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CHAPTER 3

FUNCTIONAL ASPECTS OF THE THREE EXTRACELLULAR DOMAINS OF DIPEPTIDYL PEPTIDASE IV: CHARACTERIZATION OF GLYCOSYLATION EVENTS, OF THE COLLAGEN-BINDING SITE AND OF ENDOPEPTIDASE ACTIVITY

Werner Reutter, Oliver Baum, Klemens Löster, Hua Fan, Jens Peter Bork, Kathrin Bernt, Christoph Hanski, Rudolf Tauber

Dedicated to Professor Dr. Karl Decker on the occasion of his 70th birthday

The integral membrane protein dipeptidyl peptidase IV (DP IV) is a well characterized serine protease which liberates dipeptides from the N-terminus of peptides, provided the penultimate amino acid residue is proline or alanine. Here we report on further properties of this ectoenzyme. The ubiquitous distribution of DP IV in rat tissues was studied by using Northern blot techniques and immunohistochemistry. This study revealed that in adult rats the expression is mainly regulated at the mRNA level while during development it is controlled by additional mechanisms.

The biological function of the eight *N*-glycans is only partly understood. Total inhibition of *N*-glycosylation by tunicamycin dramatically reduced the cell-surface expression of DP IV suggesting a regulatory effect of oligosaccharides on protein stability. Moreover, *N*-glycosylation is shown to have an impact on intracellular trafficking

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transduction by tyrosine phosphorylation of a subset of small molecular mass proteins.[11] The association of DP IV with adenosine deaminase (ADA) and CD45 also has been reported, but the functional relevance of these interactions is still unknown.[3,12] Fifth, DP IV mediates the binding of activated T lymphocytes to collagen[13] and interacts as a cell adhesion molecule (CAM) with substrate molecules of the extracellular matrix (ECM) as shown.[14-20]

The molecular mass of the denatured and reduced glycoprotein as determined by SDS-PAGE was shown to range from 110-150 kD in different cell types from several species.[14,17,21,22] Smaller differences in the M_r of DP IV expressed in different rat tissues reflect organ-specific glycosylation patterns. Mouse fibroblast DP IV differs from all other forms by its very low M_r of 50 kD when separated by SDS-PAGE.[19] However, in its native state in vivo DP IV is regularly present as a homodimer, as demonstrated by electron microscopy and gel filtration.[23,24] This was recently confirmed by chemical cross-linking experiments. These further indicated that the association of the two DP IV-monomers occurs in the late Golgi compartment.[25]

An important step in DP IV research was achieved by the determination of the primary structure of the enzyme by cDNA sequencing after cloning from rats, mice and men. The amino acid sequences display similarities of about 85% between rat and man and 92% between rat and mouse.[26-31] By computer analysis of these data, it was possible to deduce five structural domains/regions within the amino acid sequence of DP IV (Fig. 3.1). There is an N-terminal cytoplasmic tail only six amino acids long and a single 22 amino acid trans-

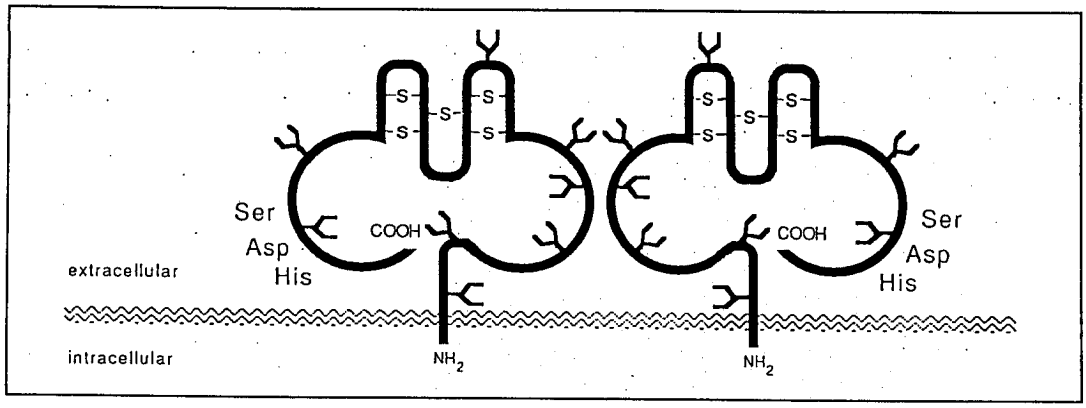


Fig. 3.1. Schematic representation of DP IV domains deduced from its primary structure. The amino acid sequence of rat DP IV has been determined by cDNA cloning and sequencing [Hong et al,[26] Ogata et al,[27] Xu et al[28]]. Five structural domains/regions can be recognized: a short intracellular N-terminal domain, one transmembrane domain and an extracellular domain consisting of three regions with structural peculiarities as indicated: a glycosylation site-rich region (296 amino acids), a cysteine-rich region (228 amino acids) and the C-terminal region (214 amino acids) containing the catalytic triad (Ser/Asp/His) of the ectopeptidase active site. DP IV is expressed on cell surfaces as a dimer of two identical protein chains which both possess enzymatic activity. Although known to be non-covalent, the exact mechanism of association is still unclear.

membrane domain representing the N-terminal signal peptide. Both are retained during biosynthesis and function as the membrane-anchoring domain, resulting in expression as a type II membrane glycoprotein.[32] The 739 amino acid extracellular domain of rat DP IV can be further divided into three regions with prominent structural features. First, adjacent to the transmembrane domain, is a region harboring a cluster of five out of eight potential *N*-glycosylation sites of the DP IV-monomer including a stalked structure characteristic for ectoenzymes. Second, towards the C-terminus a cysteine-rich region follows expressing nine of the twelve cysteines which probably form disulphide bridges. Third, the C-terminus itself includes the active site of the serine protease. Sequencing of the 90 kb large mouse gene revealed the existence of 26 exons.[33]

Until now, with the exception of post-prolyl endopeptidase,[34] no proteins have been identified with noticeable sequence similarities to DP IV suggesting that the enzyme does not belong to a larger gene family, as do other serine proteases[35] and CAMs such as integrins, cadherins or members of the immunoglobulin superfamily.[36-39] However, the C-terminal region of the active site is a characteristic feature of non-classical serine hydrolases.[29,33]

In this chapter, we focus on our own investigations designed to characterize structural features of the three extracellular regions of DP IV employing immunological, biochemical and molecular biological methods. In addition, we describe the expression patterns of DP IV-mRNA and -protein in the rat and typical features of the glycosylation of DP IV. For further characterization as a CAM, we demonstrate the involvement of the cysteine-rich region of DP IV in collagen binding. Additionally, we provide evidence for the ability of DP IV to perform proteolytic processing of collagen. These results should contribute to the understanding of the structure function relationships of DP IV.

