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Cloning, expression and chromosomal localization of a novel human dipeptidyl peptidase (DPP) IV homolog, DPP8

Catherine A. Abbott[1], Denise M. T. Yu[1,*], Erica Woollatt[2], Grant R. Sutherland[2], Geoffrey W. McCaughan[1] and Mark D. Gorrell[1]

[1]*A. W. Morrow Gastroenterology and Liver Centre, Royal Prince Alfred Hospital, Centenary Institute of Cancer Medicine and Cell Biology and The University of Sydney, NSW, Australia;* [2]*Centre for Medical Genetics, Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, Adelaide, SA, Australia*

Dipeptidyl peptidase (DPP) IV has roles in T-cell costimulation, chemokine biology, type-II diabetes and tumor biology. Fibroblast activation protein (FAP) has been implicated in tumor growth and cirrhosis. Here we describe DPP8, a novel human postproline dipeptidyl aminopeptidase that is homologous to DPPIV and FAP. Northern-blot hybridization showed that the tissue expression of *DPP8* mRNA is ubiquitous, similar to that of DPPIV. The *DPP8* gene was localized to chromosome 15q22, distinct from a closely related gene at 19p13.3 which we named *DPP9*. The full-length *DPP8* cDNA codes for an 882-amino-acid protein that has about 27% identity and 51% similarity to DPPIV and FAP, but no transmembrane domain and no N-linked or O-linked glycosylation. Western blots and confocal microscopy of transfected COS-7 cells showed DPP8 to be a 100-kDa monomeric protein expressed in the cytoplasm. Purified recombinant DPP8 hydrolyzed the DPPIV substrates Ala-Pro, Arg-Pro and Gly-Pro. Thus recombinant DPP8 shares a postproline dipeptidyl aminopeptidase activity with DPPIV and FAP. DPP8 enzyme activity had a neutral pH optimum consistent with it being nonlysosomal. The similarities between DPP8 and DPPIV in tissue expression pattern and substrates suggests a potential role for DPP8 in T-cell activation and immune function.

Keywords: dipeptidyl peptidase; fibroblast activation protein; postproline peptidase; prolyl oligopeptidase; serine proteinase.

Very few enzymes are able to cleave the prolyl bond. The most widely studied of these enzymes is a 766-amino-acid, type-II integral membrane protein dipeptidyl peptidase (DPP) IV (EC 3.4.14.5). DPPIV also has a soluble form that lacks the transmembrane domain [1,2]. The tertiary structure of DPPIV contains an N-terminal seven-blade β-propeller domain and a C-terminal α/β-hydrolase domain [3]. DPPIV cleavage of specific substrates results in alterations in their biological activities. These substrates include certain chemokines, growth factors such as glucagon and glucagon-like peptides 1 and 2, neuropeptides including neuropeptide Y and substance P and vasoactive peptides [4–6]. The N terminal truncation of the chemokines RANTES (regulated on activation normal T cell expressed and secreted), eotaxin, macrophage-derived chemokine and stromal-cell-derived factor-1 alters the receptor specificities of these chemokines, potentially altering the Th1/Th2 balance of an immune response towards Th1 [5].

DPPIV/CD26 expression is ubiquitous but is significantly upregulated on activated T, B and NK cells [7–11]. The role of DPPIV/CD26 in immune activation involves both its enzyme activity and its non-catalytic activity, which is the ability to bind adenosine deaminase [5,12–14]. DPPIV is in the peptidase family S9b, which along with prolyl endopeptidase (PEP, S9a) and acylaminoacyl peptidase (S9c) [15] form the prolyl oligopeptidase family. We have proposed that DPPIV, fibroblast activation protein (FAP) [16,17] and dipeptidyl aminopeptidase-like protein (DPP6) [18] form a *DPPIV*-like gene family which corresponds to peptidase family S9b [19]. We have compared DPPIV and FAP in detail [5]. FAP is selectively expressed by stromal fibroblasts of tumors and healing wounds [16] and by activated hepatic stellate cells [20]. FAP has postproline dipeptidyl aminopeptidase activity similar to but distinct from that of DPPIV, has gelatinase activity and binds to $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrin [20–24].

Recently, novel enzymes have been reported with substrate specificities similar to DPPIV. DPPIV-β is a cell surface glycoprotein of 82 kDa that binds DPPIV inhibitors but with less affinity than DPPIV, and no sequence data for this enzyme is available [25]. Attractin (DPPT-L) is a 175-kDa soluble glycoprotein reported to hydrolyze Gly-Pro [26]. Attractin

Correspondence to C. A. Abbott, Centenary Institute of Cancer Medicine and Cell Biology, Locked Bag No. 6, Newtown, NSW 2042, Australia.
Fax: 61 2 95656101, Tel.: 61 2 95656161,
E-mail: c.abbott@centenary.usyd.edu.au

Abbreviations: DPP, dipeptidyl peptidase; EST, expressed sequence tag; FAP, fibroblast activation protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NA, nitroanilide; PEP, prolyl endopeptidase; PBMC, peripheral blood monocyte; stPBMC, peripheral blood monocyte stimulated with phytohaemagglutinin.

Enzymes: dipeptidyl peptidase IV (P27487, EC 3.4.14.5); prolyl endopeptidase (P48147, EC 3.4.21.26); acylaminoacyl peptidase (P13798, EC 3.4.19.1); fibroblast activation protein (GenPept: g1888316); DPPIV-β; attractin (GenPept: g3676347); quiescent peptidyl peptidase (GenPept: g6465985); *N*-acetylated alpha-linked acidic dipeptidase II (GenPept: g4539525).

Note: nucleotide sequences reported in this paper have been submitted to the GenBank™ databank with accession numbers AF221634, AF221635, AF221636, AF221637.

**Present address*: Department of Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney, NSW, Australia.

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contains a kelch repeat domain [27] and shares no significant sequence homology with DPPIV or any other peptidase. Two other peptidases have been reported to hydrolyze Gly-Pro, but are structurally unrelated to DPPIV: the carboxypeptidase, quiescent peptidyl peptidase [28], and the glutamate carboxpeptidase, N-acetylated alpha-linked acidic dipeptidase II [29]. Thus, new enzymes of this family or enzymes of similar function but different structure are emerging. Judging from the critical roles of DPPIV and FAP in biological processes of cellular activation, inflammation and tissue remodeling, additional family members are likely to play a similar role in these and other biological processes. Hence the aim of this study was to characterize a novel peptidase related to DPPIV in both substrate specificity and structure.

This study describes the cloning of a full-length cDNA, chromosomal localization, expression and functional characterization of a novel human peptidase, dipeptidyl peptidase 8 (DPP8). Biochemical characterization of this novel protein revealed that it has enzyme activity similar to that of DPPIV and FAP and is upregulated during immune activation.

