Sialoforms of dipeptidylpeptidase IV from rat kidney and liver

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Dipeptidylpeptidase IV (DPP IV, CD26), a serine-type exo- and endopeptidase found in the cell surface membrane of many tissues, was employed as a model membrane glycoprotein to study the expression of sialoforms on cell surface glycoproteins. Native, enzymatically active DPP IV was purified from plasma membranes of kidney and liver by lectin affinity chromatography in conjunction with crown ether anion exchange chromatography. The enzyme was gradient-eluted in continuous fractions, all showing a single polypeptide band of about 100 kDa when separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing, denaturing conditions. Analysis of the purified DPP IV by isoelectric focusing (IEF) showed that it consists of several polypeptides of different isoelectric points (IP) ranging from 5.5 to 7.0. In vitro-desialylation of the enzyme and subsequent isoelectric focusing revealed that the differences in isoelectric points were due to differences in the degree of sialylation. Differences in the degree of sialylation between the fractions were also demonstrated by SDS–PAGE under nonreducing and nondenaturating conditions. Increased sialylation of the enzyme as demonstrated by isoelectric focusing resulted in increased migration velocity in nonreducing and nondenaturing SDS–polyacrylamide gels. In vitro-desialylation of the enzyme and its resialylation confirmed that sialylation was responsible for this extraordinary migration behavior. The native enzyme was predominantly sialylated via α2,6-linkage, as shown by lectin affinity blotting employing Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin (MAA). These findings demonstrate that a distinct membrane glycoprotein may exist in various sialoforms, distinguished from each other by a different number of sialic acid residues. Moreover, these sialoforms can be individually purified by crown ether anion exchange chromatography.

Key words: cell surface glycoproteins/glycosylation/iseoelectric focusing/sialic acid

Introduction

Sialylation has been shown to be fundamentally important in determining various biological properties of glycoproteins and glycolipids. Thus sialylation may mask the D-galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) residues involved in the clearance of serum glycoproteins by asialoglycoprotein receptors (Kawasaki and Ashwell, 1977; Ashwell and Harford, 1982; Il et al., 1990; Spiess, 1990; Chiu et al., 1994), and it determines the specific binding affinity of cell surface glycoconjugates for various lectins (Varki, 1992). Moreover, sialic acids may modulate the biological activity of coagulation factors and coagulation inhibitors such as fibrinogen (Dang et al., 1989), von Willebrand factor (Berkowitz and Frederici, 1988), protein C (Hau and Salem, 1991), and plasminogen (Stack et al., 1992), and of hormones such as erythropoietin (Imai et al., 1990). Sialic acids are also involved in infectious diseases, e.g. by acting as binding sites for viruses or by reducing the antigenicity of parasites such as Trypanosoma cruzi (Colli, 1993). Changes in sialylation of cell surface glycoconjugates occur during development and in malignancy and have been shown to influence cellular functions such as growth, differentiation, adhesion, and invasiveness (Saitoh et al., 1992; Jorgensen et al., 1995; Kopitz et al., 1996; Le Marer and Stehelin, 1995; Wieser et al., 1995). The biological significance of sialylation is reflected in the widespread occurrence of sialic acid residues on a large number of different soluble and membrane-bound glycoconjugates.

Transfer of sialic acid residues to newly synthesized glycoproteins and glycolipids during biosynthesis has been shown to be precisely controlled by the cellular activity of various sialyltransferases (Paulson et al., 1989). In addition, cell surface glycoproteins may be de- and resialylated during endocytosis and recycling, representing a mechanism of postbiosynthetic adaptation (Volz et al., 1995). Structural analysis of glycoprotein glycans has shown that the oligosaccharides of a given glycoprotein may differ in their extent of sialylation. This is true not only for the oligosaccharides of the different glycosylation sites, but also for the oligosaccharides bound to one distinct glycosylation site (Kornfeld and Kornfeld, 1985; Paulson and Colley, 1989; Schachter, 1995), giving rise to a microheterogeneity in glycan sialylation. Whether a different degree of sialylation of oligosaccharides may, furthermore,
lead to the generation of differently charged isoforms of a glycoprotein distinguished by the number of sialic acid residues has so far been studied almost exclusively for soluble glycoproteins, including serum transferrin (de Jong and van Eijk, 1988), thyroxin-binding globulin (Lasne et al., 1982), thyrotropin (Papandreou et al., 1993; Szukudinski et al., 1993), prolactin (Price et al., 1995), lithostatine (De Reggi et al., 1995), and human chorionic gonadotropin (Amano et al., 1989; Nemansky et al., 1995). As has been elegantly shown for serum transferrin (de Jong and van Eijk, 1988) sialylation apart from genetic polymorphism and other forms of postbiosynthetic modification, like phosphorylation and sulfatation, may significantly influence the isoelectric point of serum glycoproteins. Studies on the sialylation of membrane glycoproteins have, however, examined samples of purified glycoproteins that were not separated according to charge differences beforehand. These studies could, hence, not address the issue, whether the observed microheterogeneity of glycans sialylation does result in the formation of differently charged isoforms of a membrane glycoprotein. It is, therefore, still largely unknown whether similar to serum glycoproteins membrane glycoproteins may exist in different isoforms in the same tissue, distinguished by the number of sialic acid residues per protein molecule.