RESULTS AND DISCUSSION

Expression of DP IV in Rat Tissues

A panel of mAbs recognizing four different epitopes expressed on DP IV has been developed in our laboratory.[40,41] Three of these mAbs recognize epitopes on the protein portion of DP IV designated epitopes A, B and C, respectively, whereas the fourth, mAb 25.8, binds to a carbohydrate epitope D found to be expressed on DP IV as well as on several other proteins in rat liver.[41] This neuraminidase-sensitive 25.8-epitope does not appear on proteins before they have undergone glycosylation reactions in the Golgi compartment including trimming and transfer of outer sugar residues (Fig. 3.2).

Using these four mAbs and a specific polyclonal antiserum, the appearance of the DP IV protein in different adult rat tissues has been investigated intensively by immunohistochemistry and histochemical

analysis.[2,42] Based on these qualitative results and additional quantitative data obtained e.g., by determination of DP IV-activity in homogenates and crude membrane extracts of various rat organs (Fig. 3.3A), the expression patterns of DP IV can be summarized as follows: DP IV is most intensively expressed on the apical surface of epithelial and endothelial cells, but is also present on fibroblasts, lymphocytes and even on a few neuronal cells. The highest relative concentration of DP IV is found in the kidney, where the enzymatic activity is primarily located to the brush border of proximal tubules. In the liver, DP IV is predominantly present in the microvilli of interlobular as well as common bile ducts and in a high concentration in the apical (canalicular) domain, but also in the basolateral (sinusoidal) domain of hepatocytes.[20,22,42-45] Significant quantities of DP IV are also expressed in the microvilli of small intestine, colon and pancreatic ducts, in acinar structures of salivary glands and on thymocytes, as well as in T-cell areas of the spleen and lymph nodes. A staining of capillary endothelial cells has also been observed in, e.g., lung, kidney, heart, skeletal muscle and spleen. Additionally, DP IV-immunoreactivity was found in the connective matrix of some tissues especially skin, oesophagus,

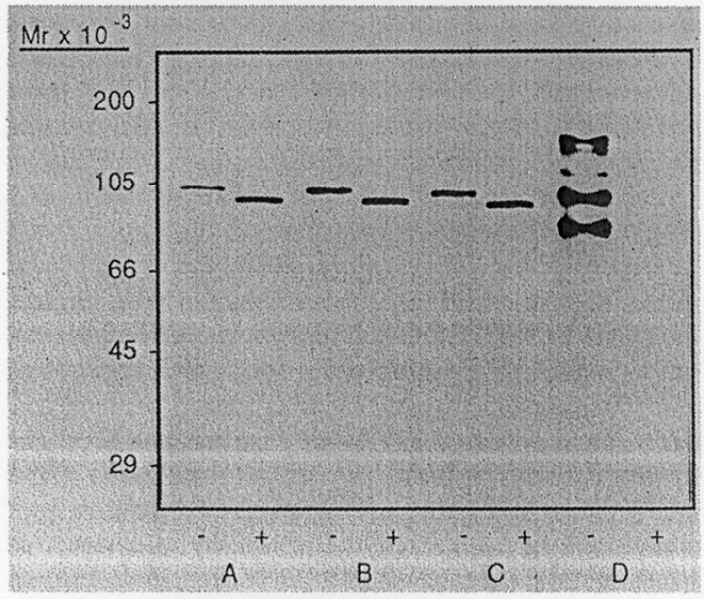


Fig. 3.2. Role of high-mannose-type oligosaccharides in the precipitation of the epitopes A-D. Primary cultured hepatocytes were labeled overnight with ^{35}S-methionine with (+) or without (-) prior treatment with deoxymannojirimycin (a potent inhibitor of mannosidase I which is active in the processing of N-glycans so that they accumulate high-mannose-type sugar chains) as described [Hartel-Schenk et al[41]]. The cells were solubilized, and DP IV was precipitated with the mAbs against epitopes A-D. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

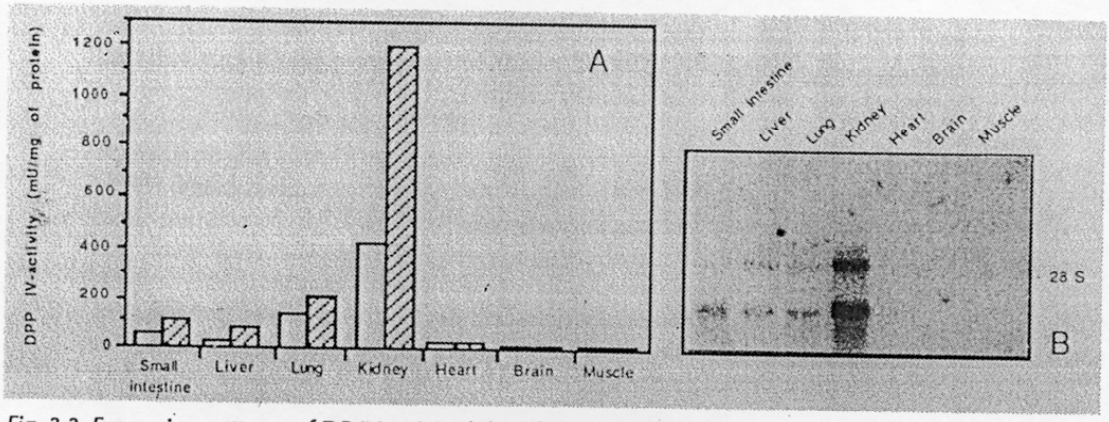


Fig. 3.3. Expression patterns of DP IV-activity (A) and DP IV-mRNA (B) in organs of adult rats. DP IV activity in tissue homogenates (□) and crude membrane extracts (▨) was quantified with Gly-Pro-nitroanilide-tosylate as substrate (A). For northern blot analysis (B), 20 µg of total RNA from each organ were hybridized with a 60 bp ^{32}P-labeled oligonucleotide which was complementary to bp 780-840 of the DP IV cDNA. The specific DP IV-mRNAs bands at 3.2 and 4.8 kb were detected by autoradiography as described [Hildebrandt et al[47]].

