Dipeptidyl peptidases 8 and 9: specificity and molecular characterization compared with dipeptidyl peptidase IV

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Dipeptidyl peptidases 8 and 9 have been identified as gene members of the S9b family of dipeptidyl peptidases. In the present paper, we report the characterization of recombinant dipeptidyl peptidases 8 and 9 using the baculovirus expression system. We have found that only the full-length variants of the two proteins can be expressed as active peptidases, which are 882 and 892 amino acids in length for dipeptidyl peptidase 8 and 9 respectively. We show further that the purified proteins are active dimers and that they show similar Michaelis–Menten kinetics and substrate specificity. Both cleave the peptide hormones glucagon-like pep-

tide-1, glucagon-like peptide-2, neuropeptide Y and peptide YY with marked kinetic differences compared with dipeptidyl peptidase IV. Inhibition of dipeptidyl peptidases IV, 8 and 9 using the well-known dipeptidyl peptidase IV inhibitor valine pyrrolidide resulted in similar K_i values, indicating that this inhibitor is non-selective for any of the three dipeptidyl peptidases.

Key words: dipeptidyl peptidase, glucagon-like peptide, neuropeptide Y, peptide YY, S9b family, valine pyrrolidide.

INTRODUCTION

The heterogenous S9b family of DPPs (dipeptidyl peptidases) is a growing family of serine peptidases, which is characterized by the first identified member DPP-IV (also called CD26; EC 3.4.14.5) [1]. DPP-IV was first identified by Hopsu-Havu and Glenner [2] as an enzyme that possesses a glycylproline β -naphthylamidase activity, and it was later shown that DPP-IV has a general ability to cleave prolyl and alanyl peptide bonds at the penultimate position from the N-terminus [3–6]. Initially, the observed specificity of DPP-IV was assumed to be unique, but other DPPs and related peptidases with similar specificity have since been identified [7,8].

More recently, two members of the S9b family DPP-8 and DPP-9 have been discovered and pinpointed *in silico* to the two human chromosome loci 15q22 and 19p13.3 respectively [9,10]. The ORF (open reading frame) of DPP-8 codes for 882 amino acids and has been reported to be a monomeric protein of approx. 100 kDa. Based on similarities with the tissue expression distribution of DPP-IV, hypothetical functions of DPP-8 in relation to T-cell activation and other immune functions have been suggested [9], but still no clear physiological roles have been reported. At least one DPP-8 splice variant of possible functional significance has been identified in the EST (expressed sequence tag) database (hEST identifier 8047491), comprising a sequence which, from alignments with DPP-IV, lacks part of the α/β hydrolase domain including the catalytic active-site residue Ser⁷³⁹ (Figure 1).

DPP-9 was identified *in silico* by Abbott et al. [9] and was first cloned and expressed recombinantly by Olsen and Wagtmann [10]. An 863-amino-acid cDNA variant was expressed, giving an

enzymatically inactive protein, which migrated with a molecular mass of approx. 98 kDa as determined from SDS/PAGE analysis [10]. No *in vivo* functions have been described for DPP-9, and an enzymatically active variant of a recombinantly expressed variant of DPP-9 has been reported in two studies with an apparent ORF of 863 amino acids [11,12]. Analysis of the upstream cDNA sequence of DPP-9 has revealed another ATG translation start site to the one reported by any of the previous studies (see Figure 1), thus prolonging the ORF to a total of 892 amino acids (GenBank[®] accession number NM_139159).

A counterscreen strategy designed for development of specific DPP-IV inhibitors, involving cloning, expression and characterization of the specificity profiles of DPP-8, DPP-9 and DPP-IV, was undertaken. We used the baculovirus expression system for production of His₆-tagged constructs of both the splice-variant $\Delta 657$ –757 and full-length 882-amino-acid variants of DPP-8 (DPP-8 $_{\Delta 657-757aa}$ and DPP-8 $_{882aa}$ respectively), and the 863- and 892-amino-acid variants of DPP-9 (DPP-9 $_{863aa}$ and DPP-9 $_{892aa}$ respectively) (see Figure 1). In contrast with previous reports [11,12], we found that only the full-length variants of both DPP-8 and DPP-9 were enzymatically active, and both were able to cleave the naturally occurring hormones GLP-1 (glucagon-like peptide 1), GLP-2, NPY (neuropeptide Y) and PYY (peptide YY). Although the truncated form of DPP-9 could be expressed, no enzymatic activity could be detected using different putative substrates. Thus these results indicate that only full-length expression of the paralogous genes of DPP-8 and DPP-9 produce enzymatically active proteins and that the mature proteins are able to cleave naturally occurring peptides of the incretin and pancreatic hormone families.

Abbreviations used: DLS, dynamic light scattering; DPP, dipeptidyl peptidase; EST, expressed sequence tag; GLP, glucagon-like peptide; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; NPY, neuropeptide Y; ORF, open reading frame; pNA, *p*-nitroanilide; PYY, peptide YY; ValPyr, valine pyrrolidide.

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The nucleotide sequence data reported for the full-length 892-amino-acid variant of dipeptidyl peptidase 9 have been deposited in the DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession number DQ417928.

Figure 1 Protein sequence alignment of human DPP-IV, DPP-8 and DPP-9

Bold residues indicate 100% identity of at least two residues. Boxes indicate 100% identity throughout the residue. Important active-site residues (see [15] and [17] for further details of the active site of DPP-IV) are indicated accordingly: ♦, catalytic residues Tyr³⁴⁷, Ser⁵³⁰, Asp⁷⁰⁸ and H⁴⁰; ▼, N-terminal co-ordinating residues Glu²⁰⁵ and Glu²⁰⁶; ●, residues of the S1 pocket Tyr⁶³¹, Val⁶⁵⁶, Trp⁶⁵⁹, Tyr⁶⁶², Tyr⁶⁶⁶ and Val⁷¹¹. The prolonged ORF of DPP-9-892aa is indicated by an underline. DPP-8-Δ657-757aa is indicated by a broken underline.