In the present study we have addressed this question by studying the sialylation of dipeptidylpeptidase IV (DPP IV, CD 26), a serine-type exo- and endopeptidase, cleaves N-terminal dipeptides from polypeptides with proline or alanine as the penultimate amino acid (Hopsu-Havu and Sarimo, 1967; Kenny et al., 1976; Bermpohl et al., 1998), e.g., substance P, β-casomorphine and the fibrin α-chain. DPP IV is expressed in all tissues so far investigated, in particular in the brush border membranes of small intestine, kidney and bile canaliculi (Gossrau, 1979a; Hartel et al., 1988). The cDNA for DPP IV was cloned in rat (Hong et al., 1989), mouse (Marguet et al., 1992), and human (Misumi et al., 1992; Tanaka et al., 1992; Darmoul et al., 1992). DPP IV is an integral type II membrane protein anchored to the membrane by the signal peptide sequence (Ogata et al., 1989; Hong and Doyle, 1990) and is present in the plasma membrane as a homodimer (Jascur et al., 1991). DPP IV has eight consensus sequences for N-glycosylation (Hong and Doyle, 1987) which are all N-glycosylated (Petell et al., 1987). Except for one oligosaccharide chain, which retains a high mannose structure, DPP IV N-glycans mature to complex-type structures on their way through the endomembranes (Yamashita et al., 1988; Hartel-Schenk et al., 1991). The oligosaccharides of DPP IV from kidney exhibit extensive structural heterogeneity (Yamashita et al., 1988).

It was the aim of the present study to examine whether cellular membrane-bound DPP IV does in vivo exist in differently charged isoforms distinguished from each other by the number of sialic acid residues per protein molecule. This was investigated in that native, enzymatically active DPP IV was purified from plasma membranes of rat liver and kidney employing a purification protocol mainly based on crown ether ion exchange chromatography. By this method, indeed, differently charged forms of native DPP IV could be separately purified. Further analysis showed that they differ in their degree of sialylation.

Results

Native, enzymatically active DPP IV was purified from plasma membranes of rat kidney and liver by lectin affinity chromatography in conjunction with ion exchange chromatography on crown ether-silica gel columns (Figure 1A,B). As a first step, isolated brush border membranes of kidney and liver were solubilized with the nonionic detergent Triton X-100, which has been shown to be suitable for the solubilization of enzymatically active DPP IV (Josc et al., 1985). Triton X-100 extracts were sequentially separated first on concanavalin A (Con A)-Sepharose and then on wheat germ agglutinin (WGA)-agarose. Con A has binding specificity for glycoproteins with oligosaccharides of the oligomannosidic or the biantennary complex type (Baenziger and Fiete, 1979; Narasimhan et al., 1979), whereas WGA binds oligosaccharides having chitobiose sequences and terminal N-acetyl-d-glucosamine (GlCNAc) residues (Debray et al., 1981). Moreover, terminal sialic acid can also account for affinity to WGA due to the common structural element of an acetalamido group (Monsigny et al., 1980).

DPP IV from both kidney and liver membranes was almost completely bound to Con A and eluted with 0.2 M methyl-α-D-mannopyranoside, resulting in 8-fold and 6-fold enrichment of kidney DPP IV and liver DPP IV, respectively.

DPP IV from liver membranes showed high affinity for WGA. Bound DPP IV activity could be eluted with 0.2 M GlcNAc resulting in 2-fold enrichment. By contrast, only 60% of the total activity of kidney DPP IV bound to WGA. Therefore, WGA affinity chromatography was finally omitted in the purification of kidney DPP IV.