ureter and penis, suggesting that DP IV might associate particularly with a certain type of collagen.[2]

As shown in Fig. 3.3B, the Northern blot analysis with specific oligonucleotide probes derived from the DP IV-cDNA sequence revealed a level of mRNA-expression comparable to DP IV protein concentration in each of the investigated adult rat tissues.[46,47] Hence, in the adult rat mRNA levels seem to correlate with protein expression, suggesting a transcriptional control of DP IV-expression.

During ontogeny, however, both transcriptional and post-transcriptional factors influence DP IV-expression in the lung, but not in the kidney providing evidence for different organ-specific mechanisms operative in expression control.[47] In addition, a systematic immunohistochemical comparison of DP IV appearance during pre- and postnatal development in rats revealed that DP IV is expressed on fetal cells and on cell surface domains where DP IV-activity has not been observed in adult organisms, e.g., in brain tissues and basolateral plasma membranes of epithelial cells.[48] According to these results, a post-translational modulation of DP IV enzymatic activity also seems to be possible. Interestingly, the ontogenic investigations suggested that DP IV-expression in the rat liver correlates with the age and differentiation status of hepatocytes.[46,47]

In the rat liver, a soluble form of DP IV was detected representing 15% of total hepatic activity, which was not found in Morris hepatoma 7777.[2,40] Other studies showed that this soluble form lacks the N-terminal domain suggesting that this form might be derived from the integral form by proteolytic processing of the N-terminal signal peptide.[23,27]

Possible implications of expression patterns for in vivo functions are restricted, because one rat substrain (Fisher rat 344) supplied from German and Japanese sources does not express DP IV protein.[49,50] However, with the exception of renal and intestinal resorption of short peptides, the inherited lack of DP IV does not lead to any obvious pathological disorders.[6,51] Recently, additional studies demonstrated that the DP IV gene has been altered in this rat substrain by a one-point-missense mutation in the active site. This alteration led to a complete and efficient degradation of the translated protein in the Golgi compartment and, therefore, prevented the cell-surface expression of DP IV in any tissue.[52,53]

The distribution of DP IV in human tissues summarized by ref. 4 shows no particular species-specific differences in the expression patterns of rat and man.

Characteristics of DP IV-glycosylation

From the observation that the glycosylated and the enzymatically *N*-deglycosylated form of cell surface DP IV from rat liver exhibit a difference in M_r of 24 kD, it was inferred that DP IV possesses at least eight *N*-glycosylation sites.[22,54] *O*-Glycosylation of DP IV was not detected in these studies. Subsequently, the determination of the DP IV primary structure actually confirmed the presence of these eight *N*-glycosylation sites.[26,27] *N*-Linked oligosaccharides of DP IV do not possess a uniform configuration but display various structural features. This conclusion is partly based on the analysis by 2D-PAGE separating e.g., renal DP IV into more than 12 isoforms with pI-values between 4.8 and 5.3, as shown in Fig. 3.4. These variants presumably differ in their content of negatively charged sialic acid residues (Zeilinger et al, in preparation). An analysis by lectin blotting suggests that in DP IV from rat liver, *N*-glycans are predominantly of the complex type. The results of metabolic labeling and N-glycan analysis confirm this.[55,56] In contrast, DP IV from Morris hepatoma cells 7777 contains a higher proportion of *N*-glycans of the oligomannosidic or hybrid type.[57] Finally, structural analysis of the carbohydrate moiety of rat kidney DP IV revealed the presence of di-, tri- and tetraantennary complex-type structures containing a bisecting *N*-acetyl-D-glucosamine residue.[58]

The biological role of DP IV-*N*-glycosylation is only partly understood. Previous studies have shown that the stability of non-glycosylated DP IV obtained in the presence of tunicamycin is dramatically reduced resulting in rapid intracellular degradation.[57] In order to determine the functional importance of the single *N*-glycosylation sites, molecular biological experiments by site-directed mutagenesis were performed (Fan et al, in preparation). Three cDNA mutants were constructed, each with a single nucleotide substitution resulting in the loss of an Asn residue at different *N*-glycosylation sites. Two *N*-glycosylation

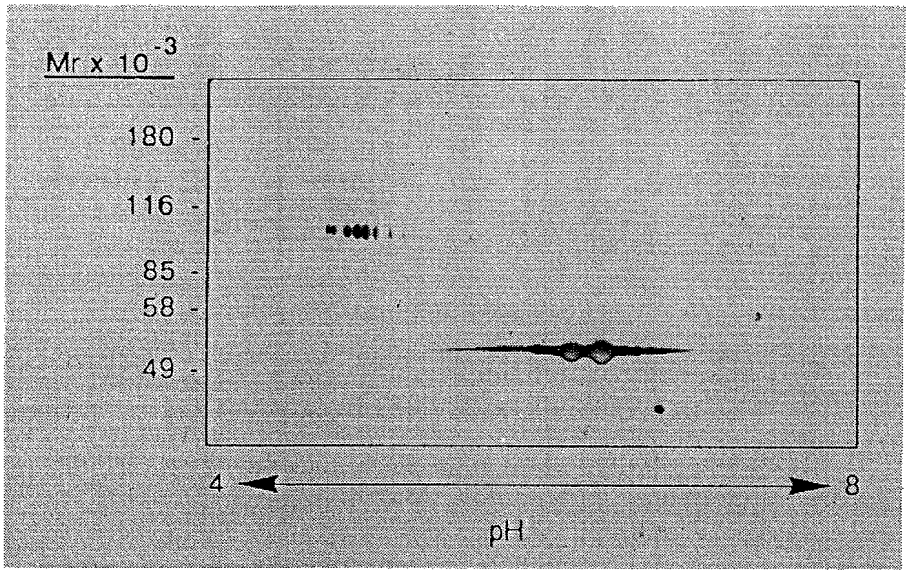


Fig. 3.4. Microheterogenity of DP IV-glycosylation. DP IV was immunoprecipitated from 100 µg of kidney plasma membrane protein extract with mAb 13.4. Immunoprecipitated proteins were separated by 2D-PAGE, using immobilized pH-gradient gel electrophoresis (IPG) in the first dimension according to Righetti,[81] and visualized by silver staining. DP IV-glycosylation variants can be detected as at least 12 protein spots covering a pI range from 4.8-5.3. The protein at 50 kD represents the heavy chain of the mAb 13.4.