EXPERIMENTAL

Bioinformatics

Searches of EST cDNA clones were performed using the NCBI EST database (GenBank[®] accession numbers for DPP-8 and DPP-9 are AF221634 and NM.139159 respectively). The Vector NTI Suite 6.0 (InforMax) was used for sequence analysis, gene alignments and primer design. Analyses of the X-ray structure of recombinant human DPP-IV (PDB code 1N1M) were performed using Quanta and WebLab ViewerPro (Accelrys).

Cloning

DNA sequences of DPP-8_{882aa} and DPP-9_{892aa} were obtained using specific primers (obtained from TAG Copenhagen; Table 1) for PCR from purified HeLa cDNA as described previously [13] and the EST clone 8047491 (obtained from I.M.A.G.E. Consortium) containing the splice variant sequence of DPP-8. Primers were designed from DPP-8 and DPP-9 cDNA sequences with flanking restriction sites for directional cloning. Obtained PCR products were cloned in a pCR2.1 vector (Invitrogen) and verified by full-length DNA sequencing. Correct clones were subcloned to pBacPAK-His3 transfer vector (Clontech) for recombinant virus production. In this way, pBacPAK-His3 constructs of DPP-8_{882aa}, DPP-9_{892aa} and DPP-8_{Δ657-757aa} were con-

Table 1 Primers used for cloning

Italic residues indicate engineered restriction sites. Underlined residues indicate start methionine-encoding sites.

Primer name	Sequence
DPP-8.forward.Sall	5'-GTCCGACCATGGCAGCAGCAATGGAACAGAA-3'
DPP-8.revers.XbaI	5'-TCTAGATTATTATATCACTTTTAGAGCAGCAATA-3'
DPP-9.forward.1'ATG.Sall	5'-GTCCGACCATGGCCACCACCGGACCCCAACGG-3'
DPP-9.revers#1.EcoRI	5'-GAATTCCTATTATCAGAGGTATTCCTGTAGAAAGTGC-3'
DPP-9.forward.2'ATG.NdeI/Sall	5'-CATATGGTCGACCATGCGGAAGGTTAAGAACTGCGC-3'
DPP-9.revers#2	5'-CTCAGAGTAGAGGAGGAGTCTCTCTCG-3'

structed using the multilinker site 94 bp from the start of the polyhedrin promoter. In this way, the N-terminal His₆-tag was constructed with the start sequence Met-Gly-His-His-His-His-His-Gly-Ser-Thr-Met (the underlined section signifies the beginning of the recombinant sequence). A C-terminal His₆-tagged construct of the 863-amino-acid variant of DPP-9 (DPP-9_{863aa}) was obtained from a pCDM8-DPP9/His construct (kindly provided by Christina Olsen, Department of Pharmacology, Panum Institute, Copenhagen, Denmark) as a BamHI/EcoRI digest and was subcloned into baculovirus transfer vector pVL1393

in the multilinker site 179 bp from the start of the polyhedrin promoter (Invitrogen).

Recombinant baculovirus production and protein expression

Recombinant baculovirus was produced using derived *Autographa californica* nuclear polyhedrosis virus DNA and the baculovirus transfer vector constructs as described previously [13]. In short, purified transfer vectors with recombinant inserts were mixed with purified virus DNA and were transfected into Sf9 (*Spodoptera frugiperda* 9) cells using Lipofectin™ (Gibco/BRL). Virus was amplified for production of high-titre stocks using Sf9 cells. Intracellular expression of recombinant DPP-8 and DPP-9 was performed in large cell dishes (25 cm × 25 cm; Nunc) using High5 insect cells. Typically, expressions of 10⁹–10¹¹ cells were used per batch. All insect cells were grown in Grace Insect medium supplemented with 0–10% foetal calf serum, yeastolate, 20 mM L-glutamine and 50 µg/ml gentamycin in either tissue culture flasks or glass spinner bottles at 28 °C.

Protein purification

Insect cells were harvested after 48 h of incubation by centrifugation at 400 g for 10 min, washed three times with PBS and resuspended in hypotonic buffer (25 mM Hepes, pH 7.5, 5 mM KCl and 1.5 mM MgCl₂) for 10 min. Cells were lysed with 15–20 strokes in a Dounce homogenizer, incubated for 20 min and adjusted slowly to 0.4 M NaCl. Extracts were cleared by centrifugation at 20 000–30 000 g for 30 min, adjusted to 5 mM imidazole, filtered through a 0.45 µm filter and applied directly to a TALON™ resin (BD Biosciences) column of different sizes depending on cell expression magnitude. Typically, for larger expressions of more than 10¹⁰ cells, approx. 1 ml of resin was used, while for expressions of less than 10⁸ cells, a 0.3–0.5 ml column was prepared. The column was equilibrated with 25 mM Hepes, pH 7.5, 0.4 M NaCl and 5 mM imidazole (designated buffer A). The column was first washed with 10 vol. of buffer A and then with 10 vol. of 50 mM NaCl-adjusted buffer A. Elution was performed using 25 mM Hepes, pH 7.5, 50 mM NaCl and 100 mM imidazole. Subsequent ion-exchange purifications of the eluted fractions were performed using a MonoQ column (Amersham Biosciences) on an Äkta Purifier low-pressure chromatographic apparatus (Amersham Biosciences). A gradient of 0–0.5 M NaCl in a 50 mM Tris/HCl, pH 8, buffer was used. The fractions were concentrated on Centrprep YM-10 and Centricon YM-10 filter devices (Millipore Corporation).