EXPERIMENTAL PROCEDURES

General

Restriction and other enzymes used in cloning were obtained from Boehringer Mannheim Roche. Standard molecular biology techniques were used [30] unless indicated otherwise. The collection of specimens used in this study was approved by the Royal Prince Alfred Hospital human ethics committee.

Cell culture and RNA preparation

Human peripheral blood monocytes (PBMCs) were isolated by Ficoll-Hypaque density-gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden) of blood obtained from healthy donors. The PBMCs were incubated in AIM-V medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 2 mM L-glutamine and were stimulated with either 1 $\mu g \cdot mL^{-1}$ phytohaemagglutinin (Wellcome) or 100 $ng \cdot mL^{-1}$ OKT3 (Orthoclone, Ortho Biotec Ravitan, NJ, USA) for 72 h. The human cell lines Jurkat, CCRF-CEM, Raji, Daudi and HepG2 were grown to confluence in Dulbecco's modified Eagle's medium (Trace Biosciences, Sydney, NSW, Australia) supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

Liver and placental RNA were prepared from snap-frozen human tissue as described previously [31]. However, RNA was prepared from PBMCs and cell lines using an RNAeasy kit (Qiagen, Hilden, Germany).

Bioinformatics

BLAST searches [32] and all multiple sequence alignments were performed through the Australian National Genomic Information Service (ANGIS, Sydney, NSW, Australia). PILEUP (GCG Version 8, Genetics Computer Group, Madison, WI, USA) was used for multiple sequence alignments of proteins.

A BLAST search was performed on the public expressed sequence tag (EST) database using the complete human *DPPIV* (GenBank™ accession number X60708) and *FAP* (accession number U09278) nucleotide sequences as query sequences. An EST clone (accession number AA417787) was obtained from the American Type Culture Collection. The DNA insert of this clone was sequenced on both strands using automated sequencing at SUPAMAC (Sydney, NSW, Australia). Because of its homology with DPPIV, this new gene was named dipeptidyl peptidase 8 (DPP8).

Cloning of *DPP8* cDNA

The DPP8 EST sequence was used to design the forward primer, DPP8-pr1 (CAAATAGAAATTGACGATCAGGTG) and the reverse primer DPP8-pr2r (TCTTGAAGGTAGTGCAAAAGATGC) for PCR from the EST cDNA. The PCR conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s and 70 °C for 1 min. This 484-bp PCR product was gel-purified, ^{32}P-labeled using a Megaprime Labeling Kit (Amersham Pharmacia Biotec) and hybridized to a Master RNA blot (Clontech, Palo Alto, CA, USA), which contained poly(A)-rich RNA from 50 adult and fetal tissues immobilized in dots, in accordance with the manufacturer's instructions. The 484-bp DPP8 PCR product was used to screen a human placental λ STRETCH PLUS library (Clontech) using standard methods. For the tertiary screen, the clones contained in λTriplEx were converted into pTriplEx plasmids and transformed into BM25.8 *Escherichia coli* recipient bacteria. The clones were retransformed into MC1061 *E. coli* for subsequent DNA purification. The DNA was then sequenced using M13 forward and reverse and primer walking.

5′ RACE

A 5′ RACE version 2.0 kit (Life Technologies) was applied to phytohaemagglutinin-stimulated PBMC (stPBMC) and placental RNA according to the kit instructions. The sequence of the T8 library clone was used to design GSP1 (TCCTTCCTTCAGCATCAATC) and GSP2 (CTTAAAAGTGACTTTAGGATTTGCTGTACC). 5′ RACE PCR products were cloned into pGEM-T Easy® vector (Promega, Madison, WI, USA) and sequenced using M13 forward and reverse primers and by primer walking. To confirm that the 5′ RACE product was *DPP8*, RT-PCR on stPBMC RNA used the primers DPP8-pr23 (GGAAGAAGATGCCAGATCAGCTGG) and DPP8-pr19r (TCCGTGTATCCTGTATCATAGAAG) to span the junction between the 5′ RACE product and the EST clone AA417787. This PCR yielded two products, stPBMCdy3-2-1 (1602 bp) and stPBMCdy3-3-10 (1083 bp), which were gel purified and cloned into pGEM-T Easy® and subsequently sequenced.

Subcloning of *DPP8* cDNA into a pcDNA3.1/V5/His expression vector

The stPBMC RACE product, the stPBMCdy3-2-1 (1602 bp) junction fragment and the library clone T21 were joined together and cloned into the expression vector pcDNA3.1/V5/HisA (Invitrogen BV, Groningen, the Netherlands). This created a *DPP8* cDNA of 3.1 kb with an ORF of 882 amino acids. The first construct was made using three sequential cloning steps. First, an *Eco*RV–*Xba*I fragment of T21 (containing 3′ DPP8, stop codon and 3′ UTR on *DPP8* cDNA) was ligated into *Eco*RV–*Xba*I-digested pcDNA3.1/V5/HisA vector. Secondly, an *Eco*RI–*Eco*RV fragment of stPBMCdy3-2-1 was then added to this construct digested with *Eco*RI–*Eco*RV. Finally the 5′ RACE product was cut with *Eco*RI and cloned into the *Eco*RI site of the previous construct to form the complete 3.1-kb *DPP8* cDNA. This construct, pcDNA3.1–DPP8, expressed DPP8 protein with no tag.

In addition, the stop codon in the DPP8 expression construct in pcDNA3.1/V5/HisA was altered using PCR to

create a C-terminal fusion with the V5 and His tag contained in the vector. This construct was named pcDNA3.1-DPP8/V5/His. All expression constructs subcloned into pcDNA3.1/V5/His were verified by full sequence analysis.

Chromosomal localization of *DPP8* by fluorescence *in situ* hybridization analysis

DPP8 was localized using two different probes, the *DPP8* EST and the T8 clone. The probes were nick-translated with biotin-14-dATP and hybridized *in situ* at a final concentration of 10 ng·μL^{-1} to metaphases from two normal males. The fluorescence *in situ* hybridization method was modified from that previously described [33] in that chromosomes were stained with both propidium iodide and 4,6-diamidino-2-phenylindole.

DPP8 gene expression analyzed by Northern blot

Human multiple-tissue Northern blots (Clontech) containing 2 μg poly(A)-rich RNA were prehybridized in Express Hybridization solution (Clontech) for 30 min at 68 °C. Both the DPP8 484-bp product and the 5′ RACE stPBMC product were radiolabeled using the Megaprime Labeling kit and [^{32}P]dCTP (NEN Life Science, Boston, MA, USA). Unincorporated label was removed using a NICK column (Amersham Pharmacia Biotech), and the denatured probe was incubated with the blot for 2 h at 68 °C in Express Hybridization solution. Washes were performed at high stringency, and blots exposed to BIOMAX MS film overnight with a BioMax (Kodak, Rochester, USA) MS screen at −70 °C.