mutants expressed DP IV on their cell surface and showed enzymatic activity, suggesting that these glycosylation sites do not significantly influence protein stability. In contrast, the third *N*-glycosylation mutant exhibited no enzymatic activity and was not detectable on the cell surface. We, therefore, suggest that different glycosylation sites on DP IV have different biological functions (Fan et al, in preparation).

Role of Glycosylation in Intracellular Trafficking of DP IV

In polarized epithelia such as hepatocytes, enterocytes or renal tubular cells, DP IV undergoes extensive intracellular trafficking including biosynthetic transport from the endoplasmic reticulum to the cell surface, internalization into the endosomal compartment and recycling to the cell surface. Since DP IV is an apical surface protein in polarized epithelia, intracellular trafficking of DP IV is intimately related to the sorting of the newly synthesized molecules to the apical surface domain. Studies in different model systems show that the sorting of DP IV may take place on the exocytic as well as on the endocytic route.[59] Whereas in MDCK cells the sorting of newly synthesized DP IV takes place on the exocytic route upon exit from the *trans*-Golgi network (TGN), newly synthesized DP IV in hepatocytes is first routed to the basolateral membrane and then sorted to the apical domain via the endocytic-transcytotic route. In Caco-2 adenocarcinoma cells of

intestinal origin, DP IV may be sorted on both the exocytic and the endocytic-transcytotic route. DP IV is subject to internalization even in non-polarized cells including CHO and Hep G2. It is tempting to speculate that apart from sorting, internalization and recycling may serve additional functions such as the up- and down-regulation of cell surface expression of DP IV or signal transduction.

During biosynthesis, glycosylation seems to be pivotal for the formation of functionally stable molecules and for surface transport of newly synthesized DP IV, similar to other cell surface glycoproteins. Subsequent to co-translational *N*-glycosylation in the rough endoplasmic reticulum, the oligomannosidic glycans of newly synthesized DP IV are extensively processed during transport to the cell surface. The fundamental processing reactions include removal of D-glucose and D-mannose residues from $Glc_3Man_9GlcNAc_2$ and the transfer of terminal sugar residues, *N*-acetyl-D-glucosamine, D-galactose, L-fucose and sialic acid, by an array of processing glycosidases and glycosyltransferases. At the cell surface, processed mature DP IV is not detectable before 60 minutes after biosynthesis in the endoplasmic reticulum.[60] As shown after inhibition of *N*-glycosylation with tunicamycin in rat hepatocytes,[60] non-glycosylated DP IV is rapidly degraded within 1 hour after synthesis and fails to reach the cell surface. On the other hand, a processing of the oligomannosidic precursor oligosaccharides into complex structures is not a prerequisite for surface transport. This indicates that it is the transfer of $Glc_3Man_9GlcNAc_2$ oligosaccharides to *N*-glycosylation sites and not their subsequent processing which is essential for the folding of the nascent polypeptide chain and/or for stabilizing of the conformation of the folded protein.

Reactions modifying the oligosaccharide pattern of cell surface DP IV are not restricted to the biosynthetic pathway, but may also occur during endocytosis and recycling.[61] This conclusion is based on the following evidence: Previous measurements of the turnover rates of the different sugar residues of DP IV in the liver and in different cell types demonstrated that sugars in terminal and penultimate positions, L-fucose, sialic acid and D-galactose, may be removed from the non-reducing end of oligosaccharides even after transport to the cell surface.[55,62-65] Post-biosynthetic processing of DP IV by a series of trimming reactions was confirmed by a structural analysis for oligomannosidic glycans of cell surface DP IV. As shown in hepatocytes and Morris hepatoma 7777 cells, oligomannosidic glycans on surface DP IV synthesized in the presence of the mannosidase I inhibitor, deoxymannojirimycin (dMM), are trimmed from $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ by mannosidase(s) sensitive to dMM during endocytosis and recycling (Loch et al, in preparation). Although not proven unequivocally, it is very likely that trimming of terminal sugars of complex-type glycans as well as of mannose residues of oligomannosidic structures takes place either in the plasma membrane or in endocytic compartments, or even after the return to the *trans*-Golgi or *trans*-Golgi network. Unlike DP IV,

several surface glycoproteins including the transferrin receptor, escape trimming of their oligosaccharides, although they are also rapidly internalized and recycled to the surface. This indicates that trimming is selective. Experiments demonstrating that DP IV is subject to oligosaccharide trimming with the same kinetics and to the same extent after expression in different cell lines support the assumption that the propensity to undergo oligosaccharide trimming is controlled by inherent properties of surface DP IV (Loch et al, in preparation).

Studies designed to examine the recycling of surface glycoproteins to compartments of the biosynthetic route show that DP IV actually returns to glycosyltransferases in the *trans*-Golgi and *trans*-Golgi network, and acquires the terminal sugars, L-fucose and sialic acids, while trafficking through these compartments.[64,65,65a] Like post-biosynthetic trimming reactions, recycling to compartments of the biosynthetic route and re-glycosylation is selective. Apart from DP IV, only distinct surface glycoproteins may return to the *trans*-Golgi and the TGN. Interestingly, no return of DP IV to mannosidases IA/IB in *cis*-Golgi elements was observed, indicating that return to the biosynthetic route is restricted to medial/*trans* elements of the Golgi and to the *trans*-Golgi network (Volz et al, submitted).