SDS/PAGE and Coomassie Blue analyses were performed using 4–12% gradient NuPAGE® Bis-Tris Gels (Invitrogen) and GelCode Blue Stain Reagent (Pierce).

Protein concentrations were determined spectrophotometrically using UV280/254 nm absorbance and molar absorption coefficients of 186 920, 138 130 and 148 330 M⁻¹ · cm⁻¹ for DPP-IV, DPP-8 and DPP-9 respectively.

Size-exclusion chromatography

Size-exclusion experiments performed using a Superdex 200 10/300 GL column (Amersham Biosciences). A buffer composition of 100 mM NaCl and 50 mM Tris/HCl, pH 8, was used. Flow was 0.25 ml/min throughout.

DLS (dynamic light scattering)

DLS was performed on a DynaPro Dynamic Light Scattering Instrument (Protein Solutions). All data were measured at room

temperature (20 °C), and analyses were performed using the supplied software package Dynamics version 5.

Enzymatic activity assays

Enzymatic activity was determined kinetically at 37 °C in standard buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1% Triton X-100. Different pNA (*p*-nitroanilide) substrates were used, including Ala-Ala-Pro-pNA, succinate-Ala-pNA, Arg-Pro-pNA, Ala-Pro-pNA, Val-Ala-pNA, Gly-Pro-pNA, Gly-Gly-pNA, Ala-Phe-pNA and Ala-Ala-pNA. Substrate (50 µl) and enzyme (50 µl) were used at double concentrations of substrate and enzyme, and released pNA was detected spectrophotometrically at 405 nm every 5 min for a total of 30 min in a kinetic SpectraMax 340 microplate reader (Global Medical Instrumentation). Steady-state Michaelis–Menten kinetics were determined using 2-fold dilutions of the substrate and were fitted directly to the Michaelis–Menten equation, $v = ([S] \times V_{\max}) / ([S] + K_m)$, where [S] is substrate concentration. Protein concentrations were optimized to give the best absorbance readout during the 30 min incubation and were 0.65, 13.5 and 15.5 nM for DPP-IV, DPP-8_{882aa} and DPP-9_{892aa} respectively. A molar absorption coefficient of 8.8 mM⁻¹ · cm⁻¹ at 410 nm was used for the determination of pNA concentration. The amount of liberated pNA product during incubations was determined using a standard curve obtained at 410 nm for various concentrations of a pNA stock solution in the SpectraMax 340 microplate reader. Detection limits were between approx. 1 nM and 1.5 µM.

Inhibition kinetics were performed for all purified enzymes using the well-known competitive DPP-IV inhibitor ValPyr (valine pyrrolidide) and the substrate Gly-Pro-pNA. IC₅₀ determinations were performed from measured enzyme velocities at varying concentrations of inhibitor of 30.0, 10.0, 3.33, 1.11, 0.37 and 0.12 µM and a constant substrate concentration equal to the K_m value for each of the enzymes, i.e. 0.2 mM for DPP-IV, 0.3 mM for DPP-8_{882aa} and 0.4 mM for DPP-9_{892aa}. Using the relationship $IC_{50}/K_i = 1 + [S]/K_m$, K_i values were derived from the IC₅₀ determinations. At least three measurements were performed for all kinetic determinations.

MALDI-TOF (matrix-assisted laser-desorption/ionization-time-of-flight) MS analysis

MALDI-TOF MS was performed on a Voyager RP MALDI-TOF instrument (PerSeptive Biosystems) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25 kV. Sample preparation was carried out as follows: 1 µl of sample solution (0.5–1.0 mg/ml) was mixed with 10 µl of matrix solution (α -cyanocinnamic acid dissolved in a mixture of acetonitrile/water/3% trifluoroacetic acid, 5:4:1, by vol.) and 1 µl was deposited on the sample plate (standard stainless steel) and was allowed to dry. Calibration was performed using external peptide standards resulting in a mass accuracy of 0.1% in the relevant mass area.

MALDI-TOF MS was used for analysis of enzymatic digestion of proteins by trypsin and semi-quantitative determination of cleavage rates by DPP-8_{882aa} and DPP-9_{892aa} of the peptides GLP-1, GLP-2, NPY and PYY (all from Peninsula Laboratories Europe). For determination of peptide cleavage rates, 1.0 µM peptide was incubated with 0.1 µM purified protein (i.e. 10× molar excess) final concentration in 50 µl at room temperature and in standard buffer (see the Enzymatic activity assays subsection). Samples were withdrawn at 0 min, 20 min, 4 h and 24 h without adding any stopping additive. Relative ratios and *in vitro* half-lives (*t*_{1/2})

Table 2 TALON™ purification

	DPP-8 _{882aa}	DPP-9 _{892aa}
Cell lysate		
Protein amount (mg)	72	10
Total activity (μ mol/min)	358	524
Specific activity (μ mol/min per mg)	5	52
Enrichment factor	1	1
Yield (%)	100	100
TALON™ pool		
Protein amount (mg)	0.47	0.49
Total activity (μ mol/min)	69	184
Specific activity (μ mol/min per mg)	148	376
Enrichment factor	30	7
Yield (%)	19	35

were estimated from ratios between the MS intensities of intact and cleaved peptides. Peptide sequences and molecular masses are as follows: GLP-1 (3298 Da), HAEGTFTSDVSSYLEGQAAKE-
FIWLKGR; GLP-2 (3766 Da), HADGSFSDENMTILDN-
LAARDFINWLIQTKITD; NPY (4272 Da), YPSKPDNPG-
EDAPAEADLARYYSALRHYINLITRQRY; and PYY (4310 Da),
YPIKPEAPGEDASPEELNRYASLRHYLNLVTRQRY.

