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Biochemical Engineering of the Side Chain of Sialic Acids Increases the Biological Stability of the Highly Sialylated Cell Adhesion Molecule CEACAM1

Rüdiger Horstkorte,[1] Hye-Youn Lee, Lothar Lucka, Kerstin Danker, Lars Mantey, and Werner Reutter

Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-14195 Berlin-Dahlem, Germany

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The biological half-life time of many glycoproteins is regulated via terminal sialic acids. In this study we determined the half-lives of two different cell adhesion molecules, CEACAM1 and the α1-integrin subunit, in PC12-cells before and after biochemical engineering the side chain of sialic acids by the use of *N*-propanoylmannosamine. Both are transmembrane glycoproteins. While the immunoglobulin superfamily member CEACAM1 mediates homophilic cell–cell adhesion the α1-integrin subunit is involved in cell–matrix interactions. We found that the half-life of the highly sialylated CEACAM1 is increased from 26 to 40 h by replacement of the *N*-acetylneuraminic acid by the novel, engineered *N*-propanoylneuraminic acids, whereas the half-life of the α1-integrin subunit remains unaffected under the same conditions. This demonstrates that biochemical engineering not only modulates the structure of cell surface sialic acids, but that biochemical engineering also influences biological stability of defined glycoproteins.

Key Words: **α1-integrin; CEACAM1; *N*-propanoylmannosamine; PC12-cells; sialic acid.**

Sialylation of glycoproteins and glycolipids plays an important role during development, regeneration, and pathogenesis (1). The physiological precursor of all sialic acids is *N*-acetylmannosamine (ManNAc). In previous studies we and others have shown that the novel, non-physiological *N*-propanoylmannosamine (ManNProp) is metabolized by the same route as the physiological ManNAc to *N*-propanoylneuraminic acid in *in vitro* and *in vivo* experiments (Scheme 1 and 2–6). This provides a novel tool for investigating the biological significance and prospective potency of the *N*-acyl side chain of sialic acid. This new procedure, termed biochemical engineering, applied to different cell systems, has so far revealed several unexpected biological characteristics of the *N*-acyl side chain. First, treatment of lymphoma cells with ManNProp reduced their infectibility by several sialic acid-dependent viruses, e.g., influenza A virus (3). Second, treatment of human diploid lung fibroblasts resulted in a loss of density-dependent growth control (7). Third, treatment of neural cell cultures of newborn rats resulted in a stimulation of the proliferation of astrocytes and microglia, and increased the number of oligodendrocyte progenitor cells (4). Furthermore, oligodendrocytes show calcium spiking in response to GABA, after biochemically engineering their cell surface with the novel sialic acid precursor ManNProp (5). For a detailed review see (8). The new method for the biochemical engineering of sialic acids has been extended by the group of Carolyn Bertozzi. They used *N*-acylmannosamine in which the acyl group contained a reactive ketone structure, namely *N*-levulinoylmannosamine (9).

Many studies have shown that terminal sialic acids are important determinants in the degradation of glycoproteins (10). Therefore, we asked the question whether biochemical engineering of the side chain of sialic acid on cell membranes of rat PC12-cells effect the degradation (e.g., biological half-life) of cell adhesion molecules. As model proteins we determined the half-lives of CEACAM1 and α1-integrins in normal and engineered PC12-cells.

CEACAM1 is one member of the carcinoembryonic antigen-related proteins (11). It is also a transmembrane glycoprotein containing large amounts of sialic acids (12), and it is widely expressed in the epithelial cells of multiple tissues (13). Apart from adhesion, several other CEACAM1 functions have been described. CEACAMs function as molecules with tumor suppressor activity, as receptors for bacteria and viruses, as activators of human neutrophilic granulocytes, as cytoskeletal interacting molecules and as pro-

[1] To whom correspondence should be addressed. Fax: +49/30/8445-1541. E-mail: rhorstko@zedat.fu-berlin.de.

