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Two highly conserved glutamic acid residues in the predicted β propeller domain of dipeptidyl peptidase IV are required for its enzyme activity

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Abstract Dipeptidyl peptidase IV (DPP IV) is a member of the prolyl oligopeptidase family and modifies the biological activities of certain chemokines and neuropeptides by cleaving their N-terminal dipeptides. This paper reports the identification and possible significance of a novel conserved sequence motif Asp-Trp-(Val/Ile/Leu)-Tyr-Glu-Glu-Glu (DW(V/I/L)YEEE) in the predicted β propeller domain of the DPP IV-like gene family. Single amino acid point mutations in this motif identified two glutamates, at positions 205 and 206, as essential for the enzyme activity of human DPP IV. This observation suggests a novel role in proteolysis for residues of DPP IV distant from the Ser-Asp-His catalytic triad.

Key words: Dipeptidyl peptidase IV; Prolyl oligopeptidase; β Propeller; α/β Hydrolase fold

1. Introduction

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) is a ubiquitous type II multifunctional cell surface protein with a dipeptidyl peptidase enzyme activity. DPP IV enzyme activity specifically cleaves dipeptides from the N-terminus of peptides with a Pro, Hyp or Ala in the penultimate position. Proline, due to its cyclic nature, is a unique amino acid that imposes structural restrictions on peptides and proteins in which it is found. Consequently, proline appears to be near the N-terminus of many biologically active proteins and plays a key physiological role in the protection of these molecules against enzymatic cleavage by aminopeptidases [1]. DPP IV substrates include certain chemokines, growth factors (e.g. growth hormone, growth hormone-releasing factor, glucagon-like peptides 1 and 2) and neuro- and vasoactive peptides including neuropeptide Y, peptide YY and substance P. The N-terminal truncation of the chemokines RANTES (regulated on activation normal T-cell expressed and secreted), eotaxin, macrophage-derived chemokines and stromal cell-derived factor-1α has been found to reduce their chemotactic activity and impair mobilisation of intracellular Ca^{2+} through their respective chemokine receptors [2–5]. However, these DPP IV-truncated chemokines maintain their ability to inhibit HIV-1 infection via the various chemokine receptors.

DPP IV is a non-classical serine protease as its catalytic residues are arranged in the unique order Ser-Asp-His. This order is the reverse of that found in classical serine proteases such as the trypsin and subtilisin families [6]. The 200 C-terminal residues of DPP IV (containing the catalytic triad) have homology with several other non-classical serine hydrolases including the prolyl oligopeptidases and the acylaminoacyl peptidases. These enzymes have been classified as members of the prolyl oligopeptidase (POP) family [7,8]. While members of this family have different substrate specificities, they use similar structural features to form their catalytic domains. All members of the POP family are predicted to contain an α/β hydrolase fold [6,9,10]. The three dimensional structures of proline iminopeptidase from *Xamanthomas campestris* pv. *Citri* (EC 3.5.11.5) [11] and POP (EC 3.4.21.26) [12] have been solved. Both of these structures contain an α/β hydrolase fold. The structure of the N-terminal regions of DPP IV and other members of the POP family are unknown but could include an atypical β propeller domain of four-stranded β sheets as occurs in POP [12] (Abbott et al., 1999, submitted).

DPP IV homologues have been cloned from other mammals [13,14], yeast (*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) [15,16], the fungus *Aspergillus oryzae*, *Xenopus laevis* [17], *Flavobacterium meningosepticum* [18] and *Caenorhabditis elegans* [19]. Other mammalian members of the POP family that share striking sequence homology with DPP IV are the dipeptidyl aminopeptidase short and long forms (DPPX-s and DPPX-l) [20,21] and fibroblast activation protein (FAP) [22,23]. Due to two substitutions in the serine recognition site, DPPX has no enzyme activity [24], whereas FAP has both a dipeptidyl peptidase and gelatinase activity [25–27]. We suggest that these molecules form a distinct subclass of the POP family, a DPP IV-like gene family that includes FAP and DPPX.

