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# THE CYSTEINE-RICH REGION OF DIPEPTIDYL PEPTIDASE IV (CD 26) IS THE COLLAGEN-BINDING SITE

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A remarkable property of the integral glycoprotein dipeptidyl peptidase IV (DPP IV, CD 26) is its affinity to proteins of the extracellular matrix (ECM). By *in vitro* binding assays we have shown that DPP IV binds to collagens; preferentially to the collagens I and III, which are both characterized by the formation of large triplehelical domains. No binding of DPP IV to laminin or fibronectin could be observed. Within collagen I, the α1(I) chain was found to be the most prominent binding ligand of DPP IV. A monoclonal anti DPP IV antibody (13.4) specifically inhibited the interaction of DPP IV with collagen I. Peptide mapping and N-terminal sequencing revealed that the corresponding epitope of mAb 13.4 is located in the cysteine-rich domain of DPP IV. We therefore conclude that the putative collagen binding site of DPP IV is different from the region of the catalytic site containing the exopeptidase activity, which is located at the C-terminal portion of the molecule.


In addition to its function as an exopeptidase, the integral glycoprotein dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5.) was found to be involved in cellular processes that are apparently unrelated to its enzymatic activity, the cleavage of N-terminal dipeptides from

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polypeptides in which proline is the penultimate amino acid [1]. Being identical with the differentiation antigen CD 26, which is expressed on activated T-helper lymphocytes [2], DPP IV was demonstrated to be involved in the regulation of the immune response [3] and in signal transduction events by tyrosine phosphorylation [4]. In addition, DPP IV is known to interact with several other proteins at the cell membrane. In its native state, the protein is regularly present as a non-covalently linked homodimer [5, 6], which is associated with adenosine-desaminase (ADA) and CD 45 [7, 8], when expressed on lymphocytes.

Furthermore, DPP IV is capable to interact with molecules of the extracellular matrix (ECM) suggesting an additional function as a cell adhesion molecule (CAM). In particular, it was shown that DPP IV (CD 26) mediates binding of activated T-helper lymphocytes to collagen [9] and participates in the initial spreading of rat hepatocytes on collagen [10]. Further evidences for a possible function of DPP IV as a CAM were obtained from immunohistochemical analysis when DPP IV was detected at sites of cell contacts to the ECM [11-14]. Additionally, in view of the high number of Gly-Pro-X motifs in collagens it is also conceivable that DPP IV participates in the metabolism of collagens, but there is so far no direct evidence that collagens are *in vivo* substrates of the proteolytic activity of DPP IV. However, the detailed molecular basis for binding of DPP IV to ECM proteins has not been examined in any of these studies.

The present investigation reports on the binding of purified DPP IV to ECM proteins, as studied by *in vitro*-binding assays. Among the tested proteins, collagen I and collagen III proved to be the most prominent ligands for DPP IV. By using monoclonal antibodies (mAb) and peptide mapping, it could be shown that the cysteine-rich portion and not the catalytic domain of DPP IV is involved in its interaction with collagen.

## MATERIALS AND METHODS

*Reagents:* Human collagens I-VI and collagen α1(I) and α2(I) chains were isolated and characterized as described [15]. Human fibronectin and mouse laminin were purchased from Boehringer (Mannheim, Germany); Gly-Pro-4-nitroanilide-tosylate and Gly-Pro-4-ß-naphthylamide-HCl from Bachem (Bubendorf, Switzerland); V8-*Staphylococcus aureus*-protease from Boehringer (Mannheim, Germany); peroxidase-conjugated secondary antibodies, Fast Garnet GBC salt and Ponceau S-Red from Sigma (Deisenhofen, Germany); nitrocellulose from Schleicher and Schüll (Dassel, Germany), ProBlott membranes from Applied Biosystems (Foster City, CA), $^{125}I$ (NaI in NaOH) from Amersham-Buchler (Braunschweig, Germany). The monoclonal antibodies (mAb) 13.4 and 25.8 against DPP IV and mAb 33.4 against the $\alpha_1$ subunit of rat $\alpha_1\beta_1$ integrin were described recently [16, 17].