Expression of *DPP8* in human lymphocytes and cell lines

RNA (1 μg) was reverse-transcribed using the Superscript II enzyme kit (Gibco–BRL) as described previously [34]. PCR using DPP8-pr18 (CTGTGACGCCACTAATTATCTATG) as the forward primer and DPP8-pr26R (CCTAGAGAGGCTAGGGTATTCAAG) as the reverse primer was used to detect full-length *DPP8* mRNA. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control primer set was G3PDH for (ACCACAGTCCATGCCATCAC) and G3PDHrev (TCCACCACCCTGTTGCTGTA) to give a 470-bp product.

cDNA (diluted 1 : 4; 1 μg) was amplified in a 25-μL PCR mixture which contained: 0.2 mM dNTPs, 0.125 unit Amplitaq Gold enzyme (PerkinElmer), 1 × buffer II (Perkin–Elmer), 1.5 mM $MgCl_2$ and 100 ng·mL^{-1} each primer. The 35-cycle PCR was performed as follows: denaturation at 94 °C for 1 min, primer annealing at 55 °C for 30 s, and an extension step at 72 °C for 1 min. The amplified products were analyzed by electrophoresis of 15 μL PCR mixture on a 3 : 1 Nusieve gel (FMC Bioproducts, Rockville, MD, USA) plus 0.5 μg·mL^{-1} ethidium bromide in Tris/acetate/EDTA buffer (0.04 M Tris/acetate, 0.001 M EDTA, pH 8.0).

Transfection, Western blot, immunocytochemistry, cytochemistry and flow cytometry

Monkey kidney fibroblast (COS-7) cells (American Type Culture Collection, CRL-1651) were grown and transfected as described previously [3]. For making stable cell lines, Geneticin (G418; Life Technologies) was added to the medium, beginning 24 h after transfection. COS cell extracts were prepared by sonication followed by differential centrifugation and neither boiled nor reduced before SDS/PAGE (10% gel) and transfer to nitrocellulose, as described previously [1,20]. The presence of DPP8 fused with the V5 epitope was detected using an anti-V5 mAb (Invitrogen). COS cell monolayers were fixed in cold ethanol before staining with anti-V5 mAb [3,9,20]. Some monolayers were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 [35], then double-stained with wheat germ agglutinin to label Golgi apparatus and with goat anti-mouse IgG to label DPP8, conjugated to Alexa Fluor 488 and Alexa Fluor 594, respectively (Molecular Probes, Eugene, OR, USA). Flow cytometry and confocal scanning microscopy using a Leica TCS-NT confocal microscope have been described previously [3,20].

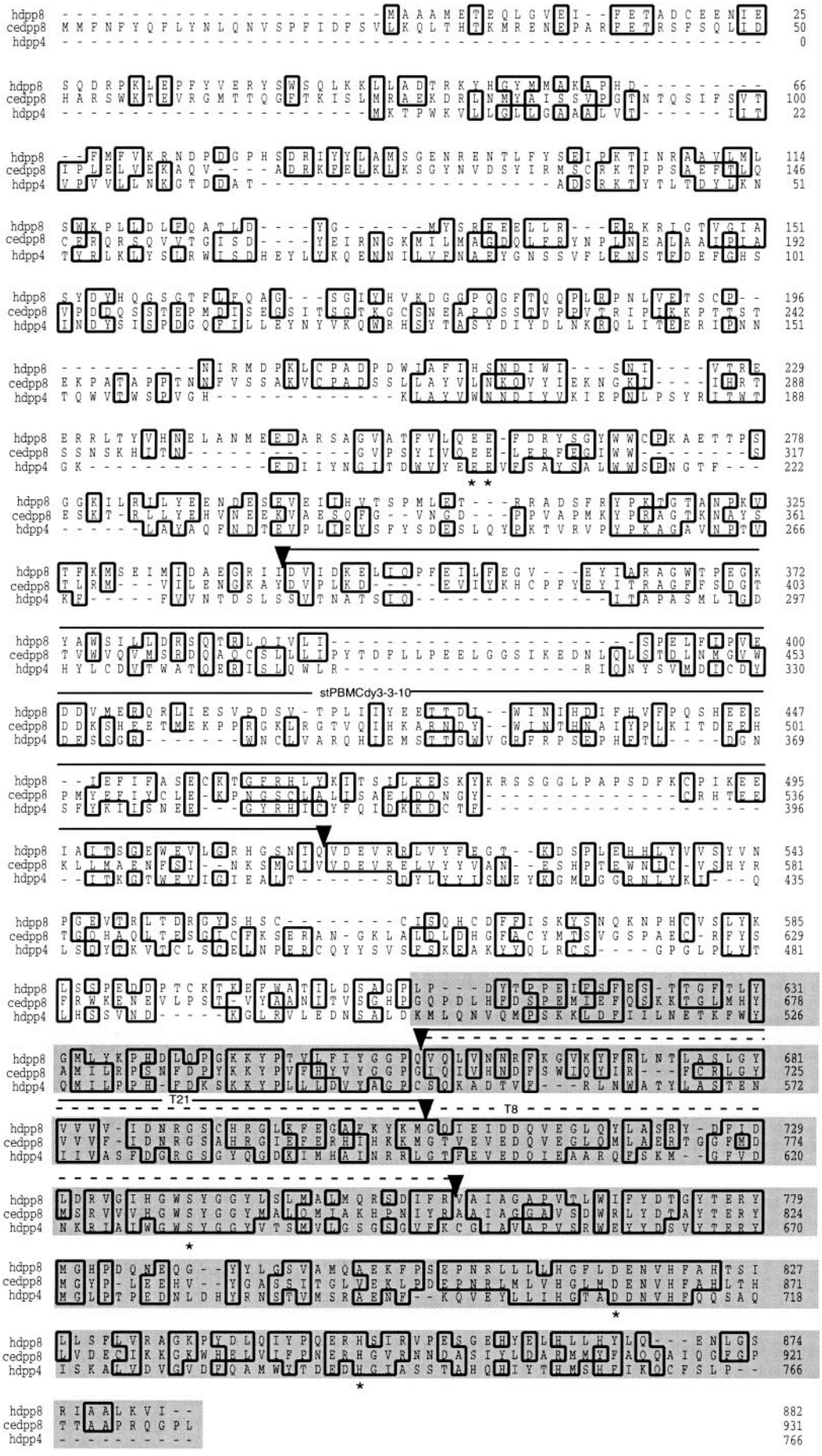
Purification of recombinant DPP8/V5/His and DPPIV/V5/His

Cells (1×10^7) expressing each protein were sonicated in native buffer (50 mM sodium phosphate, 300 mM NaCl), then treated with 700 U DNAse for 20 min at room temperature. DPPIV is expressed at the cell surface, so 1% Triton X-100 was used to solubilize DPPIV/V5/His. Insoluble material was removed by centrifugation. The supernatant was incubated with 1 mL Talon® Metal Affinity Resin (Clontech) following the manufacturer's instructions for a batch/gravity flow procedure. The resin was washed with 50 mM sodium phosphate, containing 300 mM NaCl and 5 mM imidazole, and proteins were eluted using the same buffer containing 150 mM imidazole. Enzyme activity was used to monitor eluted fractions.

