FEBS Letters 428 (1998) 152–156 FEBS 20280

Rat dipeptidyl peptidase IV (DPP IV) exhibits endopeptidase activity with specificity for denatured fibrillar collagens

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Received 30 March 1998; revised version received 20 April 1998

Abstract Dipeptidyl peptidase IV (DPP IV, CD 26) is an integral membrane serine protease exhibiting a well characterized exopeptidase activity. The present study shows that DPP IV also possesses a novel gelatinase activity and therefore endopeptidase activity, which was directly demonstrated by gelatin zymography. Protease inhibitor profile analysis showed that the endo- and exopeptidase activities of DPP IV share a common active site. Substrate specificity was detected for denatured collagen types I, II, III and V suggesting that DPP IV might contribute to collagen trimming and metabolism. On the basis of these data we propose that DPP IV and the recently sequenced gelatinolytic seprase (FAPα) represent a new subfamily of gelatinolytic integral membrane serine proteases.

Key words: Dipeptidyl peptidase IV; Endopeptidase; Collagen degradation; Gelatin zymography; Gelatinolytic integral membrane serine protease

1. Introduction

Dipeptidyl peptidase IV (DPP IV, CD 26) is a non-classical serine protease localized as a homodimeric integral glycoprotein at the cell surface of many solid tissues and different subtypes of lymphocytes in mammals [1,2]. The amino acid sequence [3] of the 110 kDa monomer reveals three domains. In addition to a short intracellular N-terminal domain and the transmembrane domain, DPP IV also contains a large extracellular domain consisting of a glycosylation-rich region, a cysteine-rich region and a region including the active site. DPP IV has a well characterized exopeptidase activity, which cleaves N-terminal dipeptides from bioactive peptides in which proline is the penultimate amino acid. Peptides in which alanine or hydroxyproline are the penultimate amino acids are also attacked, but at a significantly lower rate. In addition to its exopeptidase activity, Kenny et al. and Lojda et al. reported that DPP IV also possesses significant endopeptidase activity, and is able to hydrolyze certain synthetic N-terminal blocked peptides at the carboxyl site of proline [4,5]. However, this observed endopeptidase activity could not be confirmed by other groups [6] and is possibly due to contamination with copurified prolyl endopeptidase [7].

The physiological significance of DPP IV in vertebrates is not yet clear, although numerous possible substrates of DPP IV have been proposed, including substance P, β-casomorphine, kentsin, somatoliberin and the fibrin α-chain [8]. Ever since the discovery of DPP IV by Hopsu-Havu and Glenner [9], workers have suggested a role of DPP IV in collagen metabolism, because the numerous Gly-Pro sequences in collagen represent potential cleavage sites for a proline-specific hydrolase like DPP IV [10–17]. This hypothesis is supported by the following studies. First, it has been demonstrated by immunohistochemistry, catalytic histochemistry and affinity chromatography that DPP IV binds to collagen [13]. Second, several groups have produced evidence for the involvement of DPP IV in cell-matrix interaction [12,13,15,18–21]. In particular, Hanski et al. showed that the DPP IV substrate tripeptide Gly-Pro-Ala interferes in vitro with the initial spreading of rat hepatocytes on both a native collagen matrix and a denatured collagen-fibronectin matrix [12,13]. Third, DPP IV-negative Fischer rats were found to excrete significantly elevated quantities of proline- and hydroxyproline-containing peptides in their urine compared with control rats, indicating impaired collagen metabolism in these DPP IV-negative rats [14,22]. Fourth, amino acid sequence analysis of seprase (FAPa), an integral membrane serine protease with significant gelatinolytic activity, revealed remarkable structural similarities to DPP IV and 68% amino acid sequence identity in the carboxyl terminus containing the catalytic region [23]. Finally, in a recent study we observed a release of collagen from collagen-coated cover slips by liposomes containing DPP IV [17]. In summary, all these studies suggest that DPP IV may cleave collagen. However, no direct evidence to support this hypothesis has hitherto been obtained.

