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In Vivo Modulation of the Acidic *N*-Glycans from Rat Liver Dipeptidyl Peptidase IV by *N*-Propanoyl-D-mannosamine

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Derivatives of *N*-acyl-D-mannosamine differing in the *N*-acyl-side chain can be metabolically converted into neuraminic acids with corresponding *N*-acyl side chains. In the present study we show the *in vivo* modulation of sialic acids in membrane-bound dipeptidyl peptidase IV (CD 26) from rat liver after administration of *N*-propanoyl-D-mannosamine. Treatment of rats with this unphysiological precursor resulted in an incorporation of N-propanoylneuraminic acid into N-linked glycans of dipeptidyl peptidase IV. © 1999 Academic Press

Sialic acids[2] are closely associated with a variety of cell surface recognition functions. They mediate cell-cell, cell-matrix and other functions related to the biological stability, charge and conformation of sialoconjugates [1]. Modifications of the amino and hydroxyl groups of the common C-9 skeleton increase the structural diversity of sialic acids [2]. The specific functions of these structural alterations are only gradually understood. For example, in the uptake of influenza viruses, it has been shown that strain A and strain B bind to N-acetylneuraminic acid (NANA) [3], whereas strain C binds specifically to 9-O-acetyl-N-acetylneuraminic acid [4].

A concept of modulating the N-acyl-side chain of neuraminic acids by application of unphysiological precursors was developed in our group. Unnatural derivatives of N-acylmannosamine in which the N-acetyl-group is replaced by N-propanoyl, N-pentanoyl and N-hexanoyl are biosynthetically converted into the respective N-acylneuraminic acid [5, 6] (Fig. 1). The biochemical modulation of surface glycoconjugates have been shown to dramatically impair the uptake of sialic acid-dependent viruses in lymphoma cells, such as influenza virus and polyoma viruses [7, 8]. In addition to virus-cell interaction, these analogs also modulate cell-cell interaction [9] and cell-lectin interaction [10].

Up to now, analysis of cell surface alterations after application of these new sialic acid precursors has been restricted to the measurement of sialic acids in the total protein fraction. In the present study, the alterations occurring in the N-glycans after administration of N-propanoyl-D-mannosamine to rats *in vivo* were studied in the defined membrane glycoprotein dipeptidyl peptidase IV (DPP IV, CD 26) [11]. The quantity of N-propanoylneuraminic acid (NPropNA) in the N-glycans, determined on the basis of their charge is reported. Furthermore, we compared the glycosylation pattern of the modified and natural DPP IV.

EXPERIMENTAL PROCEDURES

Treatment of rats with sialic acid precursor analog. N-Propanoyl-D-mannosamine was synthesized and characterized as described previously [7]. Wistar rats were treated for three days by intraperitoneal injection of 200 mg/kg rat weight of N-propanoyl-D-mannosamine twice a day. The liver was removed on the fourth day under light ether anesthesia after perfusion with 40 ml of 0.9% NaCl (w/v).

Purification of DPP IV from rat liver. Plasma membranes from rat liver were isolated according to a method of Pfleger *et al.* [12] with some modifications [13]. Purification was achieved by immunoaffinity-chromatography using the monoclonal antibody 13.4 directed against rat liver DPP IV [14] coupled to protein A-sepharose (Pharmacia, Germany) as described by Schneider *et al.* [15]. The purity of DPP IV was monitored by SDS-PAGE with silver-staining.

Lectin-binding assay. The lectin-binding studies were performed using the DIG Glycan Differentiation Kit according to the protocol of the manufacturer (Boehringer Mannheim, Mannheim, Germany). These lectin-DIG-conjugates were applied: GNA (*Galanthus nivalis-agglutinin* binds Manα1-2/3/6Man), SNA (*Sambucus niger-agglutinin* binds to Siaα2-6Gal, MAA (*Maackia amurensis-agglutinin* binds to Siaα2-3Gal, PNA (*Peanut-agglutinin* binds to Galβ1-3GalNAc), DSA (*Datura stramonium-agglutinin* binds to Galβ1-4GlcNAc), AAA (*Aleuria aurantia-agglutinin* binds to Fucα1-

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[2] Sialic acid comprises all types of substituted neuraminic acids with the common structure of 5-amino-3,5-dideoxy-D-glycero-D-galactononulosonic acid.