The essential step in the purification of native DPP IV from both tissues was anion exchange chromatography on crown ether-silica gel (Pedersen and Frensdorff, 1972; Reusch, 1988; Josc et al., 1989). Kidney and liver DPP IV activity were completely bound to the column, when applied in 10 mM Tris-HCl, pH 7.2, 10 mM KCl, 0.1% Triton X-100. The column was then washed with 30 bed volumes of 10 mM Tris–HCl, pH 7.2, 10 mM KCl, containing 0.1% octylglucoside instead of Triton X-100, in order to replace the Triton X-100. Bound glycoproteins were eluted with a NaCl gradient (0–750 mM NaCl) (Figure 2A). Elution of DPP IV activity started at 65 mM NaCl and proceeded until 140 mM NaCl. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining of the eluted fractions containing DPP IV activity showed that only DPP IV was eluted under these conditions (Figure 3). Under denaturing conditions, DPP IV migrated with a relative molecular mass (M_r) of ~100 kDa for the kidney enzyme (Figure 3B) and 110 kDa for the liver enzyme (not shown), in accordance with previous reports (Kreisel et al., 1982; Tiruppathi et al., 1990). Purity of the eluted fractions was also shown by SDS–PAGE under nonreducing and nondenaturing conditions (Figure 3A). At a NaCl concentration of 120 mM and higher, additional glycoproteins including nucleotide pyrophosphatase (EC 3.6.1.9) (Figure 2) and leucine aminopeptidase (EC 3.4.11.2) (not shown) were eluted. Both nucleotide pyrophosphatase and leucine aminopeptidase were identified by functional assay of their enzymatic activities, by their M_r as determined by SDS–PAGE and by use of specific antibodies. When Triton X-100 was added to the final elution buffer (750 mM NaCl, Figure 2A; 400 mM NaCl, Figure 2B), nucleotide pyrophosphatase (Figure 2) and leucine aminopeptidase (Figure 2) were eluted.
peptidase (not shown) eluted as a sharp peak. No additional DPP IV was recovered from the column in the presence of Triton X-100.

In general, elution of DPP IV from crown ether-silica gel depended on the increasing concentration of NaCl. When a constantly increasing molarity of NaCl in the elution buffer was applied, DPP IV eluted steadily and was almost completely recovered from the column (Figure 2A). On the other hand, when a constant NaCl concentration was applied, only part of DPP IV activity was eluted, and DPP IV activity remaining bound to the column under these conditions could only be eluted with increasing NaCl concentrations (Figure 2B). These results indicated that DPP IV occurs in both liver and kidney plasma membranes in differently charged forms which could be separately purified. As was demonstrated in repeated experiments, DPP IV activity was eluted, and DPP IV activity remaining bound to the column under these conditions could only be eluted with increasing NaCl concentrations (Figure 2B). These results indicated that DPP IV occurs in both liver and kidney plasma membranes in differently charged forms which could be separately purified. As was demonstrated in repeated experiments, DPP IV activity was eluted, and DPP IV activity remaining bound to the column under these conditions could only be eluted with increasing NaCl concentrations (Figure 2B). These results indicated that DPP IV occurs in both liver and kidney plasma membranes in differently charged forms which could be separately purified. As was demonstrated in repeated experiments, DPP IV activity was eluted, and DPP IV activity remaining bound to the column under these conditions could only be eluted with increasing NaCl concentrations (Figure 2B). These results indicated that DPP IV occurs in both liver and kidney plasma membranes in differently charged forms which could be separately purified. As was demonstrated in repeated experiments, DPP IV activity was eluted, and DPP IV activity remaining bound to the column under these conditions could only be eluted with increasing NaCl concentrations (Figure 2B). These results indicated that DPP IV occurs in both liver and kidney plasma membranes in differently charged forms which could be separately purified. As was demonstrated in repeated experiments, DPP IV activity was eluted, and DPP IV activity remaining bound to the column under these conditions could only be eluted with increasing NaCl concentrations. (Hashimoto et al., 1981; Spellman et al., 1989). DPP IV from both liver and kidney showed distinct binding with SNA and lesser binding with MAA, indicating the presence of α2,6- as well as α2,3-linked sialic acids. Desialylation of purified DPP IV from liver and kidney DPP IV by sialidase caused loss of binding reactivity with both SNA and MAA (Figure 4).