In the present study, we systematically investigated the ability of DPP IV to cleave collagen. We established a gelatin zymography assay which unequivocally demonstrates a gelatinase and therefore endopeptidase activity of immunopurified DPP IV. In addition, we performed soluble proteolytic assays to characterize the active site and the substrate specificity of DPP IV endopeptidase activity.

2. Materials and methods

2.1. Purification of DPP IV

DPP IV was purified from a 1% (w/v) Triton X-100 detergent extract of a crude membrane fraction derived from rat renal cortex. The isolation was performed by affinity chromatography on concanavalin A (ConA)-Sepharose [24], followed by immunoaffinity chromatography on the monospecific mAb 13.4 [25]. Elution was performed with 0.25 M α-methyl-mannopyranoside in TBS, pH 7.8 and 50 mM diethylamine (pH gradient 10.5 to 11.5), respectively. This purification

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Abbreviations: DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; DPP IV, dipeptidyl peptidase IV; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; FAPα, fibroblast activation protein α; kDa, kilodalton; mAb, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride; M_r, relative molecular mass; NEM, *N*-ethylmaleimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris buffered saline; TPCK, tosyl-phenylalanine chloromethyl ketone

PII S0014-5793(98)00515-8

procedure yielded a pure protein preparation with a specific enzymatic activity of 45 U/mg and an enrichment factor of 160. The mAb 13.4 against DPP IV has been characterized previously [26].

2.2. Analysis of the immunopurified DPP IV preparation

Determination of DPP IV exopeptidase activity: The DPP IV exopeptidase activity was measured with the substrate Gly-Pro-4-nitroanilide-tosylate (Bachem, Heidelberg, Germany) as described before [27]. DPP IV exopeptidase overlay assay: DPP IV samples were subjected to SDS-PAGE, then transferred to nitrocellulose and incubated with Gly-Pro-2-naphthylamide-HCl/Fast Garnet GBC salt mixture (Bachem) as described by Walborg et al. [19]. Immunoblotting: SDS-PAGE and protein transfer to nitrocellulose membranes were performed according to standard protocols using a Mini-Protean II system (BioRad, München, Germany). The blots were developed with mAb 13.4 against DPP IV.

2.3. Gelatin zymography

Gelatin zymography for the detection of gelatinase activity of DPP IV was originally described by Heussen and Dowdle [28] and modified by Chen et al. [29]. In our experiments, 7.5% SDS-polyacrylamide gels were prepared containing copolymerized heat-denatured collagen type I (SIGMA, Deisenhofen, Germany, C 8897) at a final concentration of 0.5 mg/ml. The subsequent electrophoresis of purified DPP IV samples mixed with reducing SDS-PAGE sample buffer was performed at 4°C and 100 V to preserve the native conformation of DPP IV. The gels were washed with 2.5% Triton X-100 (v/v) twice for 15 min each to remove SDS, which reversibly inhibits DPP IV activity. They were then rinsed briefly with TBS, pH 7.4, incubated in TBS, 2.5 mM EDTA at 37°C for 8 h and stained with Coomassie Blue. Gelatinase activity was revealed by negative staining of transparent bands. M_r markers appeared as darker bands in the gelatin gels.

2.4. Soluble proteolytic assay

Twenty μg acid-soluble, thermally denatured collagen were incubated with 8 μg active DPP IV at 37°C in sterile TBS, 2.5 mM EDTA, pH 7.4 for different periods of time. The proteolytic reaction was stopped by boiling with reducing SDS-PAGE sample buffer. Aliquots of the mixtures were separated by SDS-PAGE and stained with Coomassie Blue. In gels, collagen degradation was manifested as a decrease in the intensity of the collagen bands. The α_1-chain bands were densitometrically quantified using an AGFA ARCUS II scanner and IP Lab Gel H software. The detection limit was about 100 ng. The amount of collagen detected at the beginning of the reaction was defined as 100%. Each value was calculated by the average of three experiments. The coefficient of variation (*cv*) was between 5% and 10% for all reported values.