Our characterisation of the human DPP IV gene structure [28] enabled us to use each exon as an independent unit to search the protein sequence databases. This analysis revealed that a region of human DPP IV exons 8 and 9 is highly conserved within members of the DPP IV-like gene family. We observed within this region a conserved sequence motif Asp-Trp-(Val/Ile/Leu)-Tyr-Glu-Glu-Glu (DW(V/I/L)YEEE). This motif is found in the N-terminal region of DPP IV, FAP and DPPX of all the above-mentioned species distant from the C-terminal region that contains the catalytic triad residues. We hypothesised that since this motif is highly conserved between species and within members of the DPP IV-like gene family, it is involved in the dipeptidyl peptidase activity of these proteins. The aim of this present study was

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Abbreviations: DPP IV, dipeptidyl peptidase IV; POP, prolyl oligopeptidase; DPP, dipeptidyl aminopeptidase; FAP, fibroblast activation protein; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; ADA-FITC, adenosine deaminase conjugated to FITC; PE, phycoerythrin; HRP, horseradish peroxidase

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to investigate the role of residues found in this novel sequence motif in the enzyme activity of DPP IV.

2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from Trace Biosciences (Sydney, Australia), culture flasks were from Nunc (Naperville, IL, USA). NuPage electrophoresis reagents were obtained from Novex (San Diego, CA, USA) and nitrocellulose from Schleicher and Schuell (Dassel, Germany). All other reagents including DPP IV substrates and adenosine deaminase (ADA) were from Sigma (St. Louis, MO, USA). Visualisation of immune complexes after Western blotting used chemiluminescence with Supersignal (Pierce, Rockford, IL, USA) and BIOMAX ML film (Kodak, Rochester, NY, USA).

The cDNA for wild-type DPP IV, ptzCD26.11, was a gift from Dr. Chikao Morimoto (Dana Farber Center, Boston, USA) [29]. The monoclonal antibody (mAb) against DPP IV, EF6/B10 [30] and the rabbit anti-ADA antiserum were gifts from Dr. Thilo Kähne and mAb 2A6 was a gift from Professor Sibrand Poppema [31,32].

2.2. Bioinformatics

Basic local alignment search tool (BLAST) programs [33–35] and all multiple sequence alignments were performed through the Australian National Genomic Information Service (ANGIS, Sydney, Australia). BLAST protein (BLASTP) searched non-redundant PDB, SwissProt, PIR and GenPept databases. PileUp (GCG Version 8, Genetics Computer Group, Madison, WI, USA) was used for multiple sequence alignments of the amino acids of DPP IV, FAP and DPPX proteins.

2.3. Preparation of DPP IV mutants

Full length DPP IV cDNA was excised with *Xba*I from ptzCD26.11 and ligated into the transient expression vector pEF-BOS [36]. Point mutations were performed in the pEF-BOS/DPP IV construct using a Quick Change Mutagenesis kit (Stratagene, La Jolla, CA, USA). Eight mutant plasmids were prepared using the appropriate oligonucleotides to direct mutagenesis as listed in Table 1. The mutagenic oligonucleotides were purchased from GeneWorks (Adelaide, Australia). After mutagenesis, plasmid DNA was prepared using either a RPM kit (BIO 101, Vista, CA, USA) or a QIAGEN kit (Hilden, Germany). Introduced point mutations were verified using automated sequencing with fluorescent dideoxy terminators (SUPAMAC, Sydney, Australia).

2.4. Transient expression of DPP IV mutants

Monkey kidney fibroblast, COS-7 cells (ATCC, CRL-1651), were grown in Dulbecco's MEM medium supplemented with 10% foetal calf serum and 2 mM glutamine. For flow cytometry and Western blot analysis, COS-7 cells were transfected in a subconfluent 75 cm^2 flask using 15 μg DNA and 48 μl Fugene-6 (Roche, Palo Alto, CA, USA) following the manufacturer's instructions.

