48 h, respectively. Total sialic acids were quantified using the periodate/resorcinol assay. Bars represent mean values of three independent experiments.

β1-integrins. Since intracellular Ca^{2+}-fluxes activate the Ca^{2+}-requiring enzymes, such as calpain, which regulates cluster formation of leukocyte integrins (for review see: [16]), we measured the intracelluar Ca^{2+}-concentration of HL60-cells cultured in the presence or absence of ManNAc or ManNProp (Fig. 3). When cells were cultured in the presence of ManNProp and extracellular Ca^{2+} was removed by EGTA, we observed a slightly higher intracellular Ca^{2+}-concentration (Fig. 3 EGTA). As a control, intracellular Ca^{2+}-concentration was measured in the presence of ionomycin. No difference could be observed after application of the Ca^{2+}-ionophore ionomycin, which is used to obtain maximal intracellular Ca^{2+}-concentration, when cells were cultivated in the absence or presence of ManNAc or ManNProp (Fig. 3 Ionomycin). However, when HL60-cells were cultured in the presence of ManNProp, application of thapsigargin, an inhibitor of SERCA Ca^{2+}-ATPases, which transport Ca^{2+}-ions from the cytosol to intracellular stores, a dramatic increase of intracellular Ca^{2+}-concentration was observed (Fig. 3, thapsigargin). Since application of ManNProp interferes with the transport of Ca^{2+} into the ER lumen, it can be postulated that ManNProp, e.g., incorporation of Neu5Prop, interferes with multiple cellular signaling cascades.

3.3. *Application of thapsigargin increases the adhesion of HL60-cells to fibronectin after incorporation of Neu5Prop*

In order to investigate whether the increased Ca^{2+}-concentration after application of thapsigargin in Neu5Prop-modified cells has functional consequences, we performed cell adhesion assays on fibronectin substrates. HL60-cells were treated with ManNAc or ManNProp and allowed to adhere to fibronectin in the absence or presence of thapsigargin (Fig. 4). HL60-cells did not adhere to fibronectin in unstimulated status of β1-integrins (Fig. 4, no addition). As a positive control, cells were incubated with the phorbolester PMA known to activate integrin-mediated adhesion of HL60-cells to fibronectin [13]. When stimulated with PMA, HL60-cell adhered to fibronectin, no matter whether modified with Neu5Prop or not. However, when HL60-cells were incubated with thapsigargin, we observed a 50% increase in adhesion only in the case of