For protease inhibitor profile analysis of DPP IV endopeptidase activity, specific inhibitors were added to the soluble proteolytic assay before incubation for 80 h. The concentrations used are listed in Table 1. Pepstatin, diprotin A and diprotin B were purchased from Bachem, EDTA and SDS from Roth, Karlsruhe, Germany. Iodoacetamide, leupeptin, NEM, 1,10-phenanthroline, DFP, PMSF, aprotinin and DTT were obtained from SIGMA. Stock solutions of TPCK, PMSF and phenanthroline were prepared in DMSO. At the concentrations used, DMSO has no effect on the endo- or exopeptidase activity of DPP IV.

For investigation of DPP IV endopeptidase substrate specificity, denatured collagen of different types (SIGMA, C 8897, C 1188, C 4407, C 7521, C 3657) was used for the soluble proteolytic assay. The incubation times were 5, 10, 20, 40, 60 and 80 h, respectively. The reaction mixtures were compared with respective controls without DPP IV incubated for the same period of time.

3. Results and discussion

In this paper we describe an endopeptidase activity of immunopurified DPP IV as demonstrated by the digestion of heat-denatured fibrillar collagens.

3.1. Isolation of DPP IV from rat kidney

To ensure homogeneity of our DPP IV preparation, we purified the enzyme in two chromatographic steps. A solubilized crude membrane fraction of rat renal cortex was submitted to preparative ConA-Sepharose chromatography, followed by immunoaffinity chromatography on the DPP IV-specific immobilized mAb 13.4. The immunopurified DPP IV was analyzed by SDS-PAGE under non-denaturing reducing conditions, followed by silver stain, immunoblotting and DPP IV exopeptidase overlay assay (Fig. 1). The 140 kDa and 110 kDa band represent the active and inactive form of the DPP IV monomer, respectively, as demonstrated by immunoblot analysis with mAb 13.4 and by exopeptidase overlay assay using the DPP IV-specific substrate, Gly-Pro-2-naphthylamide. Additionally, sometimes traces of the 290 kDa active dimeric form of DPP IV as well as a 60 kDa protein appeared in the immunopurified DPP IV preparation. N-terminal amino acid sequence analysis revealed that the 60 kDa protein represents a fragment of DPP IV starting at amino acid 281 (Bermpohl and Weise, unpublished results), confirming earlier observations [30].

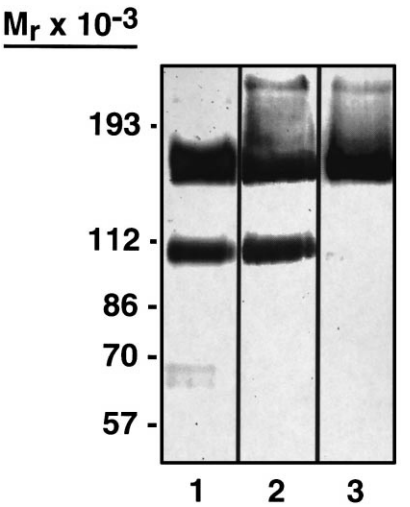

Fig. 1. Immunopurification of DPP IV monitored by SDS-PAGE. Five μg of the immunoaffinity chromatography eluate were separated under non-denaturing reducing conditions, followed by silver staining (lane 1), immunoblotting with mAb 13.4 (lane 2), or the DPP IV exopeptidase overlay assay using Gly-Pro-2-naphthylamide/Fast Garnet (lane 3).