In conjunction with the data demonstrating post-biosynthetic trimming of the oligosaccharides of surface DP IV, the results on reglycosylation support the concept that cell surface DP IV does undergo continuous reprocessing of the *N*-linked oligosaccharides by partial de- and reglycosylation, as schematically shown in Fig. 3.5. The physiological significance of oligosaccharide re-processing of DP IV during endocytosis and recycling is unknown at present. Trimming and reglycosylation might represent the occasional removal of outer sugar residues by glycosidases present in the cellular environment or encountered during internalization and recycling, followed by subsequent repair of the glycoproteins. Alternatively, re-processing could provide means to modify oligosaccharide structures of mature surface DP IV in response to cellular or extracellular stimuli, and may represent a mechanism of cellular adaptation.

Turnover studies revealed that the peripheral monosaccharides of the *N*-glycans of DP IV (*N*-acetylneuraminic acid, L-fucose and D-galactose) turn over much faster than the core-sugars (D-mannose and the *N*-acetyl-D-glucosamine) and the polypeptide backbone as well.[55,56] However, it is still in question in which subcellular compartment the enzymes responsible for this post-processing event are localized. We suggest that they might be active in either the plasma membrane, in endosomes or in the *trans*-Golgi-network (TGN). However, subsequent to this modification, the glycoproteins are re-glycosylated in the Golgi complex and re-inserted into the cellular plasma membrane.[64] The rapid turnover of carbohydrates in malignant tissues e.g., rat hepatoma, in contrast to liver, is additionally extended to the core

mannose monosaccharides of plasma membranes glycoproteins.[66] This characteristic, designated intramolecular heterogeneous turnover or reprocessing, is is detectable in not only DP IV but also other integral membrane glycoproteins[67] and does not occur in serum glycoproteins.[68]

Involvement of the Cysteine-Rich Region of DP IV in Cell-Collagen Binding

The presence of DP IV in sites of cell contact to the ECM provided an early indication of the involvement of DP IV in cell-matrix interactions.[14,69] The ability of DP IV to interact with proteins of the ECM as a CAM was then confirmed by several groups.[13,16-20] In particular, Hanski et al[16,18] showed that the DP IV substrate tripeptide, Gly-Pro-Ala, interferes in vitro with the initial spreading of rat hepatocytes on a matrix consisting of fibronectin and denatured collagen, as well as on native collagen. The same blocking activity was also displayed by a polyclonal antiserum against DP IV.[18] In vitro immunofluores-

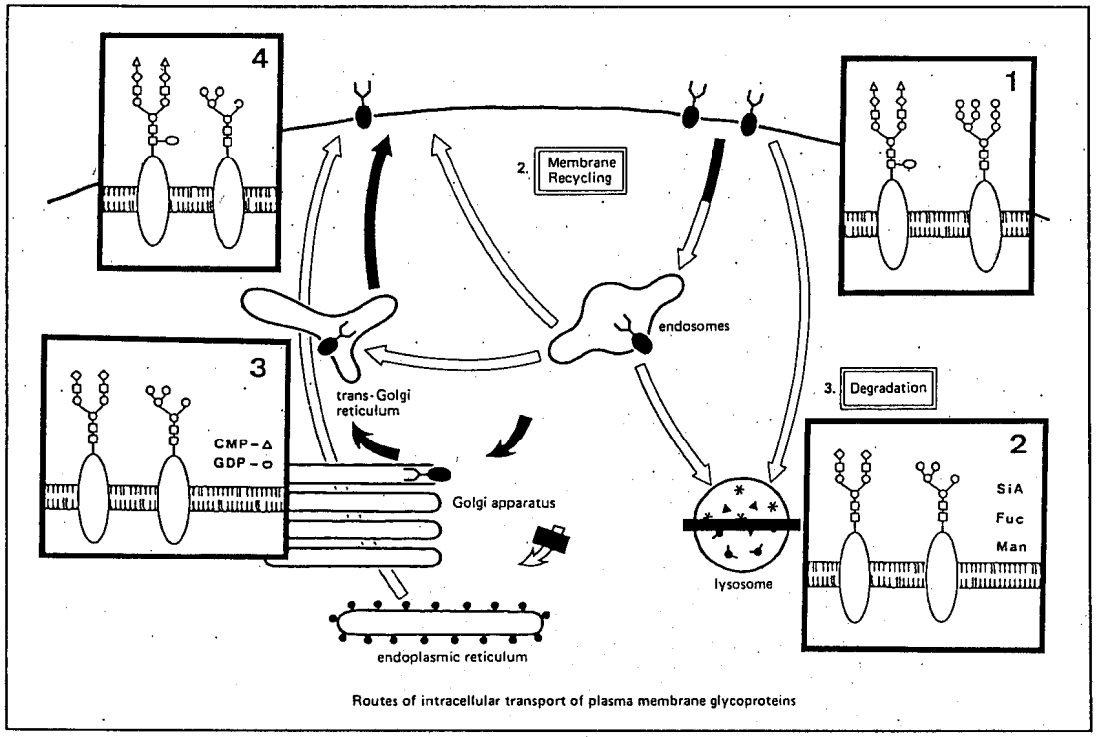


Fig. 3.5. Postbiosynthetic processing of DP IV during internalization and recycling in hepatocytes and hepatocarcinoma cells. 1,2. During internalization or in situ on the cell surface, DP IV undergoes trimming by removal of terminal and penultimate sugars, i.e. of fucose and sialic acid, from complex-type glycans and of mannose residues from oligomannosidic structures. 3,4. Partial deglycosylation is followed by transfer of sialic acid and fucose residues to complex-type glycans of DP IV while recycling through the medial/trans-Golgi and the trans-Golgi network. So far, no mechanisms have been described for transfer of mannose residues to the glycans of mature glycoproteins.

cence and catalytic histochemistry on coated native collagen, as well as affinity chromatography of plasma membrane protein extracts on collagen I-Sepharose, also indicated a DP IV-collagen interaction.[18] Piazza et al[20] reported in vitro fibronectin binding of DP IV independent of its ectopeptidase activity. Finally, Bauvois[19] demonstrated that partially purified DP IV can disrupt cell adhesion to collagen.