In order to examine whether the differently charged DPP IV fractions as separated by crown ether anion exchange chromatography represent isoforms of the enzyme, distinguished by their extent of sialylation, the fractions eluted with increasing NaCl concentrations were analyzed by SDS–PAGE under non-reducing and nondenaturing conditions and by isoelectric focusing, and compared with enzymatically desialylated DPP IV. When the various DPP IV fractions, as obtained by crown ether chromatography, were separated by isoelectric focusing (IEF) and were compared with an aliquot of enzymatically desialylated DPP IV, a pattern of DPP IV isoforms exhibiting a stepwise decrease in their isoelectric points from approximately 6.8 to 5.5 was obtained (Figure 5). Whereas DPP IV isoforms eluted at low NaCl concentration (lanes 2, 12) comigrated with the desialylated form (lane 13), DPP IV isoforms...
eluted at higher NaCl concentration migrated to the anionic part of the gel. This result demonstrates that the charge difference between DPP IV isoforms separated by elution with increasing NaCl concentrations is due to a different degree of sialylation. The different degree of sialylation also influenced the migration of DPP IV in SDS–PAGE under reducing, denaturing conditions, as well as under nonreducing, nondenaturing conditions (Figure 6). As shown for pooled samples of DPP IV from both kidney and liver membranes, enzymatically desialylated DPP IV (lanes 2, 4) migrated slightly faster than sialylated DPP IV (lanes 1, 3) when separated under reducing, denaturing conditions. The effect of desialylation on migration was reversed under nonreducing, nondenaturing conditions (lanes 5–8). Under these conditions desialylated DPP IV (lanes 6, 8) migrated more slowly than sialylated DPP IV (lanes 5, 7).

DPP IV separated under these conditions retained enzymatic activity as demonstrated by enzyme activity staining (Figure 7). The different electrophoretic behavior of sialylated and desialylated DPP IV under these conditions most probably reflects the fact that in the presence of higher concentrations of SDS charge differences due to sialic acid are masked by SDS molecules tightly bound to denatured DPP IV. Faster migration of sialylated DPP IV under nonreducing and nondenaturing conditions is caused by the higher anionic charge of the molecules at pH 8.8. Under these conditions native DPP IV
Variability within the purified enzyme

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binds SDS to a lesser extent than does denatured DPP IV. The stepwise differences in the sialylation of the DPP IV fractions separated by crown ether chromatography could be also shown by SDS–PAGE under nonreducing and nondenaturing conditions (Figure 3A). Isoforms of DPP IV eluted at low NaCl concentration (lanes 2, 11) comigrated with desialylated DPP IV (lanes 1, 12). DPP IV fractions eluted at higher NaCl concentrations (lanes 3–10) showed increased migration in the gel.

In order to examine whether the change in migration observed after enzymatic desialylation is indeed caused by the removal of sialic acid residues, desialylated DPP IV was resialylated, using α2,6-sialyltransferase (EC 2.4.99.1) from rat liver. When separated by SDS–PAGE under nondenaturing, nonreducing conditions resialylated DPP IV comigrated with the original native sialylated DPP IV, showing that the different migration of sialylated and desialylated DPP IV is solely due to the presence and absence of sialic acid residues, respectively (Figure 8).

Discussion

The present paper shows that DPP IV from plasma membranes of rat kidney and liver exists in differently charged isoforms distinguished from each other by the extent of sialylation. This assumption is based on the following evidence.

First, separation of a glycoprotein fraction of rat liver and kidney membranes by crown ether chromatography could be also shown by SDS–PAGE under nonreducing and nondenaturing conditions (Figure 3A). Isoforms of DPP IV eluted at low NaCl concentration (lanes 2, 11) comigrated with desialylated DPP IV (lanes 1, 12). DPP IV fractions eluted at higher NaCl concentrations (lanes 3–10) showed increased migration in the gel.

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than octylglucoside, indicates that the other membrane glycoproteins were retained on the column also by hydrophobic interactions.

The differently charged DPP IV fractions from the crown ether column were further analyzed by isoelectric focusing. Less charged fractions eluting at low NaCl concentrations migrated in the more basic part of the gel, while the more highly charged fractions migrated in the more acidic part. Enzymatic desialylation with sialidase from Clostridium perfringens converted charged DPP IV fractions into a form that comigrated with the DPP IV fraction that was eluted at low NaCl concentration. This indicates that the observed charge differences are due to different extents of sialylation.