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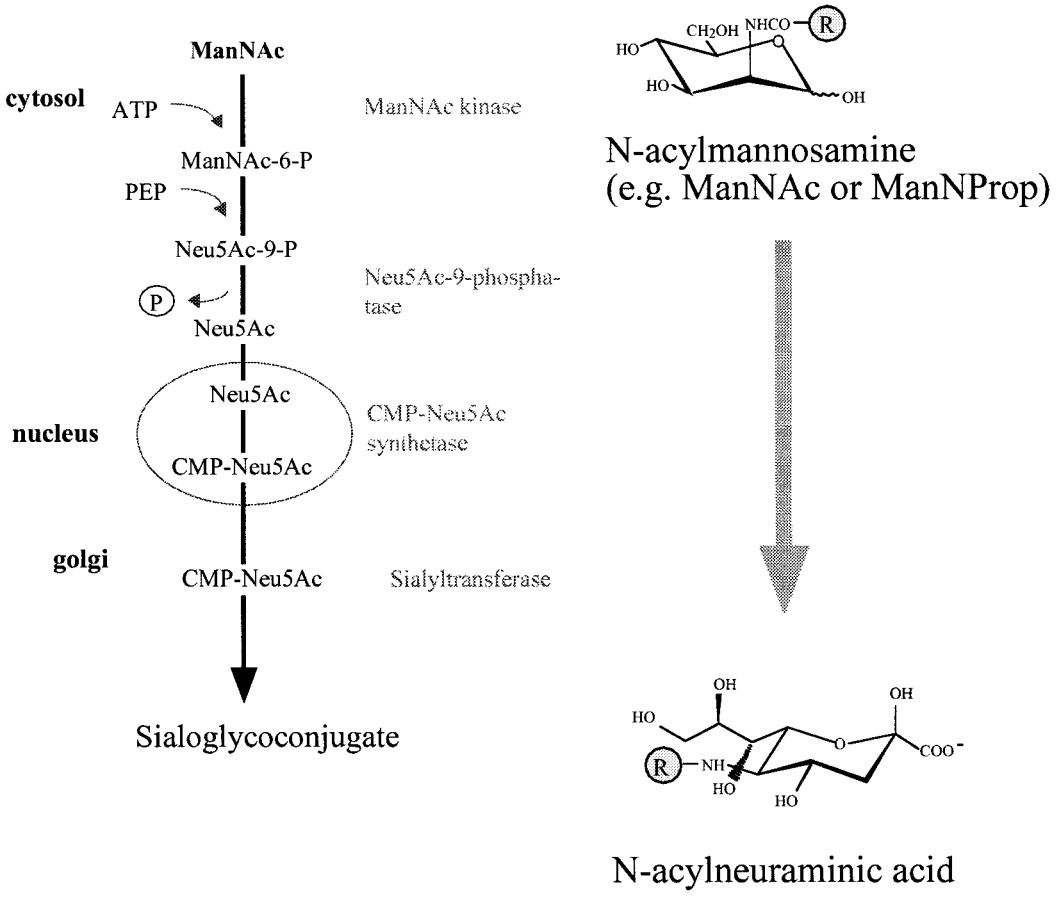

R:	formula
- acetyl (Ac)	-CO-CH_3
- propanoyl (Prop)	-CO-CH_2-CH_3

SCHEME 1. Biosynthetic pathway of physiological and engineered sialic acid precursors.

teins involved in signal transduction (for review, see 14). The integrin $\alpha_1\beta_1$ was first identified as the largest of a series of very late antigens (VLAs) expressed by T-cells and was shown to be a receptor for collagens and laminins (15).

Here we report that the biological stability of the highly sialylated CEACAM1 is prolonged whereas the stability of α1-integrin subunit is unaffected after biochemical engineering and incorporation of *N*-propanoylneuraminic acids into cell surface glycoproteins.

MATERIALS AND METHODS

Cell culture and antibodies. PC12-cells were routinely cultivated in Falcon plastic flasks using RPMI 1640 supplemented with 10% horse serum.

Monoclonal anti-CEACAM1 antibody BE 9.2 (16) and polyclonal anti α1-integrin AS2K5 (17) were used as IgG fractions. Antibodies to rabbit or mouse immunglobulins conjugated to peroxidase were obtained from Dako. AffiniPure Rabbit AntiMouseIgG was obtained by Dianova.