Enzyme assays

Enzyme assays were performed as described previously [19]. Either clarified cell extract from 1×10^4 sonicated COS-7 cells or purified protein derived from 1×10^5 cells was incubated with substrate in 70 μL phosphate buffer, pH 7.4, for 30 min at 37 °C, except where otherwise indicated. The specific DPPIV substrates Gly-Pro-toluenesulfonate, H-Gly-Pro-*p*-nitroanilide (NA)/HCl (Sigma, St Louis, MO, USA) and Gly-Pro-7-amino-4-trifluromethylcoumarin (Calbiochem, San Diego, CA, USA) were tested. Other substrates tested were H-Ala-Pro-pNA/HCl, H-Arg-Pro-pNA acetate salt, H-Lys-Ala-pNA.2HCl, H-Asp-Pro-pNA, H-Ala-Ala-pNA/HCl, H-Ala-Ala-Pro-pNA/HCl, H-Ala-Ala-Phe-pNA, succinyl-Ala-Pro-pNA, H-Ala-Phe-Pro-pNA and Z-Ala-Pro-p-NA from Bachem AG (Bubendorf, Switzerland). H-Ala-Pro-4-methoxyβNA/HCl, Z-Lys-Pro-4-methoxyβNA formate salt, H-Lys-Pro-4-methoxy

Fig. 1. Alignment of the deduced amino-acid sequence of DPP8 with the *C. elegans* homolog of DPP8 and human DPPIV. Amino-acid residues are numbered in the right margin. Amino-acid residues identical in all three proteins are boxed. Asterisks mark the putative catalytic triad residues and the two glutamates of the β-propeller domain essential for DPPIV enzyme activity [19]. The gray shading denotes the α/β-hydrolase domain of these proteins. Filled triangles joined by lines indicate starts and ends of alternatively spliced transcripts, stPBMCdy3-3-10 (solid lines), T8 (dashed lines) and T21 (solid lines). This alignment was constructed using the PILEUP program in GCG.


hdpp8
cedpp8
hdpp4
stPBMCdy3-3-10
T21
T8

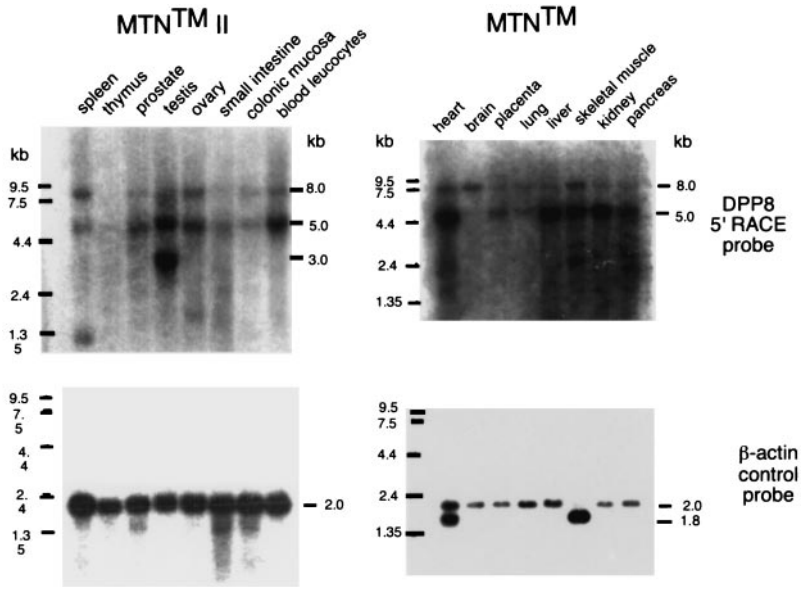


Fig. 2. Northern-blot analysis of *DPP8* expression. Human multiple-tissue Northern (MTN) blots (Clontech) containing 2 μg per lane of poly(A)-rich RNA were hybridized with a ^{32}P-labeled *DPP8* probe at 68 °C and washed at high stringency. Autoradiographic exposure was overnight at −70 °C with a BioMax MS screen. Size markers are indicated in kb on the left of each autoradiogram.

βNA/HCl, Z-Ala-Pro-4-methoxyβNA, H-Gly-Pro-βNA and H-His-Ser-4-methoxyβNA acetate salt (Bachem AG) were tested for their ability to stain unfixed transfected cells.

All inhibitors were incubated with each purified enzyme in phosphate buffer, pH 7.4, for 15 min before the addition of substrate. After the addition of 1 mM H-Ala-Pro-pNA substrate for purified DPP8 and 1 mM H-Gly-Pro-pNA substrate for purified DPPIV, samples were incubated for 60 min at 37 °C. All enzyme assays were performed in triplicate.

RESULTS

Molecular cloning and sequence analysis of *DPP8* cDNA

The EST AA417787, derived from human tonsil, contained an insert 795 bp in length, encompassing 527 bp of coding sequence, a TAA stop codon and 258 bp of 3′ noncoding sequence. The 178 amino acids encoded by this sequence had 36% amino-acid identity with and 58% amino-acid similarity to a C-terminal portion of human DPPIV. A BLASTp search into protein databases revealed that the EST AA417787 had 46% amino-acid identity with and 70% amino-acid similarity to an uncharacterized *Caenorhabditis elegans* protein which we called the *C. elegans* homolog of DPP8 (accession number g2804453).

The Master RNA blot revealed that *DPP8* had ubiquitous mRNA expression, with the most intense hybridization signals in testis and placenta (data not shown). Only two of 23 clones obtained by screening a placental cDNA library contained 5′ sequence additional to that of the *DPP8* EST. These cDNA clones were designated T8 (accession number AF221636) and T21(accession number AF221635), and were 1669 bp and 1197 bp, respectively. In addition, comparison of these sequences with the *DPP8* EST revealed that the T8 cDNA lacked a 153-bp (51-amino-acid) region that was present in both the T21 cDNA and the *DPP8* EST. Significantly, this 51-amino-acid region includes the catalytic serine (Fig. 1).