2.5. Cytochemistry

COS-7 cells grown on chamber slides (Labtek II, Naperville) were fixed in cold ethanol prior to staining for DPP IV enzyme activity, binding to biotinylated ADA or binding to anti-DPP IV mAb EF6/B10 [27,37,38]. ADA (type VI, Sigma) was coupled to sulfosuccinimidyl-6-(biotinamido) hexanoate (biotinylated) or to fluorescein isothiocyanate (FITC) by standard methods [37].

2.6. DPP IV enzyme assay

DPP IV enzyme assays were performed on trypsin/EDTA-harvested COS-7 cells 72 h after transfection and used Gly-Pro-*p*-nitroanilide-*p*-toluene sulfonate salt as the substrate [39]. Transfected cells were suspended at 20 000 cells per well in 70 μl Hanks buffer (Trace Biosciences, Australia), pH 7.0, and incubated for 30 min at 19°C. Absorbances at 690 nm were subtracted from absorbances at 405 nm to increase the specificity of measurements. Analyses of Michaelis Menten kinetics used KaleidaGraph v. 3.08 (Synergy Software, Reading, PA, USA). Assays were performed in triplicate on two transfections. Statistical analysis used Student's *t* test.

2.7. Flow cytometry

Suspensions of trypsin/EDTA-harvested COS-7 cells were stained using fluorescein-conjugated ADA (ADA-FITC) and anti-DPP IV mAb EF6/B10 followed by goat anti-mouse Ig phycoerythrin (PE) (Caltag, Burlingame, CA, USA) and analysed on a FACScan (Becton Dickinson) [38]. Forward scatter and right angle scatter were used to establish gate 1, which excluded non-viable cells and aggregates of cells. Isotype-matched controls were used in each experiment and positive controls were included for adjustment of compensation.

2.8. Western Blotting

COS-7 cell membranes were prepared and Western blotting was performed as described previously [27] except that non-boiled samples were run on either 8–12% NuPAGE (Novex) or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Gradipore, N.S.W., Australia) gels and transferred to nitrocellulose as per the manufacturer's instructions. Soluble recombinant human DPP IV used as a marker was purified from a stable DPP IV expressing cell line [40]. DPP IV was detected using mAb 2A6 and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig, catalogue number P0260 (Dako, Santa Barbara, CA, USA). The ADA overlay blot used 40 μg/ml ADA type VIII, rabbit anti-ADA antiserum (1/1500) and donkey anti-rabbit HRP (1/1500, Amersham, Buckinghamshire, UK). The ADA appeared as a single band at 43 kDa upon both SDS-PAGE and Western blot and the ADA antiserum bound only the expected 43 kDa band on Western blots, indicating purity of the ADA preparation and mono-specificity of the antiserum (data not shown).

3. Results

3.1. BLASTP searches and multiple sequence alignments revealed a DPP IV-like gene family with a novel conserved sequence motif Asp-Trp-(Val/Ile/Leu)-Tyr-Glu-Glu-Glu (DW(V/I/L)YEEE)

Sequence comparisons can help in understanding how sequences and hence proteins have evolved. Protein database searches identified a distinct sub-class of the POP family, a DPP IV-like gene family that included mammalian, bacterial, yeast, fungal, *X. laevis* and *C. elegans* DPP IV, FAP and DPPX. The characterisation of the exon structure of human DPP IV [28] enabled BLAST searches of the combined protein databases with the amino acids of each exon as discrete units of input. The major purpose of these searches was to

Table 1
The amino acid and base substitutions of each point mutation

Name of mutation	Type of substitution	Base pair changes
pEF-BOS/DPPIVTrp201Tyr	NP ⇒ P	Trp (TGG) ⇒ Tyr (ATG)
pEF-BOS/DPPIVVal202Arg	NP ⇒ B	Val (GTT) ⇒ Arg (CGT)
pEF-BOS/DPPIVVal202Ala	NP ⇒ NP	Val (GTT) ⇒ Ala (GCT)
pEF-BOS/DPPIVTyr203Val	P ⇒ NP	Tyr (TAT) ⇒ Val (GTT)
pEF-BOS/DPPIVGlu204Lys	A ⇒ B	Glu (GAA) ⇒ Lys (AAA)
pEF-BOS/DPPIVGlu204Gln	A ⇒ P	Glu (GAA) ⇒ Gln (CAA)
pEF-BOS/DPPIVGlu205Lys	A ⇒ B	Glu (GAG) ⇒ Lys (AAG)
pEF-BOS/DPPIVGlu206Leu	A ⇒ NP	Glu(GAA) ⇒ Leu (CTA)