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6GlcNAc in the core), Con A (*Concanavalin A* binds to Manα1-2/3/6Man, and biantennary comlplex-type structures.

Isolation of N-glycans from DPP IV. Purified DPP IV was digested with trypsin (Boehringer Mannheim, Germany) and the N-glycans were released by peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase F (PNGase F, Boehringer Mannheim, Germany) treatment as described previously [16]. N-Glycans were finally purified by gel permeation chromatography (Sephadex G-25, Pharmacia, Germany).

Fluorescent labeling of the glycans. Released N-glycans were fluorescently labeled by reductive amination with 2-aminobenzamide (2-AB) using a Signal Labeling Kit according the protocol of the manufacturer (Oxford GlycoSciences, Abingdon, UK). Excess 2-aminobenzamide was removed by paper chromatography as described earlier (17).

Neutral pH anion-exchange chromatography. Purified and fluorescently labeled N-glycans were separated on a Mono Q anion-exchange column (HR5/5, Pharmacia, Germany) as described previously [16].

Release of sialic acids from N-glycans. Sialic acids were released from oligosaccharides by mild hydrolysis with 2 M acetic acid at 80°C for 1 h. Samples were dried by vacuum centrifugation and redissolved in water. Free sialic acids were separated from neutral oligosaccharides on 0.5 ml anion-exchange resin (AG-3-X4, 100-200 mesh, Bio-Rad, Germany). After application of the sample the column was washed with 3 × 1 ml double distilled water, and sialic acids were eluted with 4 × 1 ml of 2 M formic acid.

Separation of sialic acids by RP18 chromatography. Released and purified sialic acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) according to the method of Hara *et al.* [17]. The chromatographic procedure was carried out on a Knauer HPLC-System (Knauer, Germany) equipped with a fluorescence detector (excitation wavelength 373 nm, emission wavelength 448 nm) and a reversed-phase column (LiChrosorb RP18, 5 μm, 250 × 3.1 mm, Bischoff, Germany). Eluent A was water while eluent B was acetonitrile:methanol (6:4 v/v). The flow rate was set to 0.5 ml/min. For all separations the column was eluted for the first 10 min in the isocratic mode with 10% B, which was then increased to 25% B in 45 min as a linear gradient. N-glycolylneuraminic acid and sodium pyruvate were used as internal standards.

MALDI-TOF mass spectrometry. The sialic acids were characterized by MALDI-TOF mass spectrometry as described recently for O-acetylated neuraminic acids [19]. MALDI-TOF mass spectrometry of neutral N-glycan mixtures was performed as described previously [16].

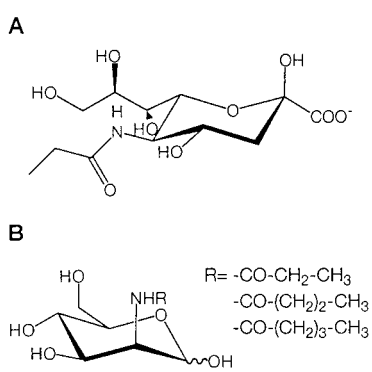

FIG. 1. (A) Structure of the unphysiological *N*-propanoyl-neuraminic acid, biosynthesized by the incorporation of *N*-propanoyl-D-mannosamine. (B) Structure of *N*-acyl-D-mannosamines. R represents the three different residues (propanoyl, butanoyl, and pentanoyl) used in a variety of experiments.

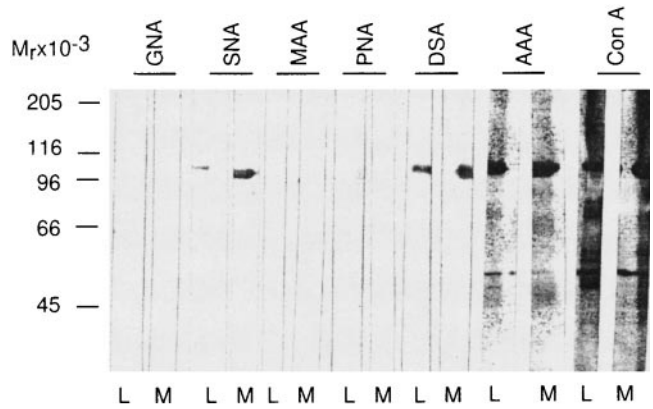

FIG. 2. Lectin-binding assay. Purified normal (N/DPP IV) and modulated DPP IV (M/DPP IV) were applied to SDS-PAGE (each 0.5 μg/lane), blotted on nitrocellulose, and incubated with DIG-conjugated lectins. N, N/DPP IV; M, M/DPP IV. Specifically, bound lectins were visualized by a staining reaction using an anti-digoxygenin FAB fragment conjugated with alkaline phosphatase. For the abbreviations and the specificity of the lectins see Experimental Procedures. The protein band at 110 kDa corresponds to DPP IV. Protein bands of 50 kDa are due to proteolytic degradation of DPP IV.