The 5′ RACE technique was applied to both stPBMC RNA and placental RNA to obtain the 5′ end of the *DPP8* gene. The 5′ RACE product obtained from stPBMC RNA was 0.2 kb larger than that from placental RNA but otherwise identical. The first methionine within a Kozak sequence was found 214 bp from the 5′ end of the stPBMC RACE product. To confirm the identity of the 5′ RACE product as the 5′ of *DPP8*, RT-PCR was carried out on a region spanning the junction between the 5′ RACE product and the T8 cDNA library clone. The RT-PCR on stPBMC RNA produced two clones, stPBMCdy3-2-1 and stPBMCdy3-3-10. Compared with T8 and T21, both clones had an additional insert region of 144 bp (48 amino acids) immediately adjacent to the splice site of T8. Sequence homology analysis of this additional insert region found an homologous region in both DPPIV and the *C. elegans* homolog of DPP8. This indicated that the library clones T8 and T21 represented splice variants of *DPP8*. Furthermore, the smaller clone stPBMCdy3-3-10 (accession number AF221637) was found to represent a third splice variant of *DPP8* as it contained a 519-bp deletion at the 5′ end which would result in a deletion of 175 amino acids.

A full-length *DPP8* clone (accession number AF221634) was constructed using the larger 5′ RACE product, stPBMCdy3-2-1, and clone T21. This generated a putative *DPP8* cDNA of 3.1 kb (including 5′ and 3′ UTRs) with an ORF of 882 amino acids. This 882-residue putative *DPP8* protein contained no N-linked or O-linked glycosylation sites, and Kyte–Doolittle hydrophobicity analysis showed no potential transmembrane domain, unlike DPPIV, FAP and DPP6.

Human DPP8 protein is homologous with both human DPPIV (51% amino-acid similarity, 27% amino-acid identity) and human PEP (46% amino-acid similarity, 21% amino-acid identity). Significant homology (55% similarity, 32% identity) was observed between human DPP8 and the *C. elegans* homolog of DPP8 (Fig. 1). A BLASTn search into GenBank revealed that the *DPP8* gene had high homology with two overlapping cosmids, accession numbers AC005594 and AC005783. These cosmids encode a region of the human chromosome at 19p13.3. The gene located in this region is 39.5 kb in size, contains 19 exons, and encodes a protein of ≈913 amino acids. The hypothetical protein encoded by the two cosmids shared the greatest homology (76% similarity, 58% identity) observed with *DPP8* and so was named DPP9. The gene structure of *DPP9* was elucidated (data not shown).

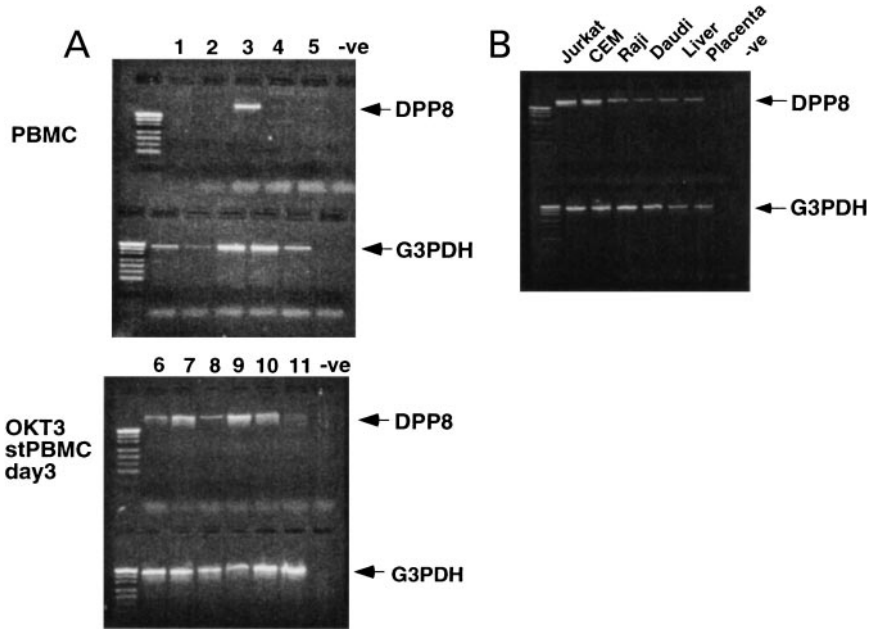


Fig. 3. RT-PCR analysis of *DPP8* expression. PCR amplifications with primers specific for either a portion of human *DPP8* that contained no alternative splicing, Val416 to Gly679 (top of each gel) or glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) (bottom of each gel). (A) Top gel, lanes 1–5 contain PCR products from unstimulated PBMC cDNA from five subjects. Bottom gel, lanes 6–11 contain PCR products from OKT3-stimulated PBMC cDNA from six subjects. (B) PCR products are from cDNA from lymphocytic cell lines, liver or placenta as indicated. Negative control amplifications contained reaction mix, enzyme and no cDNA template. Each PCR was performed for 35 cycles. The PCR products were electrophoresed on agarose gels and stained with ethidium bromide. The left lane of each gel contains PUC19 digested with *Hae*III as size markers.

The *DPP8* gene was predicted to have a structure similar to that of *DPP9* because the acceptor/donor sites of *DPP8* splice variants aligned with *DPP9* exon/intron boundaries.

Chromosomal localization of *DPP8* gene

Two probes were used for fluorescence *in situ* hybridization analysis, the *DPP8* EST and the T8 clone from the placental library. Seventeen metaphases from the first normal male were examined for fluorescent signal. All of these metaphases showed signal on one or both chromatids of 15 at band q22 (data not shown). There were a total of two non-specific background dots observed in these metaphases. A similar result was obtained from the hybridization of the probe to 15 metaphases from the second normal male. Subsequent to this experiment, we found that the *DPP8* EST had been clustered as Hs.72165 on the NCBI Unigene Database (http://www.ncbi.nlm.nih.gov/UniGene) and had been mapped on Gene Map 98 to stSG30183, Chr.15, D15S125-D15S216. This confirmed our localization to 15q22.

Tissue distribution of *DPP8* determined by Master RNA blot and Northern blot

The Master RNA blot produced similar hybridization patterns with *DPP8* and *DPPIV* probes (data not shown), indicating ubiquitous expression of both mRNAs in all human adult and fetal tissues. The most intense hybridization signals using the *DPP8* probe were in testis and placenta whereas the most intense hybridization signals using the *DPPIV* probe were in salivary gland, prostate and placenta. There were no signals over any of the negative controls on these blots.