NP = non-polar, P = uncharged polar, A = acidic, B = basic.

Fig. 1. Multiple sequence alignment of exons 8 and 9 of DPP IV with other members of the DPP IV-like gene family reveals a conserved motif Asp^{200}-Trp^{201}-Val^{202}-Tyr^{203}-Glu^{204}-Glu^{205}-Glu^{206} (DWVYEEE). GCG PileUp of DPP IV exons 8 and 9 (residues 165–258) with mammalian, *X. laevis*, bacterial, yeast, fungal and *C. elegans* DPP IV and DPP IV-like molecules FAP and DPPX. The shading represents residues that are homologous in 80% or more of sequences. The conserved sequence motif is boxed. The GenPept accession numbers for these sequences are: hdpp4, human DPP IV (g180083); mdpp4, mouse DPP IV (g550375); rdpp4, rat DPP IV (g204464); xdpp4, *X. laevis* DPP IV (g1621279); cdpp799, *C. elegans* DPP IV (g4038512); hdppxs, human DPPX short form (DPPXs, g306708); rdppxs, rat DPPXs (g408716); bdppxs, bovine DPPXs (g408720); hfap, human FAP (g1888316); mfap, mouse FAP (g1743330); xfap, *X. laevis* FAP (g1314314); ydpab841, 841 amino acids yeast gene for DPP B (g3660); ydpab818, 818 amino acids DPP B (g500698); yeast DPP A (g347197); aodpp4, *A. oryzae* DPP IV (g2924305); fmdpp4, *F. meningosepticum* DPP IV (g577284); sc793, *S. pombe* 793 amino acids DPP (g3395554); sc743, *S. pombe* 743 amino acids DPP (g2330688).

identify small regions of homology between human DPP IV and other members of the DPP IV-like gene family. Each BLASTP search produced a list of accession numbers and names of sequences that satisfied the various thresholds of the program. The most striking result of these searches was that DPP IV exons 8 and 9 consistently achieved significant BLASTP scores with each member of the DPP IV-like gene family. Human DPP IV exons 8 and 9 had 29–52% amino acid identity and 45–71% amino acid similarity with similar regions in these proteins. DPP IV exons 20–26 contain the last 200 C-terminal residues including the catalytic residues and these were the only other exons to achieve these levels of homology. This result led us to predict that residues in DPP IV exons 8 and 9 are important for dipeptidyl peptidase activity in the DPP IV-like gene family. Analysis of sequence alignments (Fig. 1) led us to focus on a highly conserved motif Asp^{200}-Trp^{201}-Val^{202}-Tyr^{203}-Glu^{204}-Glu^{205}-Glu^{206} (DWVYEEE), which lies across the exon 8/exon 9 boundary in human DPP IV.

3.2. *Certain substitutions in the conserved motif Asp^{200}-Trp^{201}-Val^{202}-Tyr^{203}-Glu^{204}-Glu^{205}-Glu^{206} influence the enzyme activity of DPP IV*

Two point mutations, Glu-205-Lys and Glu-206-Leu, specifically ablated DPP IV enzyme activity, shown by enzyme cytochemistry (Table 2) and enzyme assays of live cell suspensions (Table 3 and Fig. 2). These two mutants showed no enzyme activity by whole-cell cytochemistry and little activity in enzyme assays of cell surface-expressed DPP IV. The flow cytometry and Western blot data (see below) indicated that similar quantities of mutant and wild-type DPP IV proteins were present in these assays, suggesting that the small V_{max} of these mutants compared to wild-type DPP IV was due to the