RESULTS

Apparent molecular mass of modified and unmodified DPP IV. Rats were treated with N-propanoyl-D-mannosamine for 3 days. No indication of toxicity was observed. The DPP IV, from N-propanoyl-D-mannosamine treated and untreated rats, was purified by immune affinity-chromatography. SDS-PAGE revealed no difference in the apparent molecular weight (110 kDa) of the modulated and the normal DPP IV (data not shown).

Comparison of the lectin-binding patterns of the normal and the modulated DPP IV. The normal DPP IV (N/DPP IV) and the modulated DPP IV (M/DPP IV) were applied to SDS-PAGE, blotted on a nitrocellulose membrane and incubated with different digoxygenin-conjugated lectins (Fig. 2). These assays revealed that the modulation of the sialic acids had no influence on the binding specificity of the N-glycans to lectins. The following conclusions can be drawn from these lectin studies:

- rat liver DPP IV contains no high-mannose-type, but does contain complex-type N-glycans (binding to Con A and DSA, but non-binding to GNA),
- the sialic acids are linked α2-6 to galactose (binding to SNA),
- there was no signal for the binding of MAA, which would indicate the presence of α2-3 sialic acid linked to galactose,

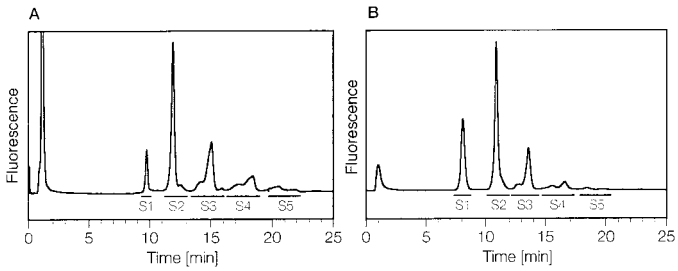

FIG. 3. Separation of sialylated oligosaccharides from N/DPP IV (A) and M/DPP IV (B) by neutral pH anion-exchange chromatography. The elution positions of mono-, bi-, tri-, tetra-, and pentacharged glycans are marked S1, S2, S3, and S4, respectively.

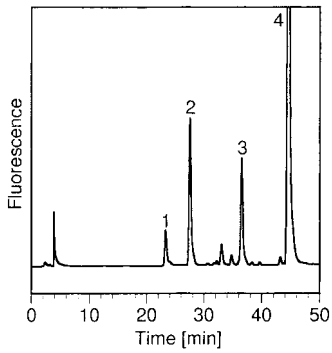

FIG. 4. Separation of DMB-labeled sialic acids from M/DPP IV on RP-18 HPLC. 1, *N*-glycolylneuraminic acid (internal standard); 2, *N*-acetylneuraminic acid; 3, *N*-propanoylneuraminic acid; 4, sodium pyruvate (internal standard).

- rat liver DPP IV carries no O-linked glycans of the mucin-type (non-binding to PNA), and
- rat liver DPP IV contains fucose linked to the core structure of the N-glycans (binding to AAA).

Separation of sialylated oligosaccharides by neutral pH anion-exchange chromatography. N-Linked glycans were enzymatically released from tryptic peptides of DPP IV by PNGase F digestion, purified by gel permeation chromatography and fluorescently labeled by 2-aminobenzamide to allow a sensitive detection. Separation of the N-glycans from N/DPP IV and M/DPP IV using neutral pH anion-exchange chromatography yielded subfractions consisting of monosialylated (S1), bisialylated (S2), trisialylated (S3), tetrasialylated (S4) and traces of pentasialylated (S5) oligosaccharides (Fig. 3A, B). Signals at 1-2 min corresponded to the void volume of the column and did not represent uncharged sugars. The two chromatograms exhibited a similar pattern of negatively charged N-glycans. However, the comparison of the ratio of the five fractions (S1-S5) from N/DPP IV and M/DPP IV showed that the more highly sialylated N-glycans are less expressed in M/DPP IV in favor of the mono- and bisialylated oligosaccharides.