Northern-blot analysis of *DPP8* used two probes; the 484-bp PCR product probe from the 3′ end of the cDNA (data not shown) gave results identical with those of an stPBMC RACE probe from the 5′ end of the cDNA (Fig. 2). Both probes indicated the presence of transcripts in all tissues examined. A major transcript of 5.0 kb and a minor transcript of 8.0 kb were present at either moderate or high levels in most tissues examined. A transcript $\approx$3.0 kb in size was detected only in testis. In the course of cloning and during RT-PCR gene expression analysis of *DPP8*, various amplified products were observed, isolated, and sequenced. These products were identified as splice variants of *DPP8*. The mRNA transcripts resulting from alternative splicing of *DPP8* would only be 148–519 bp smaller than the full-length transcript, and this difference was probably undetectable on the Northern blots. The multiple-tissue Northern blot was probed with radiolabeled human β-actin probe to verify equivalent loading of mRNA in each lane; a 2.0-kb transcript was common to all tissues (Fig. 2).

Analysis of *DPP8* gene expression by RT-PCR

DPPIV is expressed by most lymphocytes and lymphocytic cell lines but upregulated on activated lymphocytes [7,9–11]. The various splice variants of *DPP8* might not encode functional protein, so the PCR was designed to detect only mRNA that contained full-length sequence (Fig. 1). At 35 cycles, amplification product of the expected size (783 bp) was

Table 1. K_m and V_{max} values for DPP8 and DPPIV.

	K_m (mM)		V_{max} ($\Delta A \cdot min^{-1} \times 1000$)	
	DPPIV	DPP8	DPPIV	DPP8
H-Ala-Pro-pNA	0.374 ± 0.134	0.991 ± 0.171	9.6 ± 1.0	12.4 ± 0.9
H-Gly-Pro-pNA	0.347 ± 0.088	0.467 ± 0.064	7.2 ± 0.49	3.5 ± 0.14

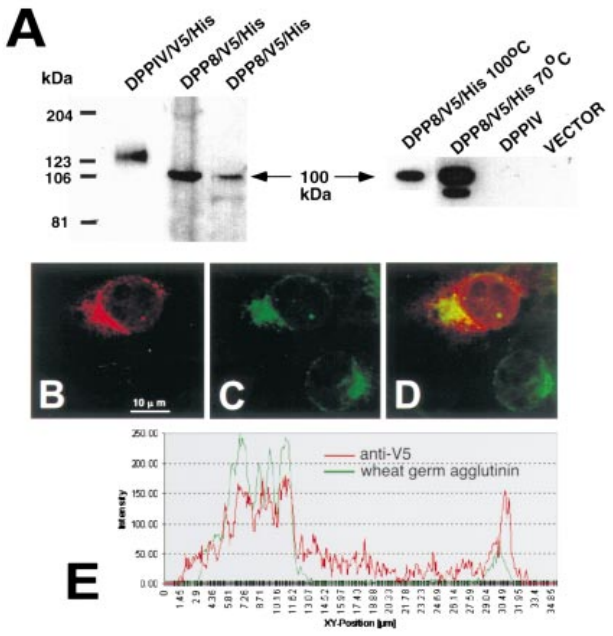


Fig. 4. Recombinant DPP8 is a 100-kDa cytoplasmic protein. Western blots of lysates (A) and permeabilized paraformaldehyde-fixed monolayers (B–E) of stably transfected COS-7 cell lines were probed with anti-V5 mAb. (A) Left hand panel, non-boiled, V5-tagged DPPIV was seen at 140 kDa, the expected mobility of DPPIV dimer [3,19,47]. DPP8 protein at 100 kDa was seen in two different cell preparations of DPP8/V5/His-transfected cells. Right hand panel, boiling (100 °C) did not alter the electrophoretic mobility of DPP8. DPP8 bands of greater mobilities were sometimes seen (third and fifth lanes) and were probably breakdown products of intact DPP8. The anti-V5 mAb is specific for tagged proteins and did not bind DPPIV-transfected or vector-transfected stable cell lines (sixth and seventh lanes). Fluorescence confocal microscopy showed that DPP8 (B, red channel) was localized to the cytoplasm including structures bound by wheat germ agglutinin (C, green channel), shown in overlay mode (D) and in a fluorescence intensity profile of the double-positive cell (E).

readily observed in OKT3-stimulated PBMCs (six of six subjects; Fig. 3B) but not in unstimulated PBMCs from most subjects (four of five, Fig. 3A), suggesting that more *DPP8* mRNA is expressed in activated T cells than in unstimulated PBMCs. Similar RT-PCR data were obtained from PBMCs stimulated with phytohaemagglutinin (not shown). In addition, *DPP8* mRNA was expressed in all B and T cell lines examined and in both liver and placenta (Fig. 3B).

Expression of DPP8 in COS-7 cells

To assess the function of DPP8 protein, the full-length *DPP8* cDNA of 3.1 kb was cloned into the *Xba*I site of the pcDNA3.1A/V5/His expression vector to produce two constructs, one of which, pcDNA3.1-DPP8/V5/His, expressed the V5 epitope and His tag fused to the C-terminus of DPP8. A 100-kDa monomer, consistent with the molecular mass predicted from the amino-acid sequence, was detected in Western blots of stably transfected COS-7 cells (Fig. 4A) but not of cell culture supernatants (data not shown). DPP8/V5/His was not detected on the cell surface by flow cytometry (data not shown). In addition, DPP8 was detected in the cytoplasmic compartment but not on the surface of either ethanol-fixed or paraformaldehyde-fixed DPP8/V5/His-expressing COS-7 cells, by both immunofluorescence confocal microscopy (Fig. 4B–E) and immunoperoxidase light microscopy (data not shown).

DPP8 is a dipeptidyl peptidase

Sequence homology between DPPIV and DPP8 suggested functional similarities, so cell lysates of DPP8-transfected cells were examined for proline-specific peptidase activity. DPPIV expressed in COS-7 cells with or without the V5/His tag were positive controls, and negative controls included vector-only transfected COS-7 cells. Extracts of DPP8-transfected COS-7 cells hydrolyzed Ala-Pro-pNA and Arg-Pro-pNA but not Gly-Pro-pNA, Gly-Arg-pNA, Gly-Pro-toluenesulfonate or Gly-Pro-7-amino-4-trifluoromethylcoumarin above the levels exhibited by untransfected COS-7 cells (data not shown). The pH optimum of DPP8 enzyme activity was 7.4 (Fig. 5A), similar to the pH 7.8 optimum of DPPIV enzyme activity [36,37]. DPP8 exhibited little activity below pH 6.3, suggesting that it is not an enzyme of the lysozome/endosome compartment. Of all the substrates tested on cell monolayers, only Ala-Pro-4MβNA/HCl stained DPP8-transfected COS cells (data not shown).