The presence of sialic acid residues in purified DPP IV could be confirmed by lectin affinity blotting using SNA and MAA. In accordance with previous reports (Yamashita et al., 1988), the results showed that the sialic acid residues are predominantly α2,6-linked to Gal with only traces of α2,3-linked sialic acid residues. Whereas sialylated and desialylated DPP IV could be clearly distinguished from each other by lectin affinity blotting, no clear distinction between the sialylated and the desialylated form could be made by SDS–PAGE under denaturing conditions. While DPP IV from liver exhibited a discrete shift from apparently 110 kDa to 100 kDa after desialylation, no clear difference in Mr was detectable for the sialylated and desialylated form of kidney DPP IV. The molecular mass determination in SDS–PAGE, however, is valid only for linear polypeptides, and deviations have been described for polypeptides that carry nonpolypeptide components such as glycans (Leach et al., 1980). Since the glycan moiety may influence the apparent Mr, changes in the glycan moiety such as desialylation may also modify the latter. Hence, deglycosylation cannot be used for the characterization of Mr values within a system that does not take into account these sources of deviation.

On the other hand, sialylation influenced the migration of DPP IV in SDS–polyacrylamide gels under nonreducing and nondenaturing conditions, in that desialylated forms migrated more slowly than sialylated DPP IV. Hence, sialylated forms having a higher molecular mass exhibit a lower apparent Mr when separated by SDS–PAGE under nonreducing, nondenaturing conditions. This is explained by the fact that nonreduced and nondenatured DPP IV does not fully bind SDS and does not fulfi l the standards of SDS–PAGE (Reynolds and Tanford, 1970a,b). As charge does influence the migration of the DPP IV molecules not associated with SDS, the migration reflects the charge and in our case the extent of sialylation. This is very effectively demonstrated by SDS–PAGE under nonreducing and nondenaturing conditions of the DPP IV fractions eluted from the crown ether column. DPP IV fractions eluting at low NaCl concentrations, and therefore possessing a low charge, migrated at the lowest velocity, DPP IV fractions eluting at high NaCl concentrations, and therefore carrying a higher charge, migrated at the highest velocity. The gradually increasing charge of the DPP IV fractions eluting at gradually increasing NaCl concentrations resulted in a gradually increasing migration velocity (Figure 3A). Enzymatic desialylation of...
charged DPP IV to produce a less charged form that comigrated with the early eluting forms of DPP IV indicates that the charge shift during elution from crown ethers is inversely related to the charge shift resulting from the loss of sialic acids. The change in migration velocity between the early eluting DPP IV fractions and the late eluting DPP IV fractions can therefore be ascribed entirely to charge differences conferred by the variable content of sialic acid residues. Further proof is given by enzymatic de- and resialylation of DPP IV. Whereas enzymatic desialylation of charged DPP IV results in a distinct reduction of migration velocity in SDS–PAGE under nonreducing and nondenaturing conditions, the enzymatic resialylation of desialylated DPP IV with sialyltransferase from rat liver leads to an increase in migration velocity exactly to the level of the originally charged form. DPP IV therefore exists in differently charged forms that can be explained by different extents of sialylation.

How can such a variety of charged forms develop within one type of tissue? Heterogeneity of sialylation may occur for various reasons. First, during biosynthesis, a different extent of branching will result in a different extent of sialylation. The microheterogeneity of oligosaccharides at a single amino acid site may be explained by competition between the glycosyltransferases on the endomembrane assembly line (for review, see Schachter, 1995). Thus, the extent of branching originates from competition of the different glucosaminyltransferases. Variety in their activities will produce various antennae. Since branching is terminated by glucosaminyltransferase III, strong expression of the latter in kidney tissues (Kobata, 1992) further contributes to the variety in branching.

Sialylation may be varied at the level of activity of sialyltransferases (Paulson et al., 1989). If the expression of sialyltransferase activities is regulated on the cellular level, the extent of sialylation on oligosaccharide structures may be varied further.

Finally, postbiosynthetic processes may also contribute to a different degree of sialylation. Thus, during endocytosis and recycling, sialylated structures are subject to enzymatic de- and resialylation (Duncan and Kornfeld, 1988; Kreisel et al., 1982; Reichner et al., 1988; Volz et al., 1995). Studies by Volz et al. on sialylated and desialylated cell surface DPP IV showed that on the other hand DPP IV is not desialylated in cell homogenates in vitro. This result rules out that partially desialylated forms of DPP IV observed in the present study were generated during the purification process. In vivo desialylation most likely occurs on the cell surface. Cell surface sialidases have been reported to desialylate oligosaccharide structures, namely to selectively desialylate gangliosides in the plasma membrane of neuroblastoma cells (Kopitz et al., 1996). Moreover, a neuraminidase has also been reported on the cell surface as part of the elastin/laminin receptor complex (Hinek, 1996), where it may be involved in the postbiosynthetic generation of desialylated structures.