RESULTS

Expression and purification of recombinant variants of DPP-8 and DPP-9

DPP-8_{882aa}, DPP-8_{Δ657–757aa}, DPP-9_{863aa} and DPP-9_{892aa} were expressed as either N- or C-terminal His₆-tagged constructs [theoretical molecular masses of His₆-tagged constructs were: DPP-8_{882aa}, 102 kDa (N-terminal His₆-tag); DPP-9_{863aa}, 99 kDa (C-terminal His₆-tag); and DPP-9_{892aa}, 103 kDa (N-terminal His₆-tag)] using the baculovirus expression system and chromatographically purified using TALON™ resin as outlined in the Experimental section. Only expression and purification of the DPP-8_{882aa} and DPP-9_{892aa} N-terminal His₆-tagged constructs produced enzymatically active proteins. From the TALON™ purification, expressions of DPP-8_{882aa} and DPP-9_{892aa} resulted in an increase in specific activities of 30- and 7-fold compared with cell lysates and yields of 19 % and 35 % respectively. Table 2 shows the purification scheme of DPP-8_{882aa} and DPP-9_{892aa}.

SDS/PAGE and Coomassie Blue analyses of the DPP-8_{882aa} purification revealed a protein band of approx. 111 kDa as compared with molecular-mass standards, and accounted for more than 90 % of the total fractionated protein (see Supplemental Figure 1 at <http://www.BiochemJ.org/bj/396/bj3960391add.htm>). DPP activity could be measured in the TALON™ fraction, but further ion-exchange purification was necessary for demonstration of co-migration between intensity of the individual protein fractions within the peak and activity levels. Following ion-exchange purification, the protein purity reached > 95 %, as determined from SDS/PAGE analysis. Approx. 0.05–0.1 mg of pure protein could be obtained per 10¹⁰ High5 cells. A trypsin digest of the 111 kDa SDS/PAGE protein band demonstrated a clear MALDI–TOF MS fragmentation pattern corresponding to DPP-8_{882aa}. More than 60 % of the sequence was covered by the analysis (results not shown).

DPP-8_{882aa} was difficult to handle and showed a high degree of lability. After 12 h of incubation of DPP-8_{882aa}, the specific activity was reduced by more than 85 % in samples stored at room temperature compared with freshly thawed stocks. Addition of

Figure 2 In-gel digestion by trypsin of excised 95 kDa DPP-9_{892aa} bands were used for MALDI–TOF MS fragment analysis

MALDI–TOF MS fragment pattern of the digest is shown. More than 50 % of the sequence was covered by the digest.

10 % (v/v) glycerol before freezing somehow preserved activity, but otherwise no actions or formulation screening were performed to preserve activity other than maintaining stocks in an ice bath during handling and before assay incubations. Expression and purification of DPP-8_{Δ657–757aa} produced no enzymatically active protein from the TALON™ elution (results not shown).

The two DPP-9 variants were expressed and purified as distinct proteins of similar molecular masses of approx. 95 kDa as determined from molecular mass standards (see Supplemental Figure 2 at <http://www.BiochemJ.org/bj/396/bj3960391add.htm>). However, a very different heterogeneity was observed for the two fractionated proteins as demonstrated from SDS/PAGE and Coomassie Blue analyses. DPP-9_{892aa} was homogeneous and more than 95 % pure after the TALON™ fractionation, whereas DPP-9_{863aa} showed a heterogeneous band pattern as evaluated from the SDS/PAGE and Coomassie Blue analyses, probably due to a poor folding ability and stability, resulting in not only a smaller amount of soluble protein, but also in incorrect folding. Activity levels co-migrated with the fractionated 95 kDa protein from the DPP-9_{892aa} expression, whereas no enzymatic activity was detected from the fractions of the expression of the DPP-9_{863aa} variant (see Supplemental Figure 2). Amounts of 0.5–1 mg of pure DPP-9_{892aa} protein could be obtained from approx. 10¹⁰ High5 cells.

In-gel enzymatic digestion by trypsin of the 95 kDa bands of both the short and long DPP-9 construct verified that the proteins were DPP-9 as determined from MALDI–TOF MS analysis (see Figure 2 for example of DPP-9_{892aa}).

DPP-9_{892aa} had a much higher stability compared with DPP-8_{882aa}, since several freeze–thaws and prolonged incubations at room temperature only affected the enzymatic activity slightly. Temperature profiling showed that both DPP-8_{882aa} and DPP-9_{892aa} lost significant activity at and above 45 °C incubation, whereas optimal activities for both of the two proteins were between 35 and 40 °C incubation. This is a clear difference compared with the very stable DPP-IV, which had a stepwise activity increase

Figure 3 Chromatographic profiles of size-exclusion analysis of DPP-IV, DPP-8_{882aa} and DPP-9_{892aa} on a Superdex 200 column

Retention volumes of each protein analysed are normalized to a high-molecular-mass standards (bottom) retention profile. Peaks were fractionated and analysed for DPP activity. Indicated peaks had DPP activity.

from 25 °C and up to 45 °C at which DPP-IV displayed 100 % activity.

Quaternary-structure analysis of DPP-8 and DPP-9 using dynamic light scattering and size-exclusion chromatography

The quaternary structures of DPP-8_{882aa} and DPP-9_{892aa} were analysed and compared with that of DPP-IV, which is known to be a catalytically active homodimer (see, e.g., [14]). DLS was used to measure the hydrodynamic radius of the purified proteins in solution. DPP-8_{882aa} and DPP-9_{892aa} displayed strong DLS signals corresponding to hydrodynamic radii of 6.8 and 5.9 nm with polydispersities of 18.1 % and 16.2 % respectively (see Supplemental Figure 3 at <http://www.BiochemJ.org/bj/396/bj3960391add.htm>). Likewise, DPP-IV displayed a monodisperse signal with a radius of 5.0 nm and polydispersity of 0.1 % (results not shown). The protein peaks of DPP-8_{882aa}, DPP-9_{892aa} and DPP-IV all corresponded to close to 100 % of total mass in solution when subtracting the solvent peak.