Both purified recombinant DPP8/V5/His and purified recombinant DPPIV/V5/His hydrolyzed H-Ala-Pro-pNA, H-Gly-Pro-pNA and H-Arg-Pro-pNA (Fig. 5B, Table 1). Transfection with DPP8 possibly causes increased dipeptidase, tripeptidase and endopeptidase activities, similar to an effect of DPPIV

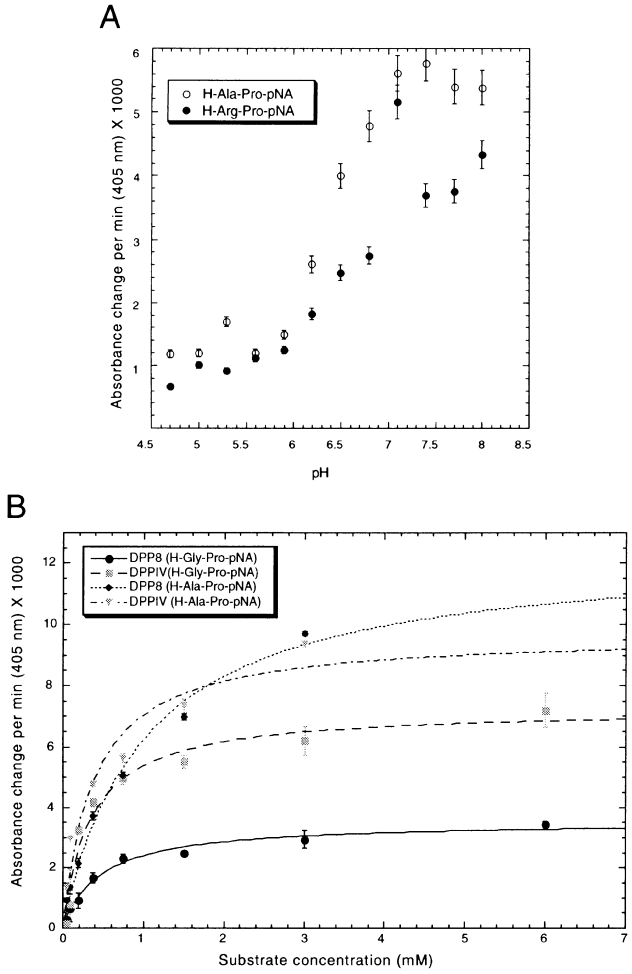


Fig. 5. DPP8 enzyme activity. (A) pH-dependence of DPP8 enzyme activity. (B) DPP8 and DPPIV enzyme kinetics. Means ± SD of absorbance change per minute, multiplied by 1000, are shown. Curve fitting assumed Michaelis–Menten kinetics.

Table 2. Inhibition of the peptidase activity of DPP8 in comparison with DPPIV. Common proteinase inhibitors of various enzyme types were incubated with the purified peptidases before assay with the substrates H-Ala-Pro-pNA on DPP8 or H-Gly-Pro-pNA on DPPIV. AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride.

Type of inhibitor	Concentration	Residual activity (% of control)	
		DPP8	DPPIV
None		100	100
Serine proteinase			
AEBSF	4 mM	40	52
Aprotinin	4 $\mu g \cdot mL^{-1}$	47	81
Benzamidine/HCl	10 mM	82	89
Bioactive and other peptides			
Gly-Gly-Gly	10 mM	99	106
Ala-Pro-Gly	10 mM	51	67
H-Lys-Pro-OH HCl salt	4 mM	63	45
Zinc sulphate	2 mM	25	0
Metalloproteinase			
EDTA	2 mM	115	99
Aspartate(acidic) proteinase			
Pepstatin	2 $\mu g \cdot mL^{-1}$	107	110
Leupeptin	0.1 mM	93	104
Cysteine(thiol) proteinase			
Iodoacetamide	2 mM	100	115
Dithiothreitol	2 mM	108	109

transfection of melanoma cells [38]. Indeed, our results showed that DPP8 transfected COS-7 cells, but not purified recombinant DPP8, exhibited tripeptidyl peptidase activity using the substrate H-Ala-Ala-Pro-pNA and endopeptidase activity using the substrate *Z*-Ala-Pro-pNA (data not shown). This was investigated further, and neither the tripeptidyl peptidase substrates H-Ala-Ala-Phe-pNA or H-Ala-Phe-Pro-pNA [39] nor the endopeptidase substrates Z-Ala-Pro-pNA or succinyl-Ala-Pro-pNA were cleaved by purified DPP8. Our data clearly demonstrate that DPP8 is a dipeptidyl peptidase and lacks tripeptidyl peptidase or endopeptidase activities.

The nature of the catalytic mechanism of DPP8 was further investigated using various inhibitors. DPP8 enzyme activity was significantly inhibited by serine proteinase inhibitors and was insensitive to inhibitors of metalloproteinases, aspartyl proteinases and cysteine proteinases (Table 2). DPP8 enzyme activity was significantly inhibited by zinc, which completely inhibits DPPIV enzyme activity [40]. The peptides Ala-Pro-Gly and Lys-Pro mimic DPP8 substrates and probably competitively inhibited DPP8.

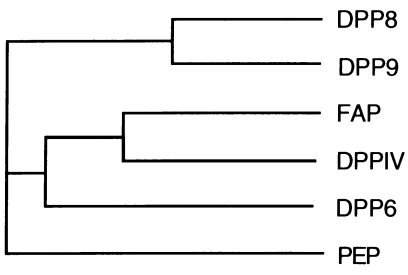


Fig. 6. Dendrogram of DPP8, DPP9, DPPIV, FAP, DPP6 and PEP. The amino-acid sequences of each protein were aligned using the GCG PILEUP program. The dendrogram was constructed using the GCG CLUSTREE program with standard parameters.

DISCUSSION

This paper reports the cloning, recombinant expression, biochemistry and tissue expression of a novel human DPPIV-related postproline peptidase that we have named DPP8. DPP8 exhibited dipeptidyl aminopeptidase but not tripeptidyl peptidase or endopeptidase activity. Like DPPIV, DPP8 was found to exhibit significant mRNA expression in activated T cells. Clear indications that DPP8 is a monomeric, nonglycosylated, soluble, cytoplasmic protein, which are characteristics of PEP but not of DPPIV, FAP or DPP6, were provided by our sequence and localization data. DPP8 enzyme activity had a neutral pH optimum, suggesting that it is not active in the acidic lysozome/endosome compartment.

By homology with *DPPIV*, *DPP8* is a member of the *DPPIV*-like gene family, a member of the prolyl oligopeptidase family S9b, and a member of the enzyme clan SC. The residues in DPP8 that potentially form the charge-relay system are Ser739, Asp817 and His849 (Fig. 1). The dipeptidyl peptidase activity of DPP8 and the absence of detectable tripeptidyl peptidase or endopeptidase activities by purified DPP8 further support its placement in the S9b family. Furthermore, the DPP8 substrate specificity was indistinguishable from that of the structurally related peptidases DPPIV and FAP.