Biotinylination of cell surface proteins. Plasma membrane proteins of PC12-cells growing on Poly-D-lysine substrate were biotinylated as described (18, 19). Sulfo-NHS biotin was purchased from Pierce.

Analytical procedures. Protein 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.

Preparation of detergent 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. Solubilisates were centrifuged at 13,000 rpm for 30 min and supernatants were collected.

Immunoprecipitation. Five milligrams of protein A-Sepharose (Sigma) were precoated with 36 μg rabbit anti-mouse IgG (Dako) or 10 μg anti-α1-integrin antibodies AS2K5 in PBS for 2 h at room temperature then washed twice with lysis buffer. Rabbit anti-mouse precoated Sepharose was further incubated with 12 μg mAb Be 9.2. Precoated protein A-Sepharose beads were added to detergent cell extracts, further incubated for 3 h at 4°C, then pelleted by centrifugation. Prior to SDS–PAGE, pellets were washed three times with lysis buffer and once with PBS.

Immunoblotting. Samples were separated on SDS–polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose filters. The blots were blocked with 10% BSA in PBS and incubated with the respective primary antibodies or with streptavidin conjugated with peroxidase (Sigma), washed with PBS, and incubated with the appropriate secondary antibodies (Dako). After washing, proteins were detected by enhanced chemiluminescence according to the manufacturer's instructions and visualized by exposing the blots to Kodak Biomax films for between 10 and 120 s.

Determination of the half-lives of CEACAM1 and α1-integrin subunit. After pulse labeling with biotin, cells were incubated for 0, 4, 12, 24, and 48 h. At each time point cells were solubilized and the molecules were immunoprecipitated as described under immunoprecipitation. The rate constants for degradation were calculated from the decay of the specific signal of immunoprecipitated CEACAM1 or α1-integrin subunit.

Quantification of N-acetyl- and N-propanoylneuraminic acid. PC12-cells were maintained for one or three days in the presence or absence of 5 mM ManNProp then harvested and pelleted. Cell pellets (10^7 cells) were lysed by hypotonic shock in distilled water and repeated freezing and thawing (two times). The crude membrane fractions were pelleted by centrifugation at 30,000*g* for 20 min (4°C), and the pellets were lyophilized. Glycolipids were extracted using three different methanol/chloroform mixtures (1:2, 1:1, 2:1, v/v) for 30 min each, followed by centrifugation at 10,000*g* (30 min, 4°C). Glycoprotein-containing pellets were hydrolyzed and sialic acids were purified and fluorescence-labeled as described (3). Labeled sialic acids were chromatographed using a reversed phase C18 column (Lichrosorb C18 5 μm 250 × 4.6 mm, Knauer, Berlin, Germany) with a flourescence detector (Ginkotek, excitation wavelength: 377 nm; emission wavelength: 448 nm). Eluent A contained distilled water while eluent B contained acetonitrile/methanol (60:40, v/v). The flow rate was 1 ml/min. Separations were carried out using a gradient running for the first 20 min in the isocratic mode with 10% B. B was then raised to 25% within 25 min and finally to 50% within the subsequent 15 min. Eluted neuraminic acids were identified by MALDI TOF MS and quantified using defined standards as already described (3).

RESULTS

PC12-Cells Metabolize ManNProp and Incorporate N-Propanoylneuraminic Acid

We first analyzed whether PC12-cells are able to metabolize ManNProp and incorporate it as *N*-propanoylneuraminic acid on their cell surface. For this purpose, PC12-cells were cultured for 1 or 3 days in the presence of 5 mM ManNProp. To test whether PC12-cells synthesize *N*-propanoylneuraminic acid (Neu5Prop) from the appropriate precursor (ManNProp), all sialic acids of membrane glycoproteins of ManNProp-treated PC12-cells were isolated and quantified by HPLC. When maintained for 1 day in the presence of ManProp, 24% of the protein-bound sialic acids consisted of *N*-propanoylneuraninic acid. After 3 days this value had risen to 35% (Fig. 1).