Table 2
DPP IV enzyme cytochemistry, mAb EF6/B10 immunocytochemistry and biotinylated ADA cytochemistry of transiently transfected COS-7 cells

Mutation	Enzyme stain	mAb binding	ADA binding
DPP IV wild-type	++	++	++
Trp-201-Tyr	++	++	++
Val-202-Arg	None	+	None
Val-202-Ala	++	++	++
Tyr-203-Val	++	++	++
Glu-204-Lys	+	++	++
Glu-204-Gln	+	++	++
Glu-205-Lys	None	++	++
Glu-206-Leu	None	++	++

Glu-205-Lys and Glu-206-Leu differed from wild-type only with respect to DPP IV enzyme cytochemistry. In contrast, Val-202-Arg poorly bound mAb and ADA binding was undetectable, suggesting a defective structure. Staining intensity equivalent to wild-type human DPP IV was scored as ++. 'None' indicates that no specific reaction product was detected.

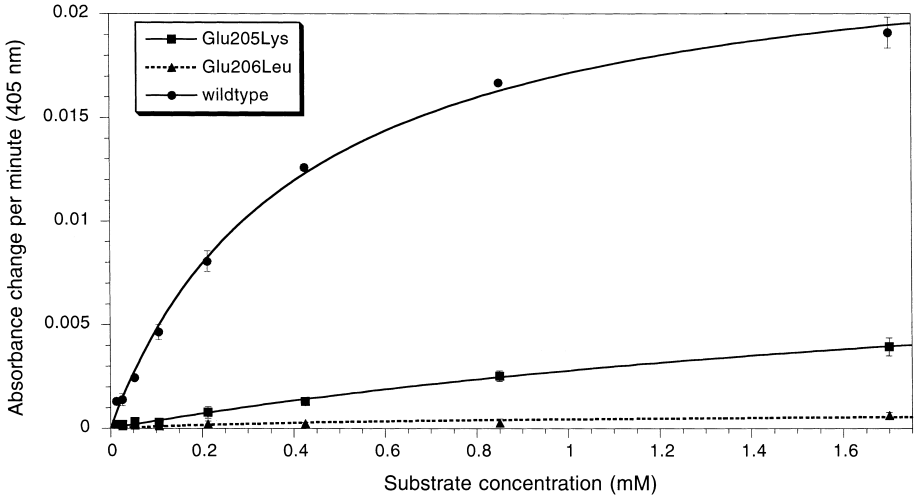

Fig. 2. Enzyme kinetics. Plots of kinetic analyses of suspensions of live cells transfected with wild-type or mutant human DPPIV constructs Glu-205-Lys or Glu-206-Leu. Means and S.D.s of the absorbance change per minute are shown. Curve fitting assumed Michaelis Menten kinetics.

impaired function of the enzyme. The Glu-205-Lys mutation introduced a charge reversal whilst Glu-206-Leu, the most dramatic substitution in this mutation study, was a change to the residue found in the equivalent position in *X. laevis* FAP [41]. The Val-201-Arg mutant ablated DPP IV enzyme activity (Table 2) but this was due to a defective structure of the expressed protein (see Section 3.3). A mutation designed to reverse the charge at an adjacent glutamic acid residue, Glu-204-Lys, did not significantly alter V_{max} or K_m (Table 3). The other mutations, Trp-201-Tyr, Val-202-Ala, Tyr-203-