The role of DPPIV in human lymphocytes has been studied in detail using enzyme inhibitors [11,12,41–44]. DPPIV-specific inhibitors suppress both DNA synthesis and cytokine production *in vitro* [10,11,42]. In addition, DPPIV-specific inhibitors decrease phorbol myristate acetate-induced tyrosine phosphorylation in human lymphocytes, further suggesting a role for DPPIV enzyme activity in lymphocyte activation [44]. *In vivo*, inhibitors of DPPIV suppress arthritis [14] and prolong cardiac allograft survival in animal models [45]. The ability of DPP8 to cleave DPPIV substrates indicates that DPPIV inhibitors may also inhibit DPP8 and that inhibitor studies may require further interpretation. Indeed, DPP8 may be responsible for some of the physiological functions that have been assigned to DPPIV.

FAP and DPPIV are integral membrane glycoproteins and require dimerization for catalytic activity [20,21,46,47]. In contrast, DPP8 and PEP are nonglycosylated cytosolic proteins that are catalytically active as monomers [48] and cleave Pro-Xaa bonds [36,49]. However, the substrate specificity of PEP is distinct from DPP8. PEP is an endopeptidase that does not cleave if a free α-amine lies N-terminal to the proline (e.g. it does not cleave H-Ala-Pro). Recently we have proposed that the tertiary structure of DPPIV is similar to that of PEP in having a seven-blade β-propeller domain and an α/β-hydrolase domain [3,5,19]. The significant sequence identity between DPP8 and DPPIV indicates that the tertiary structures of DPP8 and DPPIV are similar. However, DPP8 contains 110 amino acids more than DPPIV, so it could have an additional element of tertiary structure such as an eighth propeller blade.

Human DPP8 exhibits 76% amino-acid similarity and 58% amino-acid identity with a hypothetical human protein that we have named DPP9. The gene structures of human *DPP9* and the *C. elegans* homolog of *DPP8* were seen to be similar. Moreover, the alignment of human *DPP8* splice variants with the exon/intron boundaries of these two genes suggested that the human *DPP8* gene has a similar gene structure.

Interestingly, the serine-recognition site, GWSWG, is found in a single exon in human *DPP9*, the *C. elegans* homolog of *DPP8* and the *C. elegans* homolog of *PEP*. This differs from the arrangement in the *C. elegans* and human *DPPIV* genes [50] and human and mouse *FAP* genes [22], where the serine-recognition site spans two exons. The gene for mouse *PEP* has recently been cloned, and, at 92 kb [51], is similar in size to the *DPPIV* and *FAP* genes. However, whereas the *DPPIV* and *FAP* genes contain 26 exons, mouse *PEP* has only 15 exons, a number closer to that of the 19 exons of the *DPP9* gene. Like *DPP9*, the serine-recognition site of mouse *PEP* is in one exon. These data suggest that the gene arrangement in *PEP*, *DPP8* and *DPP9* is representative of the ancestral prolyl oligopeptidase (S9) family gene, and the arrangement in *DPPIV* and *FAP* has resulted from divergent evolution from this ancestral gene. The likely ancestral relationships between *DPP8* and *DPP9*, *DPPIV*, *FAP* and *DPP6* and *PEP* are illustrated (Fig. 6).

The ancestral relationships between *DPP8*, *DPP9*, *DPPIV* and *FAP* are also reflected in their chromosomal localization. Whereas *DPPIV* and *FAP* have both been localized to the long arm of chromosome 2, 2q24.3 [50] and 2q23 [52], respectively, *DPP8* was localized to 15q22 and *DPP9* has been localized to 19p13.3. The related genes *DPP6* and *PEP* have been localized to chromosome 7 [18] and 6q22, respectively [53].

Two human disease loci have been mapped to 15q22. These loci are an autosomal recessive deafness locus [54] and a form of Bardet–Biedl syndrome, type 4 [55]. Two of the clinical manifestations of Bardet–Biedl syndrome are obesity and diabetes. Attractin [26] and DPPIV have roles in obesity [56] and diabetes [6,57,58], respectively, and, as their substrate specificities overlap with that of DPP8, it is possible that DPP8 may be involved in Bardet–Biedl syndrome.

DPPIV is expressed on the surface of T cells and is a costimulatory molecule called CD26 [5]. CD26-negative cell lines have residual DPPIV enzyme activity and PBMCs have non-DPPIV-derived activity against Ala-Pro substrates [59], indicating the existence of other peptidase(s) with DPPIV-like activity. DPPIV-β exhibits a peptidase activity similar to DPPIV but is a 70–80-kDa cell-surface glycoprotein [25] and is therefore distinct from DPP8.

The biological significance of the three splice variants of *DPP8* that we discovered is not known. None of these splice variants result in a frame shift or premature protein termination (Fig. 1). Two of the splice variants contain all the predicted catalytic triad residues and thus potentially produce proteins with peptidase activity. Alternative-splice forms of *FAP* mRNA have also been observed [17,60]. It is possible that expression of splice variants may be used to regulate the levels of active protein. *DPP8* Northern blots revealed a number of differently sized transcripts. The predicted sizes of splice variants of *DPP8* ranged from 2.6 to 3.1 kb, whereas the large transcripts seen in most tissues examined in the Northern blots were 8.5 kb and 5.0 kb, respectively. Similarly, two other members of the *DPPIV*-like gene family, *DPPIV* and *DPP6*, exhibit mRNA transcripts in Northern blots that are much larger than the cDNA size [50,61]. We propose that the major transcripts for *DPP8* mRNA and its splice variants lie within the 5-kb band whereas the 8.5-kb transcript(s) may contain additional 5′ and 3′ untranslated sequences. *DPP8* appears to be like *DPPIV* in having a ubiquitous mRNA expression pattern by Northern-blot analysis while being upregulated in activated T cells. The similarities between DPP8 and DPPIV suggest that DPP8 may, like DPPIV, play a role in T cell costimulation and proliferation. The development of DPP8-specific antibodies or inhibitors will facilitate work in this area.

In summary, we have identified and characterized a novel human dipeptidyl aminopeptidase, DPP8, with structural and functional similarities to DPPIV and FAP. With many diverse biological roles suggested for DPPIV, particularly in the immune system, and the roles of FAP in tumor growth and liver disease, it will be interesting to investigate the roles of this new member of the *DPPIV*-like gene family in these systems. Further work in understanding this novel protein and the elucidation of inhibitors and physiological substrates will help to identify the specific functions of individual members of this gene family.

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