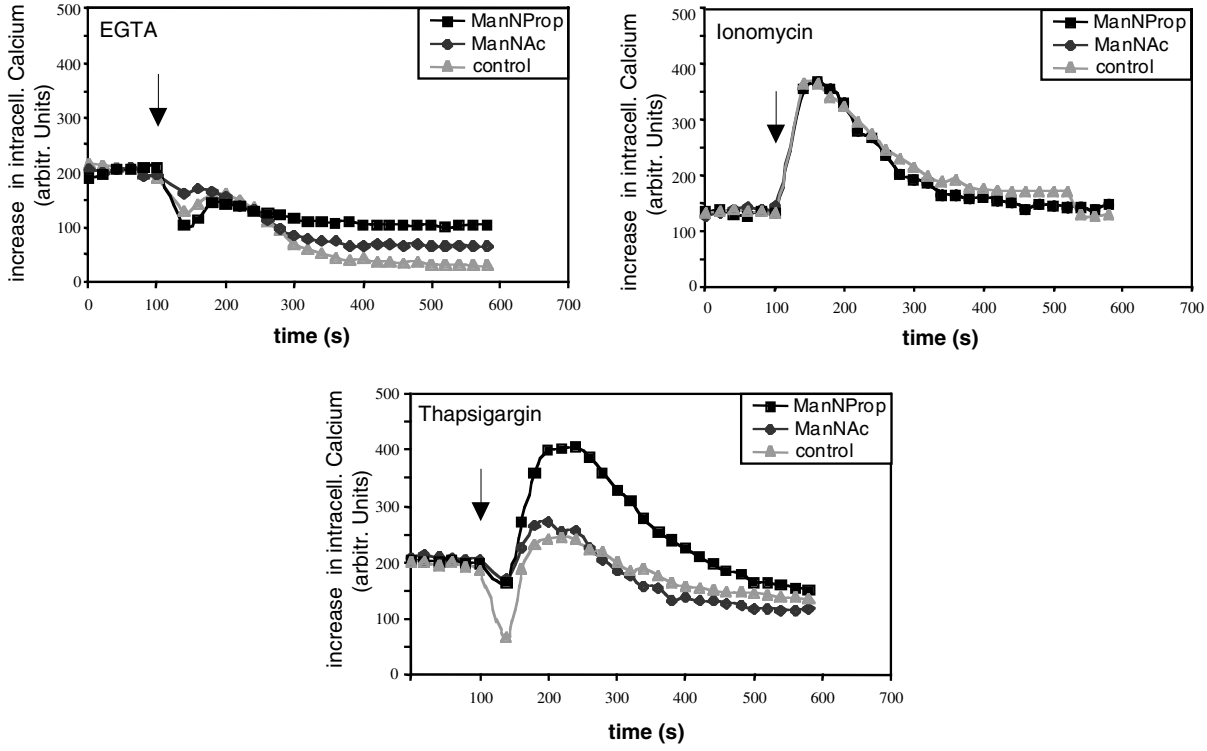

Fig. 3. Calcium signals. HL60-cells were cultured in the absence or presence of 5 mM ManNAc or ManNProp for 48 h, respectively. Cells were loaded with fluo-3 and analyzed for intracellular calcium concentration in the presence of EGTA, ionomycin or thapsigargin, respectively, on a Becton–Dickinson FACScan instrument. EGTA, ionomycin or thapsigargin were added after 100 s (arrow). Curves represent mean values of three independent experiments.

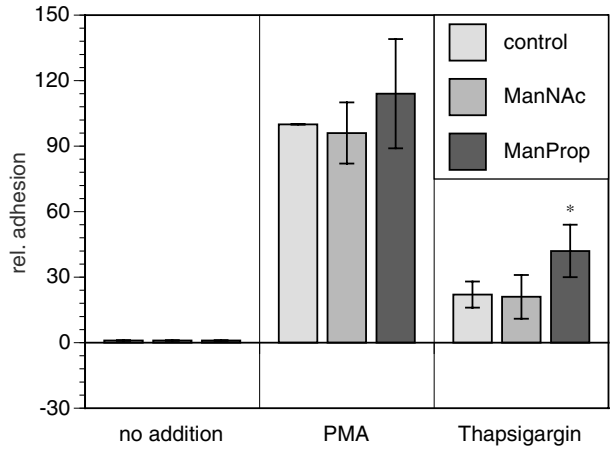

Fig. 4. Adhesion of HL60-cells to fibronectin. HL60-cells were grown in the absence or presence of 5 mM ManNAc or ManNProp for 48 h, respectively. Cells were allowed to adhere to fibronectin coated 96-well plates for 1 h in the presence of 1 nM PMA or 100 nM thapsigargin. Data were obtained from three independent experiments carried out in triplicates.

ManNProp-treated cultures (Fig. 4, thapsigargin). These data fit were to the Ca^{2+}-concentration measurements shown in Fig. 3.

3.4. ManNProp induces increased expression of galectin-3

HL60-cells are a well-known cellular system to analyze myeloid cell differentiation. It has been shown recently that expression of specific galectins describes the status of the differentiation of HL60-cells. PMA is known to promote monocytic differentiation, which goes in parallel with increased expression of galectin-3 [17]. We therefore analyzed expression of galectins in HL60-cells cultured in the absence or presence of ManNProp. No change in the expression of galectin-9 or galectin-10 could be detected (data not shown). However, after application of ManNProp expression of galectin-3 was up-regulated by 66% (Fig. 5). Since galectin-3 is a marker for monocytic differentiation of HL60-cells, we conclude that ManNProp, e.g., biochemical engineering of the *N*-acyl side chain of neuraminic acid leads to monocytic differentiation. This is further supported by the increased adhesion of ManNProp-treated HL60-cells to fibronectin.

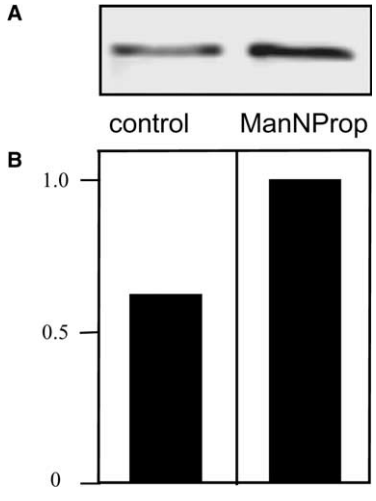

Fig. 5. Expression of galectin-3. (A) HL60-cells grown in the absence (control) or presence (ManNProp) of 5 mM ManNProp for 48 h. Cell solubilisates were analyzed by Western blot using galectin-3-specific antibodies. One out of two Western blots is shown. (B) Quantification of the representative Western blot shown in (A).

Taken together, we proposed that the *N*-acyl side chain of neuraminic acid modulates the function of the SERCA Ca^{2+}-ATPases. The fact that thapsigargin increases intracellular Ca^{2+}-concentration only after application of ManNProp raises the question of how ManNProp or Neu5Prop is able to modulate the function of SERCA Ca^{2+}-ATPases. SERCA Ca^{2+}-ATPases belong together with Na^{+}-ATPases to the P-type ATPase family (for review see: [18]). Na^{+}-ATPases are heterodimers consisting of an alpha and beta subunit (for review see: [18]). The beta subunit is highly glycosylated and removal of all carbohydrate structures results in the complete loss of enzymatic activity [19]. Since also SERCA Ca^{2+}-pumps have several potential *N*-glycosylation sites, it is possible that incorporation of Neu5Prop in SERCA Ca^{2+}-pumps interferes with its function.

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