The ability to separate and to purify various isoforms of DPP IV distinguished from each other by the extent of sialylation will form the basis for future studies on the role of sialylation in determining the biological properties and behavior of DPP IV. Bearing in mind the various biological roles of DPP IV, this may be of particular significance for sialic acid functions in the immune system, in cell adhesion and for brush border functions.

Materials and methods

Animals and chemicals

Male Wistar rats and Buffalo rats, weighing about 160–180g, were bred in our laboratory and fed a commercial diet containing 18–20% (w/w) protein (Altromin R; Altromin, Lage/Lippe, Germany), and water ad libitum. All chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), or Sigma (Deisenhofen, Germany).

Molecular mass standards, reagents for SDS–PAGE, N-acetylglucoside, concanavalin A-Sepharose, methyl-α-D-mannopyranoside, N-acetyl-D-glucosamine, leucine p-nitroanilide, thymidine monophosphate p-nitrophenyl ester and sialidase from Clostridium perfringens (EC 3.2.1.18), attached to beaded agarose, were from Sigma (Deisenhofen, Germany). Triton X-100 was from Aldrich (Steinheim, Germany). The sialic acid binding lectins of Maackia amurensis and Sambucus nigra, α-2,6 N-acetylneuraminyltransferase from rat liver (EC 2.4.99.1) and cytidine 5'-monophosphate (CMP)-N-acetylneuraminic acid were obtained from Boehringer Mannheim (Mannheim, Germany).

Tosyl-Gly-Pro p-nitroanilide was from Bachem (Bubendorf, Switzerland), Servalyte (2–11 and 4–9T), and DL-dithiothreitol (DTT) was from Serva (Heidelberg, Germany).
Nitrocellulose membranes (BA85, 0.45 µm) were obtained from Schleicher & Schuell (Dassel, Germany).

WGA was immobilized on Fractogel (Tosohaas, Yamaguchi, Japan) as described previously (Josic et al., 1987).

The 1,10-diaza-18-crown-6 ligands were immobilized on epoxy-activated silica gel (particle size 7 µm and pore size 30 nm; Eurochrom, Saulenttechnik Knauer). A semipreparative column with the dimension of 120 mm x 8.0 mm was produced and packed by Saulenttechnik Knauer (Berlin, Germany).

**Determination of enzyme activities**

DPP IV activity was measured according to Kreisel et al. (1982) with tosyl-Gly-Pro p-nitroanilide as substrate. Leucine aminopeptidase activity was determined according to Roman and Hubbard (1983) using leucine p-nitroanilide as substrate. Enzyme activities were calculated from a standard curve of p-nitroanilide at 405 nm. For assaying nucleotide pyrophosphatase activity 290 µl of buffer (150 mM Tris–HCl, pH 9.0, 10 mM MgCl₂, 0.1% (v/v) Triton X-100) was incubated for 30 min in a water bath at 37°C. The reaction was stopped by adding 600 µl of 0.1 mM NaOH. Enzyme activity was calculated from a standard curve of p-nitroanilide at 405 nm.

**Detection of DPP IV activity on nitrocellulose membranes**

DPP IV separated on SDS–polyacrylamide gels under non-denaturing conditions was blotted onto nitrocellulose membranes and stained according to Alwarg et al. (1985). Briefly, the membranes were incubated with 0.2 M Tris–HCl, pH 7.8, 1 mM glycyl-L-proline-4-methoxy-β-naphthylamide (2 mM in H₂O) for 30 min in a water bath at 37°C. The reaction was stopped by adding 600 µl of 0.1 mM NaOH. Enzyme activity was calculated from a standard curve of p-nitrophenol at 405 nm.

**Isolation of plasma membranes**

Plasma membranes from liver and kidney were prepared as described previously (Harms and Reutter, 1974; Stewart and Kenny, 1984) and frozen at −80°C. Extraction of plasma membranes was carried out according to Josic et al. (1985). Briefly, isolated plasma membranes were thawed, resuspended in buffer L (1 mM NaHCO₃, pH 7.0, 0.5 mM CaCl₂) as for liver membranes or in buffer K (2 mM HEPES, pH 7.2, 100 mM mannitol) as for kidney membranes and homogenized with five strokes in a loose fitting Dounce homogenizer and centrifuged for 30 min at 60,000 × g. The supernatant was decanted and the pellet was resuspended in buffer A (10 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1 mM CaCl₂, 1% (v/v) Triton X-100) at a concentration of 2 mg protein/ml buffer with 20 strokes in a Dounce homogenizer. After 6 h on ice the mixture was centrifuged at 50,000 × g for 30 min. The supernatant containing the solubilized proteins was stored at −80°C.