However, the detailed molecular mechanism of the interaction between DP IV and the substrate molecules of the ECM has not been examined in any of these studies. Thus, in order to characterize the binding of DP IV to ECM molecules we established an in vitro binding assay.[70] Native DP IV was isolated by sequential affinity chromatography on lectin- and Gly-Pro-Sepharose, radio-iodinated by using the chloramine-T method and incubated in a solid phase adhesion assay with different ECM proteins as a coat. In vitro binding of native DP IV depended markedly on the type of coated ECM protein, as was determined by the recovery of radioactivity bound to the substrates (Fig. 3.6). DP IV showed preferential binding to various collagens, but only low interaction with the other investigated ECM-proteins, laminin and fibronectin. Among the examined collagens, DP IV displayed its highest binding affinity for collagens type I and III, with lower binding to

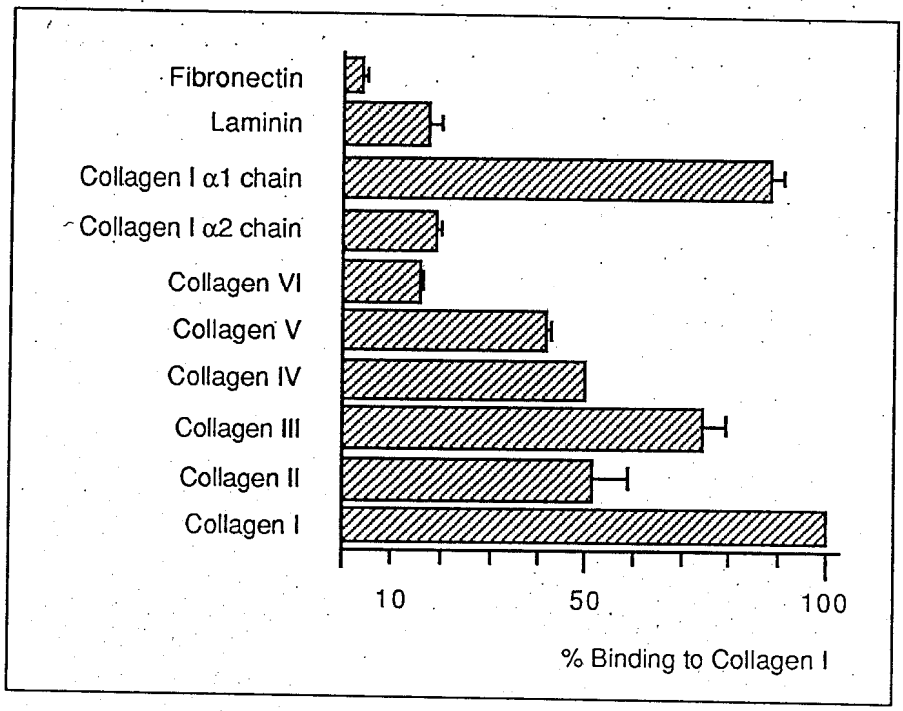


Fig. 3.6. In vitro-binding of DP IV to proteins of the ECM. 200 ng of each ECM protein were adsorbed to microtiter plates, blocked with 0.1% BSA in PBS, pH 8.0 and coincubated with both 15.000 dpm of ^{125}I-labeled and 62.5 ng of non-radioactive DP IV. n = 5.

collagens type II, IV and V, and weak adhesion to collagen type VI. In control experiments, the in vitro binding assay was performed in an inverted way by using soluble ECM-proteins and DP IV coated onto the microtitre plates as stationary phase. This approach gave similar results, with high affinity adhesion to soluble collagen type I, minor interaction with collagen type IV and negligible binding of fibronectin to immobilized DP IV (data not shown).

These results support in general the idea that DP IV can act as a collagen binding CAM. A previously reported[20] interaction of DP IV with fibronectin, however, was not observed in our laboratory. Thus, the binding specificity of DP IV is similar to that of the main cellular collagen receptors, $\alpha_1\beta_1$- and $\alpha_2\beta_1$-integrins, which bind to collagen with high affinity (especially after its denaturation), whereas laminin and fibronectin are recognized only to a small extent or not at all.[71]

The relatively low affinity to collagen type IV was surprising, because this matrix molecule is a major component of the basal membrane, being in close contact with the cell surface and, therefore, representing a potential ligand of DP IV. The affinity of DP IV to collagens might depend on the presence of the large collagenous domains found in particular in collagens type I to V but not in collagen type VI.[72] The relatively high binding constant of DP IV, especially to collagens type I and III, might be due to structural similarities shared by both collagens. In this context, the closely related primary and tertiary structures of the α1(I) chain of collagen type I and the α1(III) chain of collagen type III[72] might be important for the exposition of ligand binding sites involved in DP IV interaction.

When DP IV was incubated with isolated single chains of collagen type I, a strong binding rate to the α1(I) chain but a significantly weaker one to the α2(I) chain was observed (Fig. 3.6). These differences in binding specificity are probably due to structural features varying between the two collagen chains. They differ in the total number of amino acid residues by nearly 100 and show a variant number of lysine/hydroxylysine cross-linking sites.[72] Moreover, only the α1(I) chain contains a histidine conjugation sequence and the $\alpha_2\beta_1$-integrin recognition motif for collagen I-binding, the Asp-Gly-Glu-Ala (DGEA) sequence, located in the middle of the helical domain.[73]

To obtain structural information on the collagen binding site, the inhibitory effect of two specific mAbs on the interaction of DP IV with collagen type I was examined. Whereas mAb 13.4 directed against a protein epitope of DP IV[21] strongly inhibited binding to collagen type I, mAb 25.8, specific for the carbohydrate epitope of DP IV mentioned above, showed only a weak inhibitory effect (Fig. 3.7). Peptide mapping of DP IV using V8-*Staphylococcus aureus*-protease revealed that mAb 13.4 recognizes a 33 kD fragment.[70] This protein fragment was identified by N-terminal amino acid sequencing as peptide Tyr_{236}-Glu_{491}.[70]