*Membrane preparation and protein solubilization:* Plasma membranes were prepared from rat livers by zonal centrifugation [18]. Integral membrane proteins were solubilized for 1 h at

4°C using 0.5 % (w/v) Triton X-100 in 10 mmol/l Tris/HCl, pH 7.2, 150 mmol/l NaCl, 1 mmol/l phenylmethylsulphonyl fluoride after adjusting the protein concentration to 2 mg/ml.

*Purification of DPP IV:* DPP IV was purified from rat liver plasma membranes by sequential chromatographic steps including chromatography on concanavalin A (Con A)-Sepharose, followed by wheat germ agglutinin (WGA)-Sepharose and glycine-proline (Gly-Pro)-Sepharose. The purity of the preparation was monitored by SDS-PAGE, and enzymatic activity was determined in solution with Gly-Pro-4-nitroanilide-tosylate [19] and on nitrocellulose after protein blotting with Gly-Pro-4-ß-naphthylamide-HCl/Fast Garnet GBC salt [20]. The final chromatographic step on Gly-Pro-Sepharose yielded a nearly pure (>98 %) protein preparation with a specific enzymatic activity of 35 U/mg.

*SDS-PAGE and immunoblotting:* SDS-PAGE and protein transfer to nitrocellulose membranes were performed using a Mini-Protean II system (BioRad, München, Germany). Immunoblotting was performed according to standard protocols; antibodies were detected by chemiluminescence.

*Digestion of DPP IV and sequencing of peptides:* 50 µg of purified DPP IV were digested with 1 µg of V8-protease in SDS-PAGE sample buffer for 24 h at 37°C, subjected to SDS-PAGE, then transferred to ProBlott membranes in 10 mmol/l 3-(cyclohexylamino)-1-propane sulphonic acid, pH 11.0, 10 % (v/v) methanol. Transferred peptides were stained with Ponceau-Red. The bands were cut out, destained with PBS and washed with double distilled water before air drying. Amino acid sequences were determined in an Applied Biosystems sequenator under standard conditions.

*Labeling of DPP IV:* DPP IV was iodinated with $^{125}I$ using chloramine T. Protein-bound radioactivity ($2.5x10^5$ cpm/µg protein) was determined after precipitation in 10 % trichloroacetic acid.

*Binding assays:* Proteins were coated (200 ng/well) on 96-well microtitre plates (Nunc, Wiesbaden, Germany) in 100 µl of 5 mmol/l carbonate buffer, pH 9.5 (for collagens and laminin) or PBS (for fibronectin and DPP IV) for 2 h at 37°C. Remaining free binding sites were blocked with 200 µl/well of 1 mg/ml BSA in PBS 0.05% Tween 20 (v/v) for 30 min at room temperature. Iodinated proteins (1-2 ng, 10000 cpm/well) were mixed with unlabeled protein (5-500 ng/ml; blank with 10 µg/ml) to a final volume of 100 µl/well in 10 mmol/l Tris/HCl, pH 7.4, 50 mmol/l NaCl, and incubated with shaking for 2 h at room temperature. The microtitre plate was washed twice with PBS 0.05% Tween 20 to remove unbound radioactivity. Bound radioactivity was eluted two times with hot (50°C, 200 µl/well) 0.2% SDS (w/v) in 2 mol/l NaOH and counted.

## RESULTS

### *Collagens I and III are major binding partners of DPP IV*

The *in vitro* binding of purified, radiolabeled DPP IV depends markedly on the ECM protein coated on microtitre plates and which were used as stationary phase. The recovery of radioactivity bound to the coated substrates revealed that DPP IV binds significantly to collagens, but not to laminin and fibronectin (fig. 1). Among the different collagens, DPP IV showed greatest binding to collagens I and III, lesser binding to collagens II, IV and V and only weak binding to collagen VI. Similar results were obtained when the binding assay was performed in an inverted way by using iodinated, soluble ECM proteins and DPP IV as a binding substrate coated on microtitre plates (data not shown). Furthermore, when binding of DPP IV was tested with the separated single chains of collagen I, a strong binding of DPP

IV to the α1(I) chain and a significantly lower binding to the α2(I) chain was observed (fig. 1).