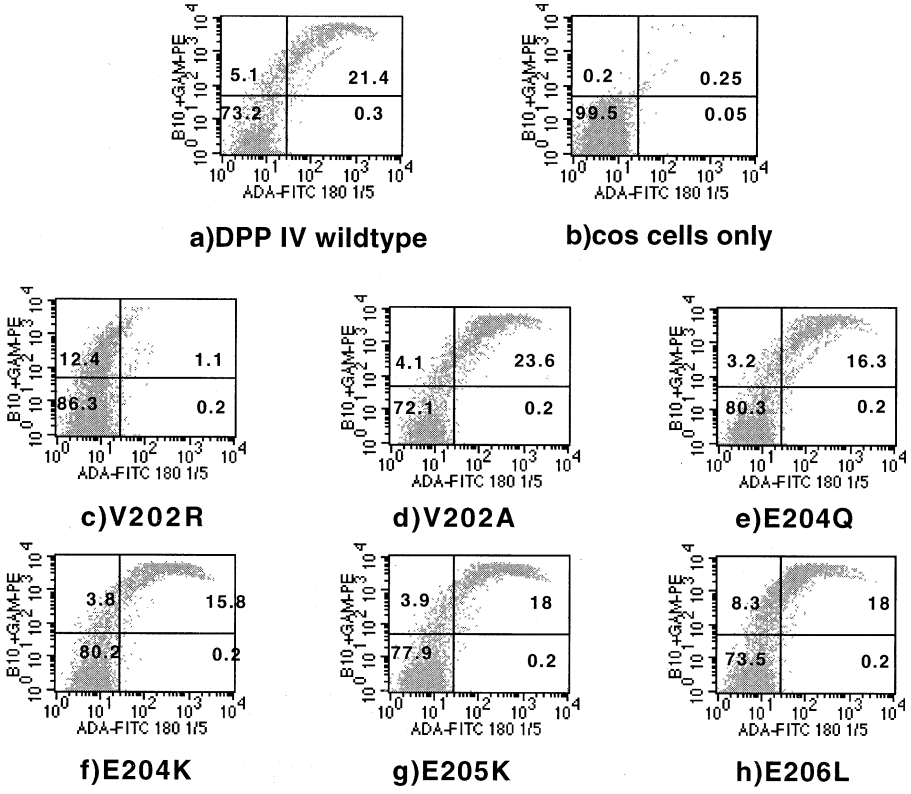

Fig. 3. Cell surface expression and ADA binding of transfected COS-7 cells. COS-7 cells transfected with wild-type (a) or mutant (c–h) DPP IV constructs were stained by two-colour cell surface immunofluorescence and analysed by flow cytometry. The green (ADA-FITC; axis) and red (anti-DPP IV mAb EF6/B10 followed by anti-mouse Ig PE; abscissa) fluorescence intensities of each event are shown. Crosshairs indicate the boundary between non-specific and specific fluorescence intensities, confirmed by inclusion of single colour and isotype-matched negative controls (data not shown). The mutants (d–h) including the two mutations that ablate enzyme activity, Glu-205-Lys (g) and Glu-206-Leu (h), bound to both mAb EF6/B10 and ADA-FITC. In contrast, mutant Val-202-Arg (c) weakly bound mAb EF6/B10 but did not bind to ADA and was thus considered to have a defective structure. The negative control was untransfected COS-7 cells (b).

Table 3
Enzyme kinetic data for wild-type and mutant DPP IV expressed on live cells, 72 h after transfection, assayed in triplicate under an eight point titration of the substrate Gly-Pro-toluene sulfonate (0.013–1.7 mM)

Mutation	V_{max}		K_m	
	Mean (ΔA_{405}/min)	S.D.	Mean (mM)	S.D.
DPP IV wild-type	0.0257	0.00055	0.458	0.02247
Trp-201-Tyr	0.0788	0.01888	0.779	0.22324
Val-202-Ala	0.1083	0.01333	0.763	0.13834
Tyr-203-Val	0.0117	0.00110	0.295	0.04375
Glu-204-Lys	0.0417	0.00298	0.972	0.11341
Glu-204-Gln	0.0485	0.00451	0.916	0.11758
Glu-205-Lys	0.0099	0.00144	2.542a	0.54840
Glu-206-Leu	0.0008	0.00035	0.739	0.70660

[a]Significantly different to wild-type DPP IV ($P < 0.005$).

Val and Glu-204-Gln, were not detectably different to wild-type DPP IV (Tables 2 and 3).