3.2. Gelatin zymography

To directly demonstrate the endopeptidase activity of immunopurified DPP IV, a gelatin zymography assay was established. DPP IV was separated under mild conditions in SDS-polyacrylamide gels containing copolymerized heat-denatured collagen. After electrophoresis gels were incubated at 37°C for 8 h and stained with Coomassie Blue. To suppress any gelatinase activity of other proteases, 2.5 mM EDTA was added to the incubation buffer. Gelatinase activity of separated proteins should lead to a disintegration of the corresponding gelatin matrix as revealed by a colorless, non-stained area in the gel. As shown in Fig. 2A, the immunopurified DPP IV fraction generated a clear 140 kDa area which corresponds to the M_r of native DPP IV. The zone of proteolytic activity became greater with increasing concentrations of DPP IV, suggesting a specific DPP IV-dependent proteolytic activity. No further gelatin degrading activity became visible, which argues for the homogeneity of our DPP IV preparation. The

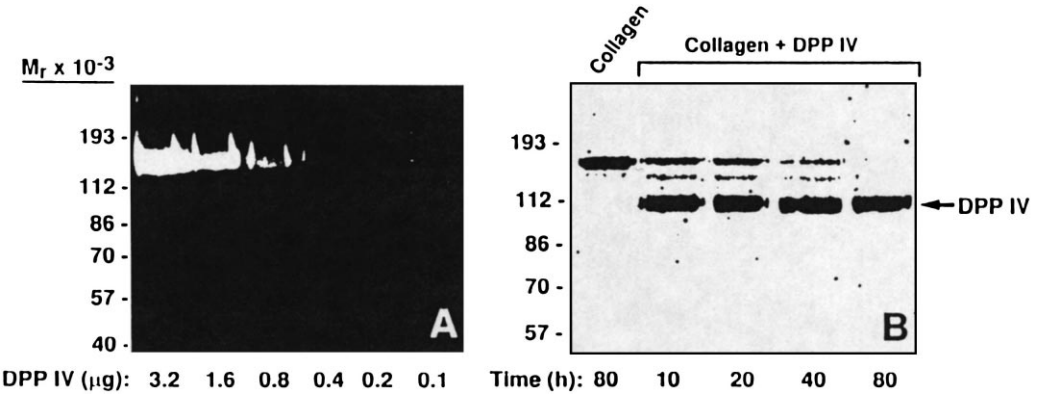

Fig. 2. Endopeptidase activity of DPP IV. A: Gelatin zymography performed with different concentrations of immunopurified DPP IV. B: Soluble proteolytic assay showing degradation of denatured collagen type II by DPP IV as a function of time.

digestion of gelatin by DPP IV provides evidence for intramolecular cleavage sites and, thus, for the endopeptidase activity of DPP IV. The presence of the Gly-X-Y motif in fibrillar collagens means that removal of the first N-terminal dipeptide would produce a peptide in which glycine is the penultimate residue. Therefore the observed gelatin degradation could not have been the result of stepwise exopeptidase digestion of the collagen chains by DPP IV. In addition, a tripeptidase activity of DPP IV has been excluded [6].

3.3. Soluble proteolytic assay

To further characterize DPP IV endopeptidase activity a soluble proteolytic assay was established, which allowed quantification of gelatin degradation by densitometric scanning of gels for the disappearance of α_1-chain bands. For this purpose, thermally denatured collagen was incubated with DPP IV in the presence of 2.5 mM EDTA at 37°C for different periods of time, then separated by SDS-PAGE and stained with Coomassie Blue. As shown in Fig. 2B, the amount of detectable collagen gradually decreased during the period of the investigation. We did not detect cleavage products in this assay, but using anti-collagen antibodies we detected fragments in immunoblots (data not shown). The detection of only small amounts of cleavage products may be due to the spreading of fragments over the gel.