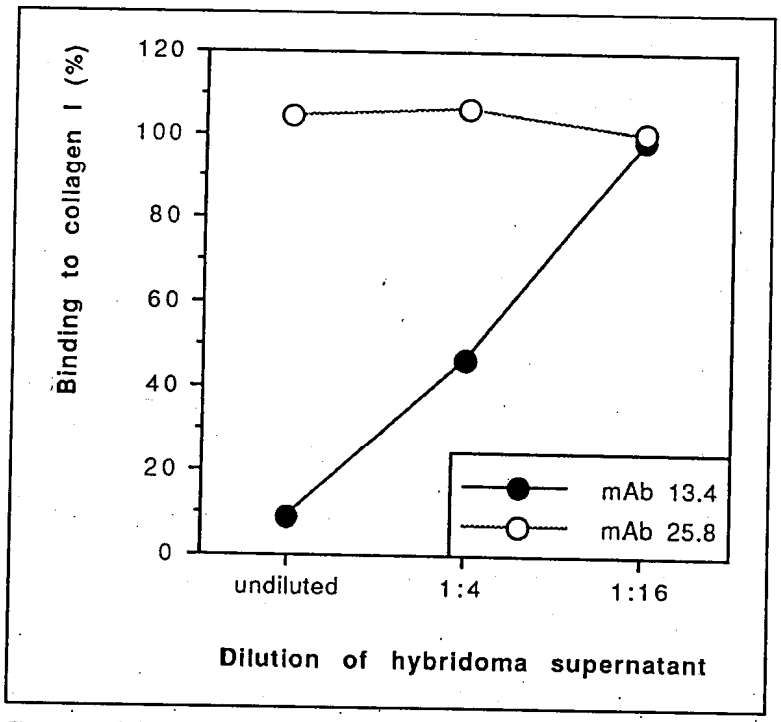


Fig. 3.7. Inhibition of DP IV adhesion to collagen in vitro by monoclonal antibodies. The in vitro adhesion assay on collagen I as described in Fig. 3.6 was performed in the presence of two mAbs specific for DP IV. The mAb 13.4 recognizes a defined protein portion whereas mAb 25.8 binds to the carbohydrate moiety of the enzyme. Both mAbs were used in the assay in dilutions from serum-free cell culture supernatants as indicated. The binding of DP IV to collagen I in a control experiment without antibody was taken as 100%. n = 3.

Since this fragment does not contain the active site of the exopeptidase DP IV (Gly_{629}-Trp-Ser-Tyr-Gly_{633}), identified by Ogata et al,[74] the collagen binding site is obviously not identical with the catalytic center of DP IV. This conclusion is supported by results obtained in DP IV-collagen I binding assays in the presence of the substrate peptide, Gly-Pro-Ala, and the competitive inhibitor peptide, Val-Pro-Leu, neither of which inhibited DP IV collagen-binding (data not shown). This hypothesis is also in accordance with the study of Hanski et al,[18] which demonstrated that DP IV cannot be eluted from collagen I-Sepharose with Gly-Pro-Ala. In addition, compared to rat hepatocytes, Morris hepatoma 7777 cells express only 11% DP IVactivity, thereby probably influencing the low adhesion ability of the metastatic cells.[2]

Other reports indicate that the enzymatic activity of DP IV is not required for additional functions related to DP IV, e.g., for T-cell ac-

tivation[75] and for association with ADA,[12] whereas the costimulatory effect for T-cell activation requires DP IV enzyme activity.[76] Thus, DP IV seems to be a multifunctional molecule in which different molecular domains are involved in different functions.

Characterization of DP IV Enzymatic Activity: Does the Enzyme Possess Endopeptidase Activity for Collagen Processing?

The pH-optimum for lamb DP IV was determined to be 7.8.[34] When the pH-range of the incubation buffer is lowered to less than pH 5.5 or elevated to more than 10.5, DP IV rapidly losses its enzymatic activity. These results suggest that the enzyme is highly sensitive to denaturation by pH-shifts. In addition, as we and others have observed, DP IV-activity is inactivated by various detergents, high salt concentrations, heating, freezing/thawing or other harsh biochemical and physical conditions, but, on the other hand, is not influenced by reducing agents. When rat liver samples are separated by SDS-PAGE under gentle conditions, the active monomeric form of DP IV appears as a 150 kD band, whereas denaturing conditions, e.g., heating for 5 minutes, converts the protein to a 105 kD band. It is not clear if this large M_r difference of 45 kD between the active and denatured form of DP IV is caused by an intramolecular unfolding or by a degradation process.[17,20] In our experiments this electrophoretic behavior was not altered by reducing agents. The demonstration of the 220 kD dimeric form of DP IV by SDS-PAGE requires the use of chemical cross-linkers.[25] Taken together, these results indicate that the two DP IV monomers are non-covalently associated and function independently of each other, but are sensitive to denaturation. To our knowledge neither the protein sequences involved in dimerization nor the biological function of this process have been characterized so far.

Inhibition of enzymatic activity by diisopropyl fluorophosphate (DIFP) provided early evidence that DP IV is a serine protease.[14,77] This was confirmed by both protein sequencing of a DIFP affinity labeled peptide derived after proteolytic digestion and transfection studies with site-directed mutated cDNAs which gave evidence, that the intact rat sequence, Gly_{629}-Trp-Ser-Tyr-Gly_{633}, is necessary for expression of DP IV activity.[74] Other investigations indicated that a steric triad consisting of the three amino acids, $Ser_{624}/Asp_{702}/His_{734}$, is involved in establishing the catalytic activity of mouse DP IV.[29,78]

In a further attempt to characterize the active site of DP IV our laboratory has performed a series of experiments employing site-directed mutagenesis and the construction of deletion mutants of rat DP IV cDNA. These variants were transfected into CHO-cells and analyzed for enzymatic activity. Any investigated alteration of the rat presumptive catalytic triad ($Ser_{624}/Asp_{702}/His_{734}$) dramatically inhibited enzymatic activity of DP IV. These results generally confirm the im-

portance of the intact primary structure in this region for enzymatic function of rat DP IV (Fan et al, in preparation).