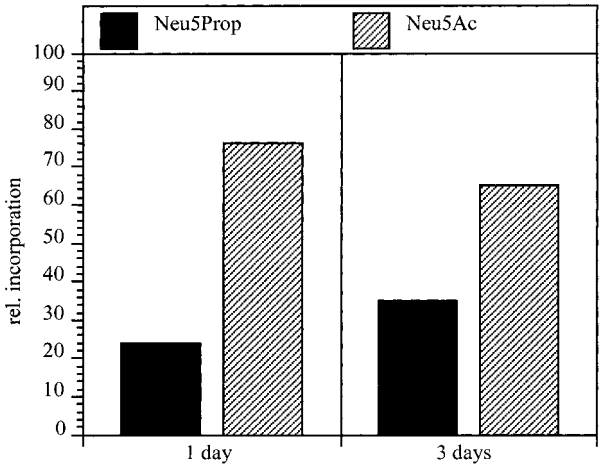

FIG. 1. Quantification of *N*-acetyl- (Neu5Ac) and *N*-propanoylneuraminic acid (Neu5Prop) in membranes of PC12-cells. PC12 cultures were maintained for 1 or 3 days in the presence of 5 mM ManNProp. Total neuraminic acids were released from membrane fractions of 10^7 cells. The neuraminic acids of the glycoprotein fraction were fluorescently labeled, analyzed, and quantitated by HPLC. Black bars represent biochemically engineered *N*-propanoylneuraminc acid. Hatched bars represent natural *N*-acetylneuraminic acid.

Turnover of CEACAM1 and α1-Integrin Subunit

The half-lives of CEACAM1 and the alpha1-integrin subunit were determined by pulse-labeling of the cell surface proteins with biotin, followed by immunoprecipitation, then detection with streptavidin-peroxidase (for details see 18). In addition to measuring turnover we also investigated the influence of biochemical engineering and *N*-propanoylneuraminic acid incorporation on CEACAM1 and alpha1-integrin subunit degradation. The nonphysiological sialic acid precursor ManNProp was applied to the medium using two strategies. First, ManNProp was added just after pulse-labeling with biotin. Second, all cells were preincubated with ManNProp for 3 days prior to pulse-labeling. After the biotinylation the cells were cultured in the presence or absence of ManNProp and collected at different time points: 0, 4, 12, 24, and 48 h. The degradation rates of the biotin-labeled molecules were determined by Western blot analysis (Figs. 2A and 2B). The calculated half-life of CEACAM1 was 26 h in PC12-cells. When cells were incubated with ManNProp after the pulse-labeling, the half-life of CEACAM1 was increased to 32 h. However, when cells were preincubated with ManNProp three days before pulse-labeling the half-life was extended to 40 h (Table 1). In contrast the half-life of the α1-integrin subunit in PC12-cells remained approximately 18 h under all experimental conditions (Table 1).