3.3.1. Influence of inhibitors on DPP IV endo- and exopeptidase activity. Using the soluble proteolytic assay, various substances were investigated for their inhibitory influence on DPP IV endopeptidase activity (Table 1). The results were compared with the effects of the same modulators on DPP IV exopeptidase activity, as determined by the standard Gly-Pro-*p*-nitroanilide assay. In summary, both the endo- and the exopeptidase activities of DPP IV were inhibited by the serine protease inhibitors DFP (0.6 mM) and PMSF (9 mM) as well as by diprotin A (13 mM) and diprotin B (13 mM), which are well characterized competitive inhibitors of DPP IV exopeptidase activity. However, neither the endo- nor the exopeptidase activity of DPP IV was significantly influenced by aspartate-,

Table 1
Effects of different types of protease inhibitors on DPP IV endo- and exopeptidase activity

Inhibitor (concentration)	Endopeptidase activity	Exopeptidase activity
Control	100.0%	100.0%
Aspartate-		
Pepstatin (10 mg/ml)	95.8%	91.0%
Cysteine-		
Iodoacetamide (10 mM)	101.9%	94.0%
Leupeptin (10 mg/ml)	88.8%	89.6%
N-ethylmaleimide (1 mM)	103.1%	87.4%
Metallo-		
1,10-phenanthroline (6 mM)	70.7%	71.9%
Serine-		
Diisopropyl fluorophosphate (0.6 mM)	10.8%	1.1%
PMSF (1 mM)	85.8%	84.4%
PMSF (9 mM)	14.2%	12.2%
DPP IV-specific		
Diprotin A (13 mM)	10.5%	14.3%
Diprotin B (13 mM)	38.6%	51.2%
Others		
1% SDS (v/v)	0.1%	1.2%
TPCK (10 mM)	90.8%	102.9%
Aprotinin (10 mg/ml)	88.8%	88.5%
Dithiothreitol (3 mM)	51.6%	84.0%

Values of DPP IV endopeptidase activity were determined by the soluble proteolytic assay followed by densitometric gel scanning. To measure DPP IV exopeptidase activity aliquots of the respective soluble proteolytic assays were applied to the Gly-Pro-2-nitroanilide assay after 1 h and 80 h of incubation. The values of DPP IV exopeptidase activity listed in the table represent the mean of both measurements. The activity obtained from DPP IV without protease inhibitor was defined as 100%. The results shown are from three separate experiments.

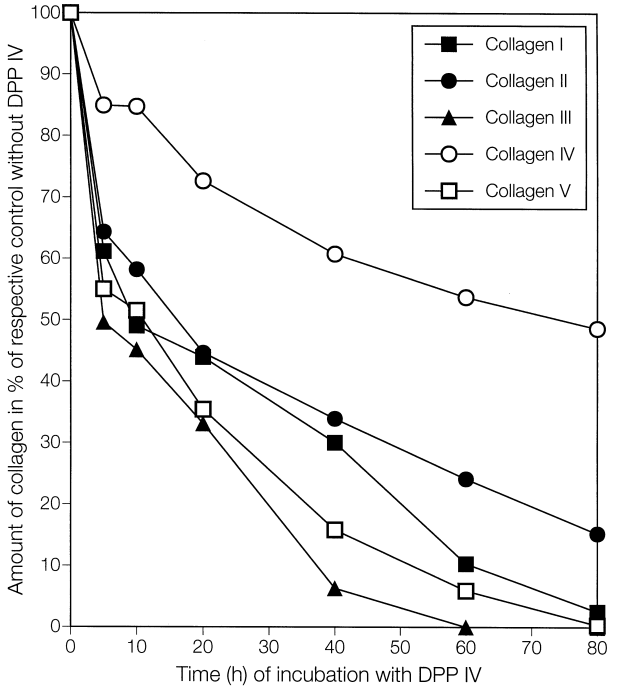

Fig. 3. Substrate specificity of DPP IV endopeptidase activity. Different types of denatured collagen were submitted to the soluble proteolytic assay with DPP IV for the periods of time indicated. Degradation was quantified by densitometric scanning of the α_1-bands. The amount of collagen detected at the beginning of the reaction was defined as 100%. Values shown represent the means of three separate experiments.