3.3. Structural integrity of mutants

The possibility that effects of mutations on enzyme activity were mediated by overall structural defects was examined in detail. Most mAbs to DPP IV, including EF6/B10, recognise epitopes in the N-terminal residues 1–356 of DPP IV (Abbott et al., 1999, submitted) [42,43]. ADA binding is in the putative β propeller domain and is dependent on the tertiary structure (Abbott et al., 1999, submitted) [44]. The DWVYEEE motif is also in this region of human DPP IV. Immunocytochemistry revealed that all point mutations bound to the anti-DPP IV mAb EF6/B10 (Table 2). In addition, all mutants except Val-202-Arg maintained binding to biotinylated ADA (Table 2) and ADA-FITC (Fig. 3). Two-colour flow cytometry demonstrated that all mutants except Val-202-Arg achieved wild-type levels of cell surface expression (Fig. 3). The Val-202-Arg mutant produced much lower levels of DPP IV expression than wild-type (Table 2 and Fig. 3c) and failed to bind ADA in a Western blot (not shown). When Val^{202} was replaced with the small uncharged residue alanine rather than the charged residue arginine, there was no effect on enzyme activity, mAb EF6/B10 binding or ADA binding (Table 2 and Fig. 3d). These data suggest that the Val-202-Arg mutation significantly disrupted the secondary and/or tertiary structure. This mutant was therefore omitted from further analysis. All other mutations exhibited the same mobility as both purified soluble recombinant DPP IV and wild-type DPP IV in Western blots, indicating intact dimerisation (Fig. 4A). In addition, an ADA overlay Western blot confirmed that all these mutants bound ADA (Fig. 4B).

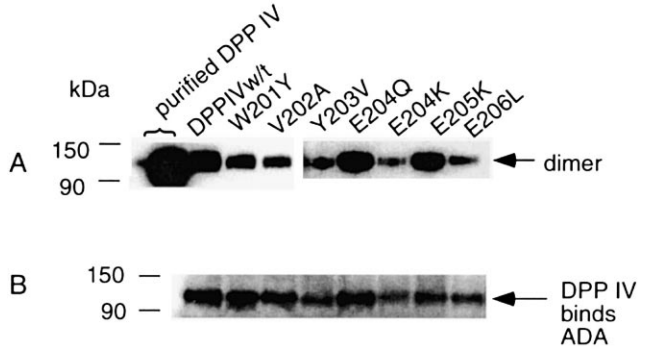

Fig. 4. Western blots of transfected COS-7 cell preparations demonstrated the structural integrity of the Glu-205-Lys and Glu-206-Leu mutations that ablated enzyme activity. (A) Western blot of 8% SDS-PAGE gel. The first two lanes contained two different quantities of purified soluble recombinant human DPP IV [40]. The other lanes contain non-boiled cell extracts. All mutants showed the same mobility as purified DPP IV, a 150 kDa dimer detected using anti-DPP IV mAb 2A6. (B) Western blot of non-boiled COS cell extracts on an 8–12% SDS-PAGE gel (NuPage) showed that all mutants bound ADA, detected by sequential incubations with ADA, rabbit anti-ADA antiserum and then anti-rabbit HRP.

4. Discussion

In this study, we found that two single amino acid point mutations, of glutamic acid residues Glu^{205} and Glu^{206} in the predicted β propeller domain of DPP IV, did not detectably alter the overall structure of the DPP IV protein but ablated its catalytic activity. In contrast, point mutations of adjacent residues did not detectably alter the catalytic activity. The residues examined form a highly conserved seven amino acid sequence motif DW(V/I/L)YEEE. We propose that a subclass of the POP family exists, the DPP IV-like gene family, and this motif will be present in all members of this subfamily. This motif is at a considerable distance in the primary structure from the established Ser, Asp, His catalytic triad that forms the charge relay system at the active site of DPP IV and other serine proteases [45]. This is the first report of a single amino acid point mutation outside the α/β hydrolase fold of a POP enzyme that disrupts only proteolysis. As the three dimensional structure of DPP IV is unknown, a mechanism for the role of these residues in proteolysis is not obvious. However, based on our sequence alignment with POP and a model of DPP IV (Abbott et al., 1999, submitted), we propose that Glu^{205} and Glu^{206} are essential for substrate entry into DPP IV.