**Lectin affinity chromatography**

Plasma membrane proteins (100 mg protein/column) from liver or kidney solubilized in buffer A were applied to a concanavalin A–Sepharose column (20 × 160 mm) equilibrated with buffer A. The column was washed with five bed volumes of buffer A. Material bound to the column was eluted with 200 ml of 0.2 M methyl-α-D-mannopyranoside in buffer A at a flow rate of 1 ml/min. Fractions of 10 ml were collected and assayed for DPP IV activity. Fractions containing DPP IV activity were pooled and exhaustively dialyzed against buffer A. The dialyzed eluate was applied to a WGA-Toyoperl column equilibrated with buffer A. The column was washed with five bed volumes of buffer A. Material bound to the column was eluted in 10 ml fractions with 200 ml 0.2 M N-acetyl-D-glucosamine in buffer A at a flow rate of 1 ml/min. Fractions (10 ml) containing DPP IV activity were pooled and exhaustively dialyzed against buffer B (10 mM Tris–HCl, pH 7.2, 10 mM KCl).

**Fractionation of DPP IV by crown ether HPAC**

A 1,10-diaza-18-crown-6-silica gel column, equilibrated in water, was saturated with 10 ml 1M KCl, and then equilibrated in buffer B (10 mM Tris–HCl, pH 7.2, 10 mM KCl, 0.1% (w/v) octylglucoside). The dialyzed eluate from WGA-agarose was applied to the column at a flow rate of 1 ml/min. The column was washed with buffer B until Triton X-100 was no longer detectable in the fluid phase (280 nm). Material bound to the column was eluted with a continuous gradient (0–750 mM NaCl) or a discontinuous gradient (0–400 mM NaCl) in buffer B. Fractions of 1 ml were collected and assayed for DPP IV activity. Hydrophobically bound material that could not be eluted with a NaCl-gradient was eluted with 0.1% (v/v) Triton X-100 in buffer B containing either 750 mM NaCl (continuous gradient) or 400 mM NaCl (discontinuous gradient).

**Sodium dodecyl sulfate polyacrylamide–gel electrophoresis (SDS–PAGE)**

Proteins were separated in 7.5% SDS–polyacrylamide slab gels according to the method of Laemmli (1970). Samples were prepared in 62.5 mM Tris–HCl, pH 6.8, 3% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol, and 0.001% (w/v) bromophenol blue and boiled for 3 min in a water bath.

For electrophoretic separation of native, enzymatically active DPP IV under non-denaturing conditions SDS–PAGE was performed in a modified manner. Samples were prepared in 62.5 mM Tris–HCl, pH 6.8, 0.1% (w/v) SDS, 10% (v/v) glycerol, and 0.001% (w/v) bromophenol blue without being boiled, and were kept at room temperature.

**Isoelectric focusing**

Isoelectric focusing was performed in 0.75-mm-thick slab gels as described by Van den Bosch et al. (1988) with the following modifications. Samples of 35 µl were incubated with 50 µl of urea sample buffer (9.5 M urea, 2% (v/v) Triton X-100, 2% (v/v) ampholines (40%, mixture of Servalyte 2–11 and Servalyte 4–9T in a ratio of 1:1) 97 mM DL-dithiothreitol) at room temperature. Vertical 4% polyacrylamide gels containing 9.0 M urea, 2% (v/v) Triton X-100, 6% (v/v) of a mixture of 40% ampholines (Servalyte 2–11 and Servalyte 4–9T in a ratio of 1:1), 0.05% (v/v) TEMED and 0.02% (m/v) ammonium persulfate, were run for 15 min at 200 V, for 30 min at 300 V, and for 1 h at 400 V, using 20 mM H₂PO₄ as anodic buffer in the lower chamber and 50 mM NaOH as cathodic buffer in the upper chamber. Thereafter, samples were applied to the gel, overlaid with 4.75 M urea, 2% (v/v) Triton X-100, 1% (v/v) ampholines (40%; mixture of Servalyte 2–11 and Servalyte 4–9T in a ratio of 1:1), 49 mM Dl-dithiothreitol, and the gels
were run for an additional 18 h at 400 V. Gels were then silver stained according to Heukeshoven and Dernick (1988) after preincubation of the gels in 10% (w/v) trichloroacetic acid (TCA) for 2 h, and twice in 5% (w/v) sulfosalicylic acid for 1 h to remove ampholines.