cysteine- and metalloprotease inhibitors. The only difference in response to an inhibitor was observed with 3 mM DTT, which inhibited DPP IV endopeptidase activity but not the exopeptidase activity. This can be explained by the reducing properties of high concentrations of DTT, which might cleave disulfide bridges in the cysteine-rich region of DPP IV leading to a conformational change of the enzyme, which presumably affects the collagen-binding property of DPP IV but not the active site of its exopeptidase activity [17]. This conclusion is in accordance with a study of Löster et al., who identified the cysteine-rich region of DPP IV as the collagen-binding site, which does not contain the active site of DPP IV exopeptidase activity itself [15]. Thus, apart from the response to DTT, the endo- and exopeptidase activities of DPP IV showed the same protease inhibitor profile, including similar inhibition by DFP and by diprotin A and B, which are specific competitive inhibitors of DPP IV exopeptidase activity. We therefore suggest that both the endo- and the exopeptidase activity of DPP IV reside in a common active site. This conclusion is supported by the observation that the active site of the enzyme is only labelled on ser_{624} by [^{32}P]Dip-F [31].

3.3.2. Determination of substrate specificity. To determine the substrate specificity of DPP IV endopeptidase activity, the soluble proteolytic assay was performed with different potential substrates. Albumin and fibronectin were not digested by DPP IV (data not shown), which, in view of the low incidence of proline residues in these proteins, suggests that DPP IV endopeptidase activity depends on the presence of a proline-specific cleavage site. This conclusion is supported by the inhibitory effect of the proline-containing tripeptides, diprotin A and B, on DPP IV endopeptidase activity. DPP IV also failed to degrade native collagens (data not shown), although it attacked denatured collagens, indicating the incapacity of DPP IV to cleave triple-helical structures. Although all the investigated types of denatured collagens were attacked by the enzyme, they were not all cleaved at the same rate; α_1-chains of the fibrillar collagen types I, II, III and V were cleaved more rapidly than the α_1-chains of basement membrane collagen type IV (Fig. 3). These differences in cleavage specificity are probably due to different structural constraints on the DPP IV molecule in its interaction with fibrillar collagen and basement-type collagen.

3.4. Physiological relevance of DPP IV endopeptidase activity

Compared with other gelatinases and collagenases and with DPP IV exopeptidase activity, DPP IV exhibits weak endopeptidase activity. Only 20 μg of denatured fibrillar collagens were digested by 8 μg active DPP IV within 80 h, which represents a very slow cleavage rate, even allowing that each collagen molecule was cleaved at many sites in the chain. Contamination with a matrix metalloprotease or a bacterial collagenase should be excluded, since both are inhibited by EDTA and by 1,10-phenanthroline [32,33] which did not influence the endopeptidase activity of DPP IV. Moreover, the largest member of the matrix metalloprotease family is the 92 kDa type IV collagenase (gelatinase B) which was not detected in the zymography [34].

This slow cleavage rate, together with the inability to cleave native collagens, suggests that the biological relevance of DPP IV endopeptidase activity should be seen in context with other collagenases and gelatinases, as well as with DPP IV exopeptidase activity. Therefore DPP IV might participate in final collagen degradation, which is generally initiated by interstitial collagenases. Alternatively, DPP IV endopeptidase activity might have auxiliary functions in cell-matrix adhesion processes by unmasking cryptic binding sites of collagen-like RGD and DGEA sequences, thus making them accessible for the integrin receptors α_2/β_1, α_3/β_1 and α_5/β_1 [35].

Recent analysis of the amino acid sequence of seprase (FAPα), a gelatinolytic endopeptidase, which is localized in melanoma, carcinoma and sarcoma cells revealed 68% amino acid sequence identity to DPP IV in their catalytic regions and a remarkably similar structural organization of both enzymes [23]. Since a common gelatinolytic activity has now been detected, we agree with Goldstein et al. in proposing that DPP IV and seprase represent a new subfamily of gelatinolytic integral membrane serine proteases [23].

Acknowledgements: We would like to thank Werner Hofmann for skilful technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 366), the Sonnenfeld-Stiftung and the Fonds der Deutschen Chemie Industrie, Frankfurt am Main.

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