*The collagen-binding site of DPP IV is not identically with the catalytic domain*

Two different monoclonal anti DPP IV antibodies were tested to interfere with binding to collagen I. Whereas mAb 13.4 with specificity for the protein part of DPP IV [16] strongly inhibited the binding of DPP IV to collagen I, mAb 25.8 showed only a weak inhibitory effect (fig. 2). This antibody recognizes a carbohydrate moiety of DPP IV. Peptide mapping of immunopurified DPP IV using V8-*Staphylococcus aureus*-protease revealed that mAb 13.4 binds to a 33 kD fragment (fig. 2). Using N-terminal amino acid sequencing, this was identified as peptide $Tyr_{236}$-$Glu_{491}$, which covers the cysteine-rich domain of DPP IV ($Ile_{325}$-$Ser_{553}$) [21] located in the middle of the extracellular domain, but not the active site ($Gly_{629}$-Trp-Ser-Tyr-$Gly_{633}$) at the C-terminus of DPP IV [22].

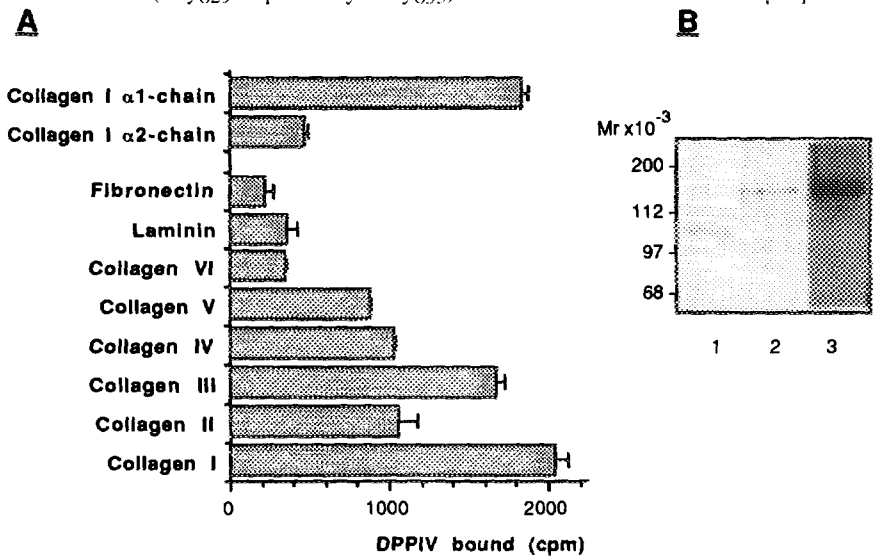


**Fig. 1.** Interaction between DPP IV and different ECM proteins.

(A) Binding of iodinated DPP IV to collagens I-VI, separated collagen I chains, laminin and fibronectin.
Each well was coated with 200 ng of different ECM proteins and incubated with 15000 cpm of iodinated DPP IV and 62.5 ng of unlabeled DPP IV. Each substrate was tested in quadruplet.

(B) Analysis of purified DPP IV by SDS-PAGE.
DPP IV migrates after denaturation (treatment with SDS/boiling prior electrophoresis) as 105 kD band (lane 1). The enzymatically active protein appears as 150 kD band (lane 2; treatment with SDS but without boiling prior electrophoresis) [12, 20, 28], which can be detected with the substrate Gly-Pro-4-ß-naphthylamide-HCl/Fast Garnet GBC salt after transfer to nitrocellulose (lane 3).
1 µg of purified native DPP IV was applied to each lane and stained with Coomassie blue R250 (lane 1,2) or enzymatically (lane 3). The separations were performed on 7.5%T acrylamide gels.