Quantification and characterization of sialic acids according to the charge of the oligosaccharide. The relative amount of NANA and NPropNA was determined for each of the collected fractions S1-S5 from M/DPP IV. For this purpose the sialic acids were released by mild hydrolysis in acetic acid, purified and fluorescently labeled with 1,2-diamino-4,5-methylenedioxybenzene [18]. Separation and quantification of the fluorescently labeled sialic acids was performed on RP18-HPLC (Fig. 4). The fractions designated 2 and 3 were collected, dried and subjected to mass determination by MALDI-TOF-MS. The pseudomolecular ions of fraction 2 (448.8 amu) and fraction 3 (462.3 amu) corresponded to the Na-adducts of NANA (448.3 amu) and NPropNA (462.3 amu). Quantification of sialic acids in the fractions S1-S5 revealed that the relative amount of the unphysiological NPropNA increases with the number of sialic acids attached to the N-glycans, as summarized in Table 1.

Comparison of the neutral N-glycans from N/DPP IV and M/DPP IV by MALDI-TOF mass spectrometry. To determine whether treatment of rats with N-propanoyl-D-mannosamine resulted in changes in the antennarity of N-glycans from DPP IV, the oligosaccharides from fluorescent labeled N/DPP IV and M/DPP IV were desialylated and submitted to MALDI-TOF mass spectrometry (Fig. 5). The most abundant ions were detected at m/z 1784 representing biantennary structures. Masses of 2149 amu and 2514 amu corresponded to tri- and tetraantennary glycans, respectively. Bi-, tri- and tetraantennary sugars carrying a fucose residue are consistent with masses of 1930 amu, 2295 amu and 2660 amu. No differences were detectable by MALDI-TOF mass spectrometry for the N-glycans from N/DPP IV and M/DPP IV, except for signals at 1665, 1811 and 2030 found for M/DPP IV. These peaks resulted from incomplete fluorescent labeling of the glycans.

The obtained masses are consistent with oligosaccharides consisting of N-acetylated aminosugars. An incorporation of N-propanoylated aminosugars would result in an increased mass of 14 amu, which was not detected. Therefore a significant incorporation of modified aminosugars into neutral N-glycans is unlikely.

TABLE 1

Relative Amount of N-propanoylneuraminic Acid in M/DPP IV Dependent on the Charge of the Oligosaccharide

Fraction #	N-propanoylneuraminic acid (% of Σ sialic acids)
S1	9
S2	8
S3	16
S4	20
S5	26

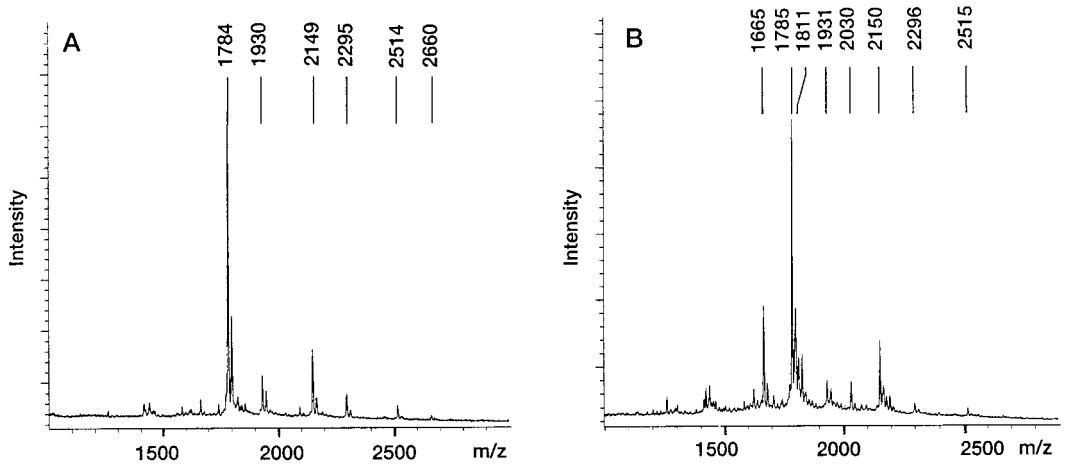

FIG. 5. MALDI-TOF mass spectra of total fluorescently-labeled desialylated oligosaccharides from N/DPP IV (A) and M/DDP IV (B). Masses of 1784, 2149, and 2514 amu correspond to bi-, tri-, and tetraantennary glycans. The respective fucosylated structures were detected at 1930, 2295, and 2660 amu. The peak doublets result from Na/K-adducts of the sugars. The signals at 1665, 1811, and 2030 amu monitored for M/DPP IV resulted from incomplete fluorescent labeling of the glycans.