To analyse further the quaternary structure, size-exclusion chromatography was performed for DPP-IV, DPP-8_{882aa} and DPP-9_{892aa}, and was compared with retention volumes of high-molecular-mass standards (Figure 3). All three proteins migrated with a retention volume of approx. 12 ml, corresponding to mol-

Table 3 Kinetic parameters of putative substrates

Assays were performed with a buffer composition of 50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1 % Triton X-100 at 37 °C. Results are means $\pm$ S.D. for three experiments.

	Substrate	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ · M ⁻¹)
DPP-IV	Gly-Pro-pNA	0.20 $\pm$ 0.01	70.6 $\pm$ 0.3	430 500
	Ala-Pro-pNA	0.05 $\pm$ 0.01	38.0 $\pm$ 2.9	688 000
	Val-Ala-pNA	0.67 $\pm$ 0.09	57.0 $\pm$ 3.7	84 900
	Arg-Pro-pNA	0.06 $\pm$ 0.02	60.6 $\pm$ 12.8	898 400
DPP-8 _{882aa}	Gly-Pro-pNA	0.33 $\pm$ 0.01	65.8 $\pm$ 0.2	199 300
	Ala-Pro-pNA	0.34 $\pm$ 0.10	65.0 $\pm$ 4.7	191 200
	Val-Ala-pNA	0.76 $\pm$ 0.08	1.5 $\pm$ 0.1	2000
	Arg-Pro-pNA	0.07 $\pm$ 0.01	2.8 $\pm$ 0.2	40 900
DPP-9 _{892aa}	Gly-Pro-pNA	0.37 $\pm$ 0.003	84.1 $\pm$ 1.1	227 100
	Ala-Pro-pNA	0.31 $\pm$ 0.13	90.0 $\pm$ 13.4	225 400
	Val-Ala-pNA	1.22 $\pm$ 0.13	2.5 $\pm$ 0.2	2200
	Arg-Pro-pNA	0.06 $\pm$ 0.01	2.4 $\pm$ 0.2	41 200

ecular masses slightly above 200 kDa. Taken together, this indicates that both DPP-8 and DPP-9 are catalytically active homodimers in solution, as in DPP-IV.

Recombinant DPP-8 and DPP-9 show distinct Michaelis–Menten kinetics and specificity for cleavage of putative pNA substrate analogues

DPP activity was demonstrated for DPP-8_{882aa} and DPP-9_{892aa} using the putative substrates Gly-Pro-pNA, Ala-Pro-pNA, Val-Ala-pNA and Arg-Pro-pNA (Table 3). No tri- or endo-peptidase activities were observed (under the incubation and assay conditions outlined in the Experimental section) as determined using the peptide analogue Ala-Ala-Pro-pNA and the full-length peptides GLP-1, GLP-2, NPY and PYY in a MALDI-TOF MS assay, which is similar to the one described by Lambeir et al. [15]. DPP-8_{882aa} and DPP-9_{892aa} were unable to cleave Gly-Gly-pNA, Ala-Ala-pNA and Ala-Phe-pNA under the given assay conditions.

Very similar Michaelis–Menten kinetic profiles of the different substrates were demonstrated for DPP-8_{882aa} and DPP-9_{892aa} with K_m values between 70 and 1220 μ M, which were similar to DPP-IV values of 50–670 μ M (Table 3). The k_{cat} values for DPP-8_{882aa} and DPP-9_{892aa} were generally lower than that of DPP-IV, giving equally lower catalytic capacities. For example, we observed for the Arg-Pro-pNA substrate 23- and 22-fold lower k_{cat}/K_m for DPP-8_{882aa} and DPP-9_{892aa} compared with DPP-IV respectively. Using the substrates Gly-Pro-pNA and Ala-Pro-pNA, k_{cat}/K_m for DPP-8_{882aa} and DPP-9_{892aa} were in the order of 10⁶ M⁻¹ · s⁻¹ for both enzymes, making them very competent in terms of catalytic performance, but poorer compared with DPP-IV for both of these and all other substrates tested.

Kinetics and specificity of recombinant DPP-8 and DPP-9

The ability to cleave the naturally occurring peptides GLP-1, GLP-2, NPY and PPY was tested *in vitro* using a MALDI-TOF MS-based assay. From semi-empirical analyses of the distribution of peptide fragments, different kinetics were observed depending on the peptide substrate used (Figure 4); however, these were very similar when comparing rates between DPP-8_{882aa} and DPP-9_{892aa}, which generally were markedly lower compared with that of DPP-IV.

Figure 4 Fragmentation diagrams of MALDI-TOF MS analysis of GLP-1, GLP-2, NPY and PYY digests by DPP-882aa and DPP-982aa

Cleaved peptides are indicated by arrows. Very similar digestion profiles were obtained for DPP-882aa and DPP-982aa. Digested GLP-1 and -2 were observed after 20 min and were almost completely digested after 24 h of incubation. NPY digestion products were observed after 20 min of incubation and were digested to the end after less than 4 h. Significant PYY digest products were first observed after approx. 4 h, and, after 24 h, approx. 25% were intact. See Table 4 for estimated $t_{1/2}$ values based on MALDI-TOF MS profiles.

Table 4 Relative $t_{1/2}$ values of peptide digests

The value in parentheses represents the $t_{1/2}$ of the degradation product GLP-1-(11–36).