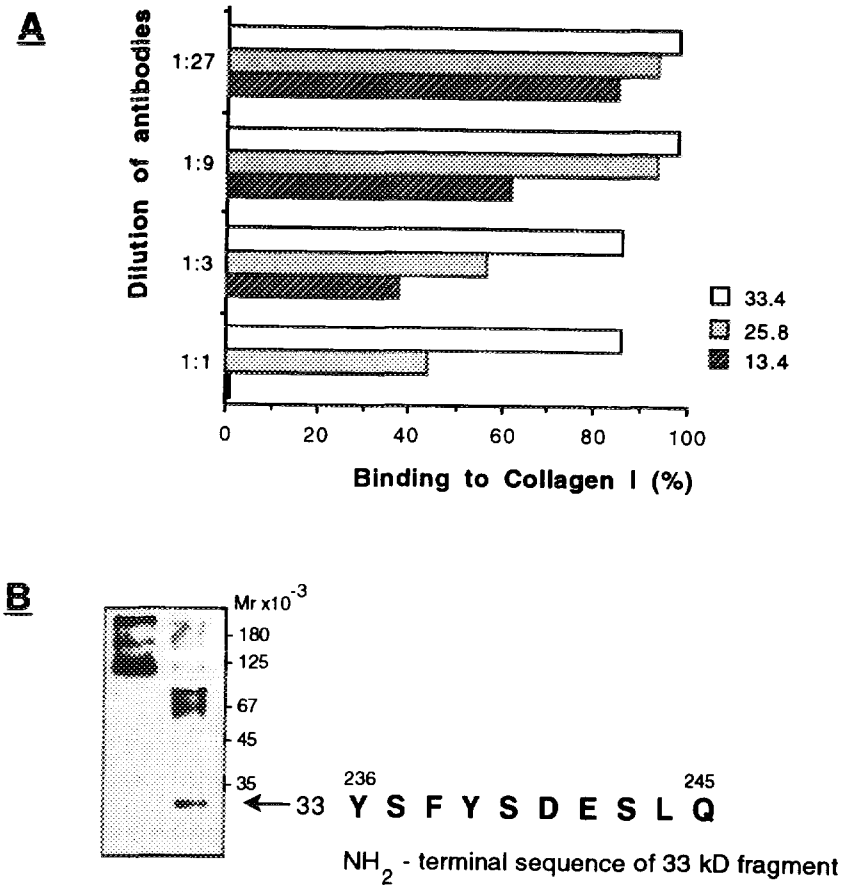


Fig. 2. Characterization of the collagen-binding site of DPP IV.

(A) Influence of different monoclonal antibodies on the binding of DPP IV to collagen I. 60 mU of native DPP IV obtained by solubilization from purified rat liver plasma membranes in 0.5 % Triton X-100 in TBS, 1 mmol/l PMSF were diluted in 500 µl of 10 mmol/l Tris/HCl, pH 7.4, 50 mmol/l NaCl, mixed with 50 µl of different mAbs (50-fold concentrated serum-free culture supernatant) and 200 µl of collagen I-sepharose, prepared as described [10] and incubated with shaking for 1 h at room temperature. Remaining DPP IV activity in the supernatant was measured and expressed as a percentage of the applied enzymatic activity. Antibodies: mAb 13.4 is directed against the protein part of DPP IV; mAb 25.8 against the carbohydrate moiety; mAb 33.4 (directed against the $\alpha_1$ subunit of rat $\alpha_1\beta_1$ integrin [17]) was used as control.
100 %: DPP IV binding to collagen I without antibodies.

(B) Analysis of DPP IV after digestion with V8-*Staphylococcus aureus*-protease. 1 µg of purified DPP IV and 5 µg of protein digest were applied to SDS-PAGE (15 %T acrylamide) and immunostained with mAb 13.4. The 33 kD band of the digest reacting with mAb 13.4 band was then subjected to N-terminal sequencing.