With respect to the DP IV-collagen interaction and the high content of Gly-Pro-X or Gly-Hyp-X motifs in collagenous substrate molecules which provide possible DP IV-cleavage sites, we tested the ability of DP IV to digest collagen at intramolecular sites and, thus, to act also as an endopeptidase. Collagen type I was purified from rat tails, labeled with ^{3}H-formaldehyde and incubated with DP IV. After fluorography of the digest separated by SDS-PAGE, several low molecular mass collagen fragments became visible, which were absent in controls (Fig. 3.8). In a second type of experiment, purified native DP IV was introduced into liposomes consisting of various lipids, and incubated with radioactive collagen coated onto the surface of cover slips. A significant release of collagen fragments into the surrounding medium was demonstrated by quantifying the recovery of radioactivity (Fig. 3.9b). As calculated from the specific radioactivity of collagen,

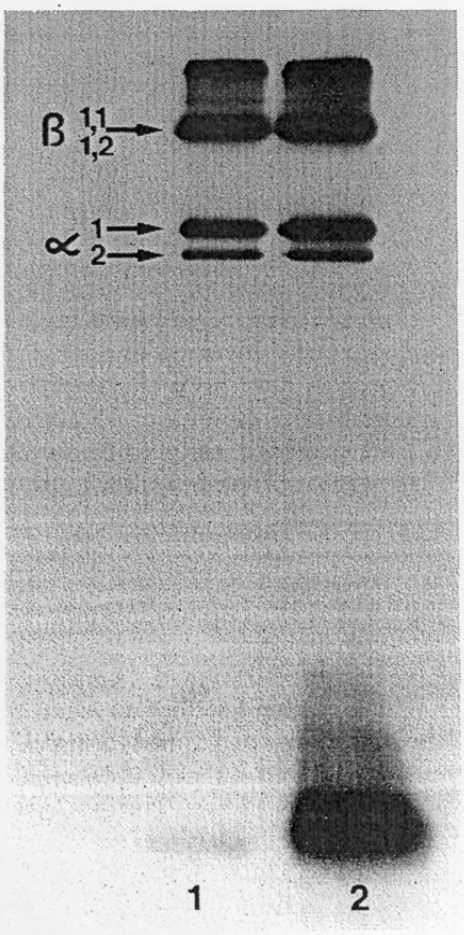
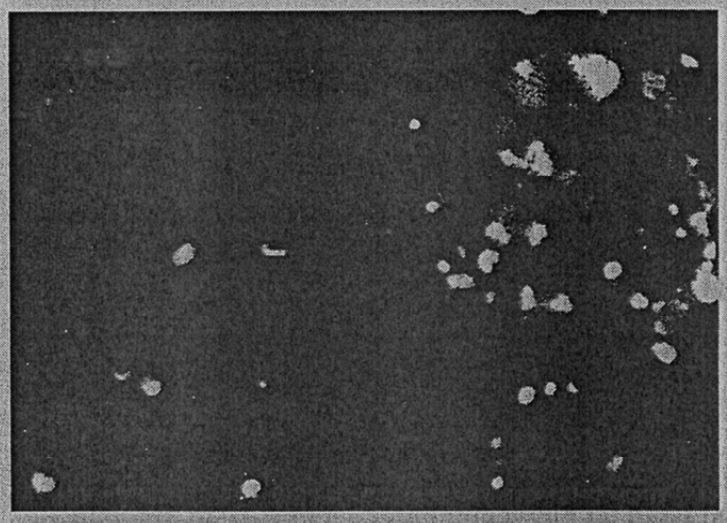


Fig. 3.8. In vitro-digestion of collagen I by DP IV. Collagen I was isolated from rat tails and labeled with ^{3}H-formaldehyde according to Barksdale and Rosenberg.[82] 250 ng collagen were incubated without (1) or with (2) 100 mU isolated native DP IV for 48 h in 10 mM Tris-HCl, pH 8.0. The samples were lyophilised and submitted to SDS-PAGE. Radioactive collagen chains (indicated on the left side) and fragments were detected by fluorography.

20 mU of DP IV (approximately 1 µg protein) caused the release of about 7.6 ng of collagen within 8 hours (Huhle, unpublished data).

Although a weak endopeptidase activity of DP IV has been reported previously,[14,20,69] it has not been excluded that this activity is derived from a copurification of prolyl endopeptidase, a serine protease with similar substrate specificity to DP IV.[34] However, since our investigations were performed with a preparation of highly purified enzyme,[79] it is likely that DP IV possesses the intrinsic properties of an endopeptidase in the proteolytic processing of collagens.



A

Radioactivity recovered in the medium (dpm)		Radioactivity recovered on the cover slip (dpm)	
- DPP IV	+ DPP IV	- DPP IV	+ DPP IV
248	2790	6880 ± 1516	4544 ± 1044

B

Fig. 3.9. Release of collagen I from collagen coated cover slips by liposomes containing DP IV. Isolated active DP IV was integrated into liposomes consisting of lecithin, phosphatidyl ethanolamine and cholesterol, as well as the fluorescence marker dansylcerebroside [Huang[83]], and incubated with cover slips coated with ^{3}H-collagen. The collagen-liposome aggregates were covered with cell culture medium for 8 h at 37°C. The specific binding of DP IV-containing liposomes to collagen I was demonstrated by immunofluorescence microscopy (A, magnification 600x). For determination of DP IV-mediated collagen release, the radioactivity in the medium and on the cover slips was quantified (B). The value shown is the mean of bound radioactivity on three cover slips ± SD.

If this was the case, it could explain the observation of increased levels of peptides containing Gly/Pro/Hyp in the urine of DP IV-negative Fisher rats, in comparison with rats expressing normal DP IV activity in their tissues.[6,7] These authors suggested that DP IV is involved in the reabsorption of collagen-derived fragments by providing digested peptides to a transport system specific for dipeptides and tripeptides.[6] The cellular origin of the collagen fragments and the proteolytic endopeptidase activity responsible for their generation were not determined. However, we suggest that the observed endoproteolytic activity of DP IV with specificity for collagen might expose cryptic binding sites like Arg-Gly-Asp (RGD)-sequences suitable for integrin receptor recognition and interaction. Such peptide sequences derived by denaturation are preferentially bound by integrins.[39] The endoprotease activity of DP IV as described in this paper could be the functional basis for a role of DP IV in the mainting tissue architecture by mediating cell-collagen binding. Although still speculative, the reduced expression of DP IV might be one of the reasons for the up-regulated fibrotic activity in the human cirrhotic liver.[80] In summary, we suggest that DP IV as a collagen-trimming-endopeptidase may have auxiliary functions in cell-matrix adhesion processes and, thus, should be considered as a cell adhesion molecule (CAM) of the plasma membrane.

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