Both natural and recombinant DPP IV glycoproteins form M_r 150 kDa dimers. The dimer is catalytically active whereas the monomer is not [46,47]. Therefore, failure to dimerise could ablate DPP IV enzyme activity. However, all of the mutants studied exhibited wild-type levels of dimerisation, so it is very unlikely that Glu^{205} or Glu^{206} are essential for dimerisation.

The three dimensional structure of pig POP has recently been solved [12] and by examining the sequence and structure of POP, we can suggest a position of the DWVYEEE motif in the human DPP IV tertiary structure. As predicted by others [6,10], POP contains a peptidase domain having an α/β hydrolase fold which contains the catalytic triad (Ser-Asp-His).

However, the central tunnel of an unusual seven blade β propeller domain covers the catalytic cleft formed by the α/β hydrolase fold. This propeller domain is thought to make POP an oligopeptidase by excluding large structured peptides from occupying the catalytic pocket. In this fashion, the propeller is thought to protect larger peptides and proteins from proteolysis. The entrance to the POP catalytic pocket is in the centre of the lower face of the β propeller. In POP, six charged residues (Lys^{82}, Glu^{134}, His^{180}, Asp^{242}, Lys^{389} and Lys^{390}) surround the narrow pore at this entrance and can be termed pore closing residues. It has been suggested that the lining of the opening of this narrow pore by charged flexible side chains allows for specific substrate entry and that this is a substrate-induced event [12].

Threading algorithms of human DPP IV have predicted that the DWVYEEE motif forms part of an α helix whereas physiochemical studies indicate that DPP IV contains more β structure than predicted [48]. Similar threading queries and a preliminary model of human DPP IV now predict this motif to be part of a β strand and a loop on the inner surface of the predicted β propeller (Abbott et al., 1999, submitted). A similar sequence in pig POP, Gly^{129}-Tyr^{130}-Ala^{131}-Phe^{132}-Ser^{133}-Glu^{134}-Asp^{135}, aligns with the Asp^{200}-Trp^{201}-Val^{202}-Tyr^{203}-Glu^{204}-Glu^{205}-Glu^{206} motif of human DPP IV. This motif in pig POP is conserved amongst POPs of other species. In POP, the residues Gly^{129}-Tyr^{130}-Ala^{131}-Phe^{132} lie at the end of the first strand of the second blade while residues Ser^{133}-Glu^{134}-Asp^{135} lie in the subsequent loop. In addition, as discussed previously, Glu^{134} is a pore closing residue [12]. Assuming that the POP structure is a prototype for DPP IV, the Glu^{205} of DPP IV may be equivalent to Glu^{134} in POP. Therefore, reversing or neutralising the charge at Glu^{205} or Glu^{206} may have ablated DPP IV enzyme activity by disallowing substrate entry into the central tunnel of the β propeller. The other residues of the conserved motif may have a role in positioning these two glutamates in the pore. Interestingly, the Glu-206-Leu substitution is found in *X. laevis* FAP. Unlike human and mouse FAP [26,27], *X. laevis* FAP has not been tested for dipeptidyl peptidase enzyme activity [41] and perhaps, this protein is enzymatically inactive.

The enzyme activity of DPP IV has roles in HIV infection, inflammatory processes and immune responses [2,49–53]. In order to design specific DPP IV inhibitors and analogues for use as therapeutics, it is essential to understand the mechanism of DPP IV substrate specificity and cleavage in more detail. Our structure-function data provide a novel perspective on the requirements for proteolysis in the DPP IV-like gene family. In particular, it pinpoints residues distant in primary structure to the C-terminal catalytic triad residues that are important for DPP IV enzyme activity. Crystallisation of DPP IV will be required to determine the precise position and nature of this motif in this family of enzymes.

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