## DISCUSSION

The aim of this study was to obtain information on the molecular basis of the interaction between DPP IV and ECM proteins, its ligand specificity and the ligand recognition site of DPP IV. The examination of different ECM proteins as potential ligands

for DPP IV revealed that collagens are main binding partners of DPP IV whereas the non-collagenous ECM proteins laminin and fibronectin showed only very weak binding. The recently described binding of DPP IV to fibronectin, which was postulated to mediate DPP IV-collagen interaction, could not be confirmed [12]. Collagen IV, a major component of the basal lamina, which is in close contact with the cell membrane was thought to represent the main ligand for DPP IV. Therefore, the preferential binding of DPP IV to the fibrillar collagens I and III and the lower binding to collagen IV was particularly surprising. In general, the interaction between DPP IV and collagens seems to be reinforced by large triplehelical domains present in collagens I to V but not in collagen VI. Structural and/or sequence similarities inside the α1 chains might be the reason for the almost equal binding of DPP IV to collagen I and III. Both chains have a closely related primary and tertiary structure and differ only in some minor posttranslational modifications [23]. Thus, the binding specificity of DPP IV resembles the ligand specificity of the main collagen receptors, $\alpha_1\beta_1$- and $\alpha_2\beta_1$-integrin. They bind strongly to collagens, whereas binding to laminin is much weaker, and fibronectin fails to interact with both $\beta_1$-integrins. [24].

The differences in DPP IV binding to single collagen I chains are probably due to presently unknown structural differences between the α1(I) and α2(I) chains. The total number of amino acid residues of the two chains differs by nearly 100 and there are differences in amino acid composition and in the numbers of lysine/hydroxylysine and histidine cross-linking sites, the latter being absent in the α1(I) chain [23]. Moreover, only the α1(I) chain contains a conformation dependent recognition site for $\alpha_2\beta_1$-integrin, which is absent in the α2(I) chain [25].

The extracellular domain of DPP IV can be divided into three regions with prominent structural features [21]: a glycosylated region, a cysteine-rich region and a C-terminal located region with the active site of the exopeptidase. Since mAb 13.4, which was shown to recognize an epitope localized in the cysteine-rich region of DPP IV, inhibited the binding of DPP IV to collagen, the collagen binding site is obviously not identical with the catalytic region of DPP IV. Sequence comparison of the primary structure of rat, mouse and human DPP IV indirectly support this conclusion. The C-terminal located catalytic domain of all proteins is the most conserved region with 97 and 95 % identity, whereas both the glycosylated and the cysteine-rich region contain several stretches of alternations [21].

Therefore, it is likely that antibodies like mAb 13.4, which react exclusively with the rat protein, recognize epitopes apart from the catalytic site of DPP IV. Other studies give further evidences that the collagen binding feature of DPP IV is independent from its enzymatic activity. Hanski et al. found that the interaction of DPP IV with collagen cannot be abolished with the substrate peptide Gly-Pro-Ala [10], and Bauvois showed that the adhesion of mouse fibroblasts on collagen I can be inhibited with a purified DPP IV preparation [26]. Inhibition of the enzymatic activity of CD 26, the human homologue of DPP IV, did not influence the collagen induced T cell activation via CD 26 [27]. In addition, we observed that the *in vitro* binding of DPP IV to collagen I was not influenced by the peptides Gly-Pro-Ala or Val-Pro-Leu, which inhibit the enzyme activity of DPP IV (data not shown).

In conclusion, DPP IV seems to be a multifunctional molecule in which different molecular domains are involved in different functions. The ability of DPP IV to bind ECM proteins suggests, that DPP IV might be presumably considered as a collagen-binding CAM with participation in the network of cell-matrix adhesion. Further work will be necessary to elucidate the role of the enzymatic activity of DPP IV in cell-matrix adhesion.