DISCUSSION

In the present study, the *in vivo* modulation of sialic acids after application of the unphysiological precursor N-acetylmannosamine was demonstrated for a defined glycoprotein, dipeptidylpeptidase IV, a membrane glycoprotein from rat liver [11, 14]. SDS-PAGE revealed no difference in the apparent molecular weight of modulated and natural DPP IV. Therefore a reduced glycosylation of M/DPP IV, due to non-occupation of some of the 8 potential N-glycosylation sites, is unlikely.

Using neutral pH anion exchange chromatography the incorporation of the modulated NPropNA was determined on the basis of the charge of the oligosaccharides. The comparison of the ratio of the charged oligosaccharides from N/DPP IV and M/DPP IV showed that the more highly sialylated N-glycans are less expressed in M/DPP IV in favor of the mono- and bisialylated oligosaccharides.

The amount of NPropNA rises with the number of sialic acids bound to the glycan. Our results suggest that NANA and NPropNA are transferred by different sialyltransferases with distinct affinities for CMP-NANA and for CMP-NPropNA and that the sialyltransferase with a higher affinity for NPropNA works after action of the sialyltransferase, which preferentially transfers NANA. So the transfer of NPropNA is favored by the presence of a NANA residue in the oligosaccharide. Various sialyltransferases have been characterized from rat liver [20]. The most common type of linkage for sialylated N-glycans from rat liver is Siaα2-6Gal catalyzed by an α2-6 sialyltransferase which acts on Galβ1-4GlcNAc [21]. We suggest that this enzyme transfers the first sialic acid residue to the N-glycan but this sialyltransferase is not supposed to have a high affinity for CMP-NPropNA. A second α2-6 sialyltransferase from rat liver has been described to act preferentially on a sialylated acceptor. The biosynthesis of the disialylated antennae Siaα2-3Galβ1-3(Siaα2-6)GlcNAc-R first requires the action of the Galβ1-3/4GlcNAc α2-3 sialyltransferase prior to sialylation of the GlcNAc residue [22]. This type of glycosylation is reported for glycoproteins from rat liver and bovine [23–26] and was recently called "oversialylation" [27]. The presence of pentasialylated structures in DPP IV as shown by anion exchange chromatography revealed that disialylated antennae contribute to sialylated N-glycans in DPP IV. It is also likely that glycans bearing a disialylated antennae are present in the fraction of tri- and tetracharged glycans, because the N-glycan moiety of DPP IV consists mainly of biantennary and much less of triantennary structures as shown by MALDI-TOF-MS. Lectin binding assays did not indicate the presence of Siaα2-3Gal in DPP IV. However we have some evidence from the analysis of CEA-CAM from rat liver that α2-3 linked sialic acids are not recognized by the lectin MAA, if the GlcNAc residue in the antennae is sialylated [27]. To our knowledge the presence of disialylated antennae was described for N-glycans bearing three, four or five sialic acid residues. As shown in Table 1 the amount of NPropNA rises from bi- to trisialylated glycans from 8 to 16%. This might support the conclusion, that the disialylated antennae Siaα2-3Galβ1-3(Siaα2-6)GlcNAc-R preferentially carries a NPropNA residue.

It is not known whether sialyltransferases from rat liver have different Km and Vmax values for CMP-NANA and CMP-NPropNA, but it has recently been demonstrated that kinetic parameters of several sialyltransferases for 5-substituted CMP-glycosides differed significantly [28].

Mass profiling using MALDI-TOF-MS of the desialylated, neutral N-glycans from normal DPP IV and

modulated DPP IV showed a very similar pattern representing N-glycans of the complex type. These oligosaccharides consist of bi-, tri- and tetraantennary structures which are partially fucosylated. These data suggest that the biosynthetic precursor N-propanoyl-D-mannosamine does not basically influence the biosynthesis of the N-glycans in rat liver, apart from the modulation of the terminal sialic acids. However a detailed structural characterization of the oligosaccharides was not performed.

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