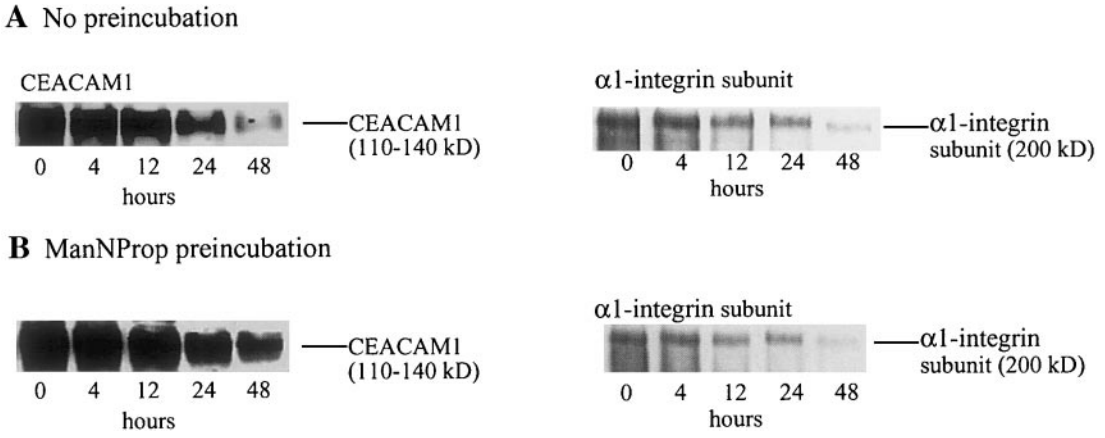

FIG. 2. Representative Western blots: Decay of specific biotin label in PC12-cells in the absence of ManNProp. (A) Plasma membranes of routinely grown PC12 cells were labeled with biotin. Then 4, 12, 24, and 48 h after labeling, CEACAM1 and α1-integrin subunit were immunoprecipitated. CEACAM1- and α1-integrin subunit-specific label was visualized by streptavidin-HRP staining. (B) Decay of specific biotin label in PC12-cells in the presence of ManNProp. PC12-cells were grown for 3 days in the presence of 5 mM ManNProp. Then plasma membranes of PC12 cells were labeled with biotin. Then 4, 12, 24, and 48 h after labeling, CEACAM1 and α1-integrin subunit were immunoprecipitated. CEACAM1- and α1-integrin subunit-specific label was visualized by streptavidin-HRP staining.

DISCUSSION

In this study we could show that in PC12-cells biochemical engineering of the side chain of sialic acid leads to an increased biological stability of the cell adhesion molecule CEACAM1. Another sialylated cell adhesion molecule, the α1-integrin subunit, does not show this increased stability after biochemical engineering.

CEACAM1 has a half-life of 26 h, which increases up to 40 h after incorporation of the unnatural *N*-propanoylneuraminic acid on the cell surface of PC12-cells using ManNProp. The half-life of α1-integrin subunit is 18 h and this time remained unchanged after biochemical engineering of the cell surface sialic acids. By having a half-life of only 18 h α1-integrin subunit is one of the fastest degraded cell adhesion molecules known so far.

CEACAM1 is very heterogeneously glycosylated and highly sialylated (13). Therefore, we assume that the expression of novel *N*-propanoylneuraminic acid on CEACAM1 specifically affects the degradation of this molecule and that the observed increase in the half-life of CEACAM1 is a specific rather than a general effect. The molecular mechanism of the increased stability of CEACAM1 might be explained by disturbed interaction with other molecules on the cell surface, which are involved in protein internalization and degradation.

Biochemical engineering using ManNProp has been used in several studies to stimulate neural cells (4, 5), but the molecular basis for the cellular stimulation of proliferation or differentiation is still unknown. In this study we have shown for the first time that by biochemical engineering of cell surfaces, the half-life of specific cell surface receptors such as the cell adhesion molecule CEACAM1, can be increased by more than 50%. Some of the observed effects of ManNProp on cells might be explained by the prolonged expression of specific cell surface receptors.

TABLE 1

Calculated Half-Lives of CEACAM1 and α1-Integrin Subunit Before and After Biochemical Engineering of the Cell Surface

		Biochemical engineering using ManNProp starting at day . . . prior to pulse-labeling	
	Control	Day 0	Day −3 (pretreatment)
CEACAM1 [h]	26 ± 3	32 ± 5	40 ± 4
α1-integrin [h]	17 ± 3	18 ± 5	18 ± 5

Note. Table shows mean values of at least four independent experiments carried out in duplicates.

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REFERENCES

1. Varki, A. (1997) Sialic acids as ligands in recognition phenomena. *FASEB J.* **11,** 248–255.
2. Kayser, H., Zeitler, R., Kannicht, C., Grunow, D., Nuck, R., and Reutter, W. (1992) Biosynthesis of a nonphysiological sialic acid in different rat organs, using *N*-propanoyl-D-hexosamines as precursors. *J. Biol. Chem.* **267,** 16934–16938.
3. Keppler, O. T., Stehling, P., Herrmann, M., Kayser, H., Grunow, D., Reutter, W., and Pawlita, M. (1995) Biosynthetic modulation

of sialic acid-dependent virus-receptor interactions of two primate polyoma viruses. *J. Biol. Chem.* **270,** 1308–1314.