	Peptide	$t_{1/2}$ (h)
DPP-IV	GLP-1	~0.2 (~1.2)
	GLP-2	~0.4
	NPY	~0.1
	PYY	~0.1
DPP-8 _{882aa}	GLP-1	~3.8
	GLP-2	~4.0
	NPY	~0.2
	PYY	~50
DPP-9 _{892aa}	GLP-1	~6.1
	GLP-2	~6.7
	NPY	~0.2
	PYY	~8

Both DPP-8_{882aa} and DPP-9_{892aa} cleaved two amino acids from the N-terminal of all four peptides and both showed a kinetic favouring of the following order NPY > GLP-2 > GLP-1 >> PYY, which differed compared with DPP-IV which showed NPY ≈ PYY > GLP-1 > GLP-2. *In vitro* $t_{1/2}$ values were determined from ratios between the MS intensities of intact and cleaved peptides (Table 4) corresponding to the following relative rates DPP-IV/DPP-8/DPP-9: 1:0.5:0.5 for NPY, 1:0.1:0.06 for GLP-2, 1:0.05:0.03 for GLP-1 and 1:0.002:0.002 for PYY. NPY was the full-length peptide substrate that was cleaved best among the four substrates tested, whereas cleavage of PYY was the lowest for DPP-8 and DPP-9 and strikingly lower compared with DPP-IV, which cleaved this substrate at the highest rate equal to the cleavage rate of NPY. Both DPP-8 and DPP-9 had lower substrate turnovers for all the full-length peptide substrates compared with DPP-IV and, furthermore, a specificity difference was observed. Only the first two N-terminal amino acids of GLP-1 were cleaved by DPP-8 and DPP-9 as opposed to DPP-IV, which cleaved sequentially two dipeptides from the N-terminal of GLP-1. This was in accordance with the inability to cleave Gly-Gly-pNA by DPP-8/-9, since the P2' residue is glycine.

Inhibition of DPP-IV, DPP-8 and DPP-9 with ValPyr

We used the competitive DPP-IV inhibitor ValPyr for inhibition studies of DPP-IV, DPP-8 and DPP-9 as outlined in the Experimental section. We found that DPP-IV, DPP-8_{882aa} and DPP-9_{892aa} had a similar kind of inhibition, resulting in very similar K_i values of 0.64 ± 0.01 , 0.63 ± 0.02 and 0.78 ± 0.06 respectively. Thus the inhibitor ValPyr was equally potent and therefore non-selective for any of the three S9b family members DPP-IV, DPP-8 and DPP-9.

DISCUSSION

We have characterized recombinant full-length variants of DPP-8 and DPP-9 as enzymes with similar kinetic, specificity and inhibition profiles, including the ability to cleave the naturally occurring peptides GLP-1, GLP-2, NPY and PYY *in vitro*. Expression of DPP-8_{Δ657–757aa} and DPP-9_{863aa} produced no enzymatically active proteins, and, regarding the latter, this result concurred with previously published [10] and unpublished (C. Olsen and N. Wagtman, unpublished work) expression studies performed

in our laboratories using both an *in vitro* translation system and COS cells. Distinct activities were observed only for the full-length variants of 882 and 892 amino acids for DPP-8 and DPP-9 respectively. These results disagree with published data of a recombinant expression construct of the short 863-amino-acid variant of DPP-9, which was reported to be active [11,12]. None of the earlier studies showed any clear co-migration between the expressed DPP-9_{863aa} protein and the DPP activity observed. In one of the studies, activity estimations were based on mammalian cell transfection experiments, making contamination of endogenous activity of DPP-IV a possibility [12]. In the present study, distinctive verifications of all the purified proteins using MALDI-TOF MS analysis of trypsin digests of the purified recombinant proteins were performed. These results indicate that DPP-9 only can be produced as an enzymatically active protein when expressed as a full-length 892-amino-acid variant. The three-dimensional structure of full-length DPP-9 is required for our understanding of the implications of the N-terminus; however, one cannot rule out the possibility that the placement of a His₆-tag can play a critical role for the missing activity of DPP-9_{863aa}. In the present study, a His₆-tag was placed in the C-terminus of the short form, whereas in the studies of Qi et al. [11] and Ajami et al. [12], the His₆-tag was placed in the N-terminus. In the construction of DPP-9_{892aa}, the His₆-tag was placed in the N-terminus.

We have found that the DPP-8_{882aa} and DPP-9_{892aa} variants are dimers, as determined using size-exclusion chromatography and DLS. Thus it appears that the general oligomeric structure of the S9b family members is a dimeric configuration, since DPP-IV, FAP α (fibroblast activation protein α) and DPP-2 are dimers like DPP-8 and DPP-9 [16,17].

Assuming overall structural conservation between DPP-8, DPP-9 and DPP-IV, the published crystal structures of recombinant human DPP-IV can be used to deduce effects of the different variants [18–20]. DPP-IV is composed of an α/β -hydrolase and an eight-bladed β -propeller domain, in the interface where the active site is located. The spliced exon $\Delta 657$ –757 of DPP-8 comprises an essential part of the protein, since the active serine residue is located here according to sequence alignments and inspection of the X-ray structure of DPP-IV. When relating to the active site of DPP-IV [defined by a sphere of 8 Å (1 Å = 0.1 nm) radius from active-site inhibitor ValPyr in DPP-IV and following pinpointing residues in alignment of DPP-8 and DPP-9], distinct identities of 74 % and 69 % can be observed between DPP-IV and DPP-8 and between DPP-IV and DPP-9 respectively, which are much higher than the relatively low overall identities of 20 % and 19 % respectively. On the other hand, DPP-8 and DPP-9 are very similar. They share an overall identity of 58 % and an identity of 92 % of essential active-site residues as compared with DPP-IV. The remaining residues within the active site are possibly related to differences in substrate selectivity between DPP-8 and DPP-9 on one hand and DPP-IV on the other. In this context, it is notable that the well-known DPP-IV inhibitor ValPyr showed equipotency for all three enzymes, indicating that the inhibitor is non-selective contrary to the DPP-IV selectivity reported before DPP-8 and DPP-9 were made available [21]. Selectivity differences were also observed in the cleavage rates of four naturally occurring peptides, GLP-1, GLP-2, NPY and PYY, which generally were similar for DPP-8 and DPP-9, but higher for DPP-IV. NPY was the best substrate for all three enzymes and was cleaved with similar rates. DPP-IV cleaved PYY with a rate similar to that of NPY cleavage, which, compared with DPP-8 and DPP-9 was approx. 500-fold higher. NPY and PYY have been reported previously as the best naturally occurring substrates for DPP-IV [15,22]. Residues in the P1, P2 and P2' positions are the same in NPY and PYY, whereas the P1' residue differs, being serine in NPY