The pH-scale was determined after focusing by slicing one lane of the gel that had been loaded with sample buffer from the top to the bottom into equal pieces and measuring the pH of each piece in 200 µl 10 mM KCl.

Staining of gels
Polyacrylamide gels were silver stained according to Heukeshoven and Dernick (1988) or according to Blum et al. (1987).

Treatment of purified DPP IV with sialidase
DPP IV purified from either kidney or liver plasma membranes was dialyzed overnight against 50 mM sodium-acetate, pH 6.5, then incubated with sialidase immobilized to agarose (from Clostridium perfringens, 0.6–1.0 U/ml gel, EC 3.2.1.18) at 37°C for 76 h under continuous agitation. Twenty units of DPP IV from kidney membranes or 1 U of DPP IV from liver membranes were incubated with 0.3 U or 0.1 U of sialidase, respectively. Before use immobilized sialidase was thoroughly washed with acetate buffer to remove the storage buffer. For control DPP IV was incubated under the same conditions without sialidase. After incubation, beaded neuraminidase was removed by centrifugation. Samples were dialysed against 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) octylglucoside, assayed for DPP IV activity, and prepared for further analysis.

Resialylation of desialylated DPP IV with sialyltransferase
Desialylated DPP IV was dialyzed overnight against sodium cacodylate buffer (50 mM sodium cacodylate, pH 6.5, 50 mM NaCl, 0.1% (w/v) octylglucoside). Likewise, sialyltransferase from rat liver (EC 2.4.99.1) (0.1U/50 µl) was dialyzed against the same buffer. CMP-N-acetylmuraminic acid was dissolved in sodium cacodylate buffer (1 mg/20 µl) and the pH was adjusted to 6.5. For resialylation, 0.2 U of desialylated DPP IV were incubated with 1 mg of CMP-N-acetylmuraminic acid and 0.02 U of sialytransferase in a final volume of 150 µl at 37°C. After incubation for 24 h, 48 h, and 72 h aliquots were withdrawn for analysis by SDS–PAGE.

Lectin affinity blotting
Sialylation of different forms of DPP IV was analysed by lectin affinity blotting using the method of Haselbeck et al. (1990). Briefly, aliquots of purified DPP IV and of desialylated DPP IV from kidney (1 µg) or from liver (0.5 µg) in 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) octylglucoside were heat denatured, then dotted onto nitrocellulose membranes (0.45 µm). 1 µg of human transferrin, having α 2,6-linked sialic acid residues (Hashimoto et al., 1981), 1 µg of bovine fetuin, having α 2,3-linked sialic acid residues (Spellman et al., 1989), and 1 µg of carboxypeptidase Y (Debray et al., 1981), lacking sialic acid residues, were then blocked by incubation for 1 h in 0.5% (m/v) blocking reagent in buffer C (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 1 mM CaCl2), membranes were incubated with the digoxigenin-labeled lectins Sambucus nigra agglutinin (SNA) (1 mg/ml) or Maackia amurensis agglutinin (MAA) (5 mg/ml), each in buffer D, for 1 h at room temperature. Membranes were then washed again three times with buffer C and incubated with sheep anti-digoxigenin Fab fragments, conjugated to alkaline phosphatase, for 1 h. Bound lectin-digoxigenin conjugates were visualized with the alkaline phosphatase reaction using the 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium chloride system by incubating the membranes in 10 ml of the following solution: 37.5 µl 5-bromo-4-chloro-3-indolyl-phosphate (50 mg/ml, in dimethylformamide) and 50 µl 4-nitro blue tetrazolium chloride (77 mg/ml in 70% dimethylformamide) in 10 ml 100 mM Tris–HCl, pH 9.5, 50 mM MgCl2, 100 mM NaCl. The membranes were rinsed with H2O to stop the reaction, and then dried.

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Abbreviations
DPP IV, dipeptidylpeptidase IV (EC 3.4.14.5.); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tx-100, Triton X-100; HPLC, high performance liquid chromatography; NPPase, nucleotide pyrophosphatase (EC 3.6.1.9.); Con A, concanavalin A; WGA, wheat germ agglutinin; MAA, Maackia amurensis agglutinin; SNA, Sambucus nigra agglutinin; TCA, trichloroacetic acid; kDa, kilodalton; Mr, relative molecular mass; CMP, cytidine 5’-monophosphate; pl, isoelectric point; CMC, critical micelle concentration; Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine.

References


Pettel,K.J., Diamond,M., Hong,W., Bujanover,Y., Amatucci,S., Pittschilleer,K. and Doyle,D.J. (1987) Isolation and characterization of a M, = 110000