4. Schmidt, C., Stehling, P., Schnitzer, J., Reutter, W., and Horstkorte, R. (1998) Biochemical engineering of neural cell surfaces by the *N*-propanoyl-substituted neuraminic acid precursor. *J. Biol. Chem.* **273,** 19146–19152.
5. Schmidt, C., Ohlemeyer, C., Kettenmann, H., Reutter, W., and Horstkorte, R. (2000) Incorporation of *N*-propanoylneuraminic acid leads to calcium oscillations upon the application of GABA. *FEBS Lett.* **478,** 276–280.
6. Collins, B. E., Fralich, T. J., Itonori, S., Ichikawa, Y., and Schnaar, R. L. (2000) Conversion of cellular sialic acid expression from *N*-acetyl- to *N*-glycolylneuraminic acid using a synthetic precursor, *N*-glycolylmannosamine pentaacetate: Inhibition of myelin-associated glycoprotein binding to neural cells. *Glycobiology* **10,** 11–20.
7. Wieser, J. R., Heisner, A., Stehling, P., Oesch, F., and Reutter, W. (1996) *In vivo* modulated *N*-acyl side chain of *N*-acetylneuraminic acid modulates the cell contact-dependent inhibition of growth. *FEBS Lett.* **395,** 170–173.
8. Keppler, O. T., Horstkorte, R., Pawlita, M., Schmidt, C., and Reutter, W. (2001) Biochemical engineering of the *N*-acyl side chain of sialic acid: Biological implications. *Glycobiology*, in press.
9. Mahal, L. K., Yarema, K. J., and Bertozzi, C. R. (1997) Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis. *Science* **276,** 1125–1128.
10. Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., and Ashwell, G. (1971) The role of sialic acid in determining the survival of glycoproteins in the circulation.. *J. Biol. Chem.* **246,** 1461–1467.
11. Beauchemin *et al.* (1999) Redefined nomenclature for members of the carcinoembryonic antigen family. *Exp. Cell Res.* **252,** 243–249.
12. Becker, A., Lucka, L., Kilian, C., Kannicht, C., and Reutter, W. (1993) Characterisation of the ATP-dependent taurocholate-carrier protein (gp110) of thehepatocyte canalicular membrane. *Eur. J. Biochem.* **214,** 539–548.
13. Becker, A., Gossrau, R., Hoffmann, C., and Reutter, W. (1989) Localization of a putative cell adhesion molecule (gp110) in Wistar and Fischer rat tissues. *Histochemistry* **93,** 55–61.
14. Öbrink, B. (1997) CEA adhesion molecules: Multifunctional proteins with signal-regulatory properties. *Curr. Opin. Cell Biol.* **9,** 616–626.
15. Hemler, M. E. (1990) VLA proteins in the integrin family: Structures, functions, and their role on leukocytes. *Annu. Rev. Immunol.* **8,** 365–400.
16. Lucka, L., Budt, M., Cichocka, I., Danker, K., Horstkorte, R., and Reutter, W. (1999) C-CAM mediated adhesion leads to an outside-in dephosphorylation signal. *Eur. J. Biochem.* **262,** 541–546.
17. Löster, K., Voigt, S., Heidrich, C., Hofmann, W., and Reutter, W. (1994) Cell-collagen adhesion is inhibited by monoclonal antibody 33.4 against the rat alpha 1-integrin subunit. *Exp. Cell Res.* **212,** 155–160.
18. Schmidt, C., Kuenemund, V., Wintergerst, E. S., Schmitz, B., and Schachner (1996) CD9 of mouse brain is implicated in neurite outgrowth and cell migration and is associated with L1 and $\alpha 6\beta 1$ integrin. *J. Neurosci. Res.* **43,** 12–31.
19. Park, T.-U., Lucka, L., Reutter, W., and Horstkorte, R. (1997) Turnover studies of the neural cell adhesion molecule NCAM: Degradation of NCAM in PC12 cells depends on the presence of NGF. *Biochem. Biophys. Res. Commun.* **234,** 686–689.