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## REFERENCES

[1] Kenny, A.J., Booth, A.G., George, S.G., Ingram, J., Kershaw, D., Wood, E.J. and Young, A.R. (1976) Biochem. J. 155, 169-182.
[2] Mentlein, R., Heymann, E., Scholz, W., Feller, A.C. and Flad, H.D. (1984) Cell. Immunol. 89, 11-19.
[3] Kubota, T., Flentke, G.R., Bachovchin, W.W. and Stollar, B.D. (1992) Clin. Exp. Immunol. 89, 4824-4833.
[4] Munoz, E., Blazquez, M.V., Madueno, J.A., Rubio, G. and Pena, J. (1992) Immunology 77, 43-50.
[5] Elovson, J. (1980) J. Biol. Chem. 255, 5807-5815.
[6] Macnair, R.D.C. and Kenny, A.J. (1979) Biochem. J. 179, 379-395.
[7] Morimoto, C. and Schlossmann, S. (1994) Immunologist 2, 4-7.
[8] Kameoka, J., Tanaka, T., Nojima, Y., Schlossmann, S.F. and Morimoto, C. (1993) Science 261, 466-469.
[9] Dang, N.H., Torimoto, Y., Schlossman, S.F. and Morimoto, C. (1990) J. Exp. Med. 172, 649-652.
[10] Hanski, C., Huhle, T., Gossrau, R. and Reutter, W. (1988) Exp. Cell Res. 178, 64-72.
[11] Thompson, N.L., Hixson, D.C., Callanan, H., Panzica, M., Flanagan, D., Faris, R.A., Hong, W., Hartel-Schenk, S. and Doyl, D. (1991) Biochem. J. 273, 497-502.

[12] Piazza, G.A., Callanan, H.M., Mowery, J. and Hixson, D.C. (1989) Biochem. J. 262, 327-334.
[13] Hartel, S., Gossrau, R., Hanski, C. and Reutter, W. (1988) Histochemistry 89, 151-161.
[14] Gossrau, R. (1985) Histochem. J. 17, 737-771.
[15] Schuppan, D., Rühlmann, T. and Hahn, E.G. (1985) Anal. Biochem. 149, 238-247.
[16] Hartel-Schenk, S., Loch, N., Zimmermann, M. and Reutter, W. (1991) Eur. J. Biochem. 196, 349-355.
[17] Löster, K., Voigt, S., Heidrich, C., Hofmann, W. and Reutter, W. (1994) Exp. Cell Res. 212, 155-160.
[18] Pfleger, R.C., Anderson, N.G. and Snyder, F. (1968) Biochemistry 7, 2826-2833.
[19] Kreisel, W., Heussner, R., Volk, B., Büchsel, R., Reutter, W. and Gerok, W. (1982) FEBS Lett. 147, 85-88.
[20] Walborg, E.F., Tsuchida, S., Weeden, D.S., Thomas, M.W., Barrick, A., McEntire, K.D., Allison, J.P. and Hixson, D.C. (1985) Exp. Cell Res. 158, 509-518.
[21] Marguet, D., Bernardt, A.-M., Vivier, I., Darmoul, D., Naquet, P. and Pierres, M. (1992) J. Biol. Chem. 267, 2200-2208.
[22] Ogata, S., Misumi, Y., Takami, N., Oda, K. and Ikehara, Y. (1992) Biochemistry 31, 2582-2587.
[23] Ayad, S., Boot-Handford, R.P., Humphries, M.J., Kadler, K.E. and Shuttleworth, C.A. (1994) The Extracellular Matrix. pp 1-29, Academic Press, London.
[24] Kühn, K. and Eble, J. (1994) Trends Cell Biol. 4, 256-261.
[25] Staatz, W.D., Fok, K.M., Zutter, M.M., Adams, S.P., Rodriguez, B.A. and Santoro, S.A. (1991) J. Biol. Chem. 266, 7363-7367.
[26] Bauvois, B. (1988) Biochem. J. 252, 723-731.
[27] Hegen, M., Mittrücker, H.-W., Hug, R., Demuth, H.-U., Neubert, K., Barth, A. and Fleischer, B. (1993) Immunobiology 189, 483-493.
[28] De Meester, I., Vanhoof, G., Hendriks, D., Demuth, H.-U., Yaron, A. and Scharpe, S. (1992) Clin. Chim. Acta 210, 23-34.