and isoleucine in PYY. Thus a polar residue in the P1' position seems to be preferred for DPP-8 and DPP-9 relative to DPP-IV. Relating to the recently published DPP-IV crystal structure with a bound NPY analogue (PDB code 1R9N), the S1' pocket appears flat and with no well-defined contacts with the P1' serine residue [23], and the large active-site cavity in itself explains the vast acceptance and high turnover of substrates. Indeed, DPP-8 and DPP-9 show differences in substrate selectivity at the P1 and P1' position compared with DPP-IV. Comparing the tested substrates there seem to be no requirements for the nature of the amino acid in the P2 residue position, as both small and bulky side chains and non-polar and charged residues are tolerated. This is analogous with what is found for DPP-IV. GLP-1 and GLP-2 were cleaved with similar rates by both DPP-8 and DPP-9, but approx. 10–30-fold lower compared with DPP-IV. Furthermore, we found that both DPP-8 and DPP-9 were unable to cleave substrates that had glycine in the P1 position, which again is a distinct difference from the specificity of DPP-IV observed previously [24] and in the present study. Given both the difference of substrate specificity between DPP-8, DPP-9 and DPP-IV and the suggested active-site composition of DPP-8 and DPP-9, it is understandable that active-site inhibitors with selectivity towards DPP-IV relative to DPP-8 and DPP-9 can be developed. These data suggest that S1 and S1' pockets might be explored for rational drug design. *In vitro* selectivity data on a series of β amino acid analogues has been reported by Kim et al. [25] and show that selectivity can be achieved. Animal data have indicated that inhibition of DPP-8 and DPP-9 produces a series of unwanted side effects [26]. The importance for development of DPP-IV-selective compounds for therapeutic use is therefore stressed.

DPP-8 and DPP-9 are examples of so-called DASH (DPP-IV activity and/or structure homologous) proteins, as they share sequence and possible structural homology as well as functional activity with DPP-IV, and so might be associated with functions previously ascribed solely to DPP-IV. DPP-8 and DPP-9 have been reported to be intracellularly located, since no trans-membrane domains or leader export signals for known cell secretion pathways have been identified in the sequence [8–10]. Thus the *in vitro* observed proteolytic cleavage of the incretin hormones GLP-1 and GLP-2 by DPP-8 and DPP-9 is unlikely to be of relevance with respect to *in vivo* physiological functions to which DPP-IV has been attributed in the vascular system. An intracellular associated regulation of GLP-1 release from L-enterocytes is unlikely, since only intact GLP-1-(7–37) has been shown to be secreted from L-enterocytes of the porcine ileum [27]. Regarding the pancreatic polypeptide family members PYY and NPY, it is interesting that these are regulated by enzymatic removal of the first dipeptide for production of selective Y receptor agonists, e.g. truncated PYY which is a highly selective Y2 receptor agonist [24,28]. However, the low cleavage rate observed for DPP-8 and DPP-9 argues against a genuine physiological role of the N-terminal processing of these pancreatic polypeptide hormones.

In conclusion, we have characterized different recombinant variants of DPP-8 and DPP-9 and found that only the full-length variants of 882 and 892 amino acids respectively were active enzymes, appearing to be homodimers in solution with very similar kinetic and substrate profiles. By analogy and comparison of the ability for DPP-8 and DPP-9 to cleave various substrates and naturally occurring peptides of the incretin and pancreatic hormone family, we have mapped requirements to amino acid nature in the proximal positions to the scissile bond and compared it with DPP-IV and conclude that development of DPP-IV-selective therapeutic inhibitors relative to DPP-8 and DPP-9 is feasible.

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REFERENCES

- Rawlings, N. D. and Barrett, A. J. (1999) MEROPS: the peptidase database. *Nucleic Acids Res.* **27**, 325–331
- Hopsu-Havu, V. K. and Glenner, G. G. (1966) A new dipeptide naphthylamidase hydrolyzing glycyl-prolyl- β -naphthylamide. *Histochemie* **7**, 197–201
- Kenny, A. J., Booth, A. G., George, S. G., Ingram, J., Kershaw, D., Wood, E. J. and Young, A. R. (1976) Dipeptidyl peptidase IV, a kidney brush-border serine peptidase. *Biochem. J.* **157**, 169–182
- Kenny, A. J., Booth, A. G., Wood, E. J. and Young, A. R. (1976) Dipeptidyl peptidase IV, a kidney microvillus serine proteinase: evidence for its large subunit molecular weight and endopeptidase activity. *Biochem. Soc. Trans.* **4**, 347–348
- Puschel, G., Mentlein, R. and Heymann, E. (1982) Isolation and characterization of dipeptidyl peptidase IV from human placenta. *Eur. J. Biochem.* **126**, 359–365
- Rawlings, N. D. and Barrett, A. J. (1993) Evolutionary families of peptidases. *Biochem. J.* **290**, 205–218
- Sedo, A. and Malik, R. (2001) Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? *Biochim. Biophys. Acta* **1550**, 107–116
- Gorrell, M. D. (2005) Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin. Sci.* **108**, 277–292
- Abbott, C. A., Yu, D. M., Woollatt, E., Sutherland, G. R., McCaughan, G. W. and Gorrell, M. D. (2000) Cloning, expression and chromosomal localization of a novel human dipeptidyl peptidase (DPP) IV homolog, DPP8. *Eur. J. Biochem.* **267**, 6140–6150
- Olsen, C. and Wagtmann, N. (2002) Identification and characterization of human DPP9, a novel homologue of dipeptidyl peptidase IV. *Gene* **299**, 185–193
- Qi, S. Y., Riviere, P. J., Trojnar, J., Junien, J. L. and Akinsanya, K. O. (2003) Cloning and characterization of dipeptidyl peptidase 10, a new member of an emerging subgroup of serine proteases. *Biochem. J.* **373**, 179–189
- Ajami, K., Abbott, C. A., McCaughan, G. W. and Gorrell, M. D. (2004) Dipeptidyl peptidase 9 has two forms, a broad tissue distribution, cytoplasmic localization and DPIP-like peptidase activity. *Biochim. Biophys. Acta* **1679**, 18–28
- Christensen, J., Colmore, S. F. and Tattersall, P. (1999) Two new members of the emerging KDWK family of combinatorial transcription modulators bind as a heterodimer to flexibly spaced PuCGPy half-sites. *Mol. Cell. Biol.* **19**, 7741–7750
- Rasmussen, H. B., Branner, S., Wiberg, F. C. and Wagtmann, N. (2003) Crystal structure of human dipeptidyl peptidase IV/CD26 in complex with a substrate analog. *Nat. Struct. Biol.* **10**, 19–25
- Lambeir, A. M., Durinx, C., Proost, P., Van Damme, J., Scharpe, S. and de Meester, I. (2001) Kinetic study of the processing by dipeptidyl-peptidase IV/CD26 of neuropeptides involved in pancreatic insulin secretion. *FEBS Lett.* **507**, 327–330
- Strop, P., Bankovich, A. J., Hansen, K. C., Garcia, K. C. and Brunger, A. T. (2004) Structure of a human A-type potassium channel interacting protein DPPX, a member of the dipeptidyl aminopeptidase family. *J. Mol. Biol.* **343**, 1055–1065
- Aertgeerts, K., Levin, I., Shi, L., Snell, G. P., Jennings, A., Prasad, G. S., Zhang, Y., Kraus, M. L., Salakian, S., Sridhar, V. et al. (2005) Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein α . *J. Biol. Chem.* **280**, 19441–19444
- Hiramatsu, H., Kyono, K., Higashiyama, Y., Fukushima, C., Shima, H., Sugiyama, S., Inaka, K., Yamamoto, A. and Shimizu, R. (2003) The structure and function of human dipeptidyl peptidase IV, possessing a unique eight-bladed β -propeller fold. *Biochem. Biophys. Res. Commun.* **302**, 849–854
- Thoma, R., Löffler, B., Stihle, M., Huber, W., Ruf, A. and Hennig, M. (2003) Structural basis of proline-specific exopeptidase activity as observed in human dipeptidyl peptidase-IV. *Structure* **11**, 947–959
- Bjelke, J. R., Christensen, J., Branner, S., Wagtmann, N., Olsen, C., Kanstrup, A. B. and Rasmussen, H. B. (2004) Tyrosine 547 constitutes an essential part of the catalytic mechanism of dipeptidyl peptidase IV. *J. Biol. Chem.* **279**, 34691–34697
- Deacon, C. F., Hughes, T. E. and Holst, J. J. (1998) Dipeptidyl peptidase IV inhibition potentiates the insulinotropic effect of glucagon-like peptide 1 in the anesthetized pig. *Diabetes* **47**, 764–769
- Mentlein, R., Dahms, P., Grandt, D. and Kruger, R. (1993) Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV. *Regul. Pept.* **49**, 133–144
- Aertgeerts, K., Ye, S., Tennant, M. G., Kraus, M. L., Rogers, J., Sang, B. C., Skene, R. J., Webb, D. R. and Prasad, G. S. (2004) Crystal structure of human dipeptidyl peptidase IV in complex with a decapeptide reveals details on substrate specificity and tetrahedral intermediate formation. *Protein Sci.* **13**, 412–421
- de Meester, I., Korom, S., Van Damme, J. and Scharpe, S. (1999) CD26, let it cut or cut it down. *Immunol. Today* **20**, 367–375

- 25 Kim, D., Wang, L., Beconi, M., Eiermann, G. J., Fisher, M. H., He, H., Hickey, G. J., Kowalchick, J. E., Leiting, B., Lyons, K. et al. (2005) (2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine: a potent, orally active dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes. *J. Med. Chem.* **48**, 141–151
- 26 Edmondson, S. D., Mastracchio, A., Beconi, M., Colwell, Jr, L. F., Habulihaz, B., He, H., Kumar, S., Leiting, B., Lyons, K. A., Mao, A. et al. (2004) Potent and selective proline derived dipeptidyl peptidase IV inhibitors. *Bioorg. Med. Chem. Lett.* **14**, 5151–5155
- 27 Hansen, L., Deacon, C. F., Orskov, C. and Holst, J. J. (1999) Glucagon-like peptide-1-(7–36) amide is transformed to glucagon-like peptide-1-(9–36) amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology* **140**, 5356–5363
- 28 Grandt, D., Teyssen, S., Schimiczek, M., Reeve, Jr, J. R., Feth, F., Rascher, W., Hirche, H., Singer, M. V., Layer, P. and Goebell, H. (1992) Novel generation of hormone receptor specificity by amino terminal processing of peptide YY. *Biochem. Biophys. Res. Commun.* **186**, 1299–1306

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