

Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders

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A B S T R A C T

DP (dipeptidyl peptidase) IV is the archetypal member of its six-member gene family. Four members of this family, DPIV, FAP (fibroblast activation protein), DP8 and DP9, have a rare substrate specificity, hydrolysis of a prolyl bond two residues from the N-terminus. The ubiquitous DPIV glycoprotein has proved interesting in the fields of immunology, endocrinology, haematology and endothelial cell and cancer biology and DPIV has become a novel target for Type II diabetes therapy. The crystal structure shows that the soluble form of DPIV comprises two domains, an α/β -hydrolase domain and an eight-blade β -propeller domain. The propeller domain contains the ADA (adenosine deaminase) binding site, a dimerization site, antibody epitopes and two openings for substrate access to the internal active site. FAP is structurally very similar to DPIV, but FAP protein expression is largely confined to diseased and damaged tissue, notably the tissue remodelling interface in chronically injured liver. DPIV has a variety of peptide substrates, the best studied being GLP-I (glucagon-like peptide-I), NPY (neuropeptide Y) and CXCL12. The DPIV family has roles in bone marrow mobilization. The functional interactions of DPIV and FAP with extracellular matrix confer roles for these proteins in cancer biology. DP8 and DP9 are widely distributed and indirectly implicated in immune function. The DPL (DP-like) glycoproteins that lack peptidase activity, DPL1 and DPL2, are brain-expressed potassium channel modulators. Thus the six members of the DPIV gene family exhibit diverse biological roles.

INTRODUCTION

Few proteinases are capable of cleaving the post-proline bond and very few can cleave a prolyl bond two positions from the N-terminus. The latter small subset of serine proteinases, the post-proline dipeptidyl aminopeptidases, consists of the four enzymes of the DP (dipeptidyl peptidase) IV gene family, DPIV, FAP (fibro-

blast activation protein), DP8 and DP9 [1], and DP-II (E.C. 3.4.14.2) [2]. DPIV (E.C. 3.4.14.5) is a ubiquitous, multifunctional homodimeric glycoprotein with roles in nutrition, metabolism, the immune and endocrine systems, bone marrow mobilization, cancer growth and cell adhesion. DPIV ligands include ADA (adenosine deaminase) [3], kidney Na^+/H^+ ion exchanger 3 [4] and fibronectin [5]. Important DPIV substrates include at

Key words: adenosine deaminase, CD26, diabetes, dipeptidyl peptidase IV, fibroblast activation protein, post-proline aminopeptidase.

Abbreviations: ADA, adenosine deaminase; DP, dipeptidyl peptidase; DPL, DP-like; ECM, extracellular matrix; FAP, fibroblast activation protein; G-CSF, granulocyte colony-stimulating factor; GIP, glucose-dependent insulintropic peptide; GKO, gene knockout; GLP, glucagon-like peptide; GRP, gastrin-releasing peptide; HSC, hepatic stellate cell; IFN, interferon; IL, interleukin; MAb, monoclonal antibody; MMP, matrix metalloproteinase; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating peptide; POP, prolyl oligopeptidase; PPAR, peroxisome proliferator-activated receptor; sCD26, soluble CD26; SREBP, sterol regulatory element-binding protein; srhCD26, soluble recombinant human CD26; uPA, urokinase plasminogen activator; VIP, vasoactive intestinal peptide.

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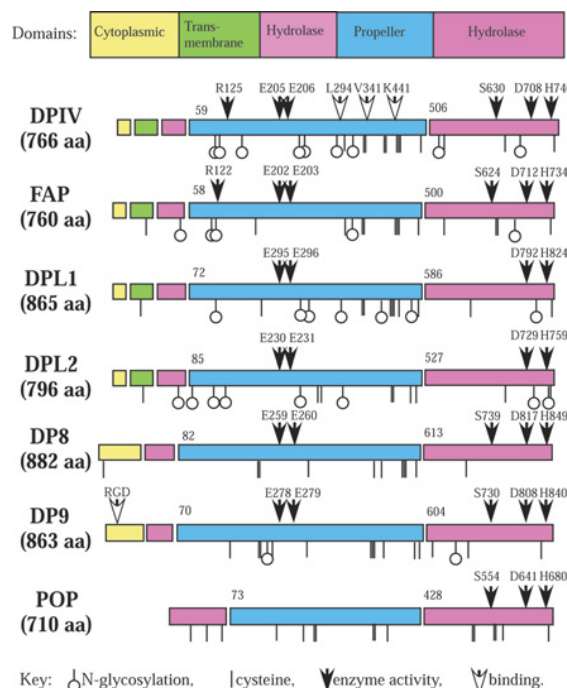
Table 1 Some natural substrates of DPIV

MDC, macrophage-derived chemokine; Mig, monokine induced by IFN γ ; IP10, IFN γ -induced protein; I-TAC, IFN-inducible T-cell α chemoattractant; IGF-I, insulin-like growth factor-I; hCG α , human chorionic gonadotrophin α chain; GHRF, growth hormone releasing factor; LH α leutinizing hormone α chain.

Substrate	N-terminus	Reference
GLP-1	His-Ala-Glu-	[19]
GLP-2	His-Ala-Asp-	[20]
GIP	Tyr-Ala-Asp-	[19]
GRP	Val-Pro-Leu-	[21,22]
Substance P	Arg-Pro-Lys-	[21]
NPY	Tyr-Pro-Ser-	[23]
Peptide YY(1–36)	Tyr-Pro-Ile-	[23]
PACAP38	His-Ser-Asp-	[22,24]
CCL5/RANTES	Ser-Pro-Tyr-	[25]
CCL11/eotaxin	Gly-Pro-Gly-	[25]
CCL22/MDC	Gly-Pro-Tyr-	[25]
CXCL9/Mig	Thr-Pro-Val-	[26]
CXCL10/IP10	Val-Pro-Leu-	[25,26]
CXCL11/I-TAC	Phe-Pro-Met-	[26]
CXCL12/SDF-1	Lys-Pro-Val-	[27]
IGF-I	Gly-Pro-Glu-	[28]
Prolactin	Thr-Pro-Val-	[21]
hCG α	Ala-Pro-Asp-	[21]
GHRF	Tyr-Ala-Glu-	[19,24]
LH α	Phe-Pro-Asn-	[28]
Thyrotropin α	Phe-Pro-Asp-	[28]
Peptide histidine methionine	His-Ala-Asp-	[19,24]
Enkephalins	Tyr-Pro-Val-	[29]
Vasostatin-I	Leu-Pro-Val-	[30]

least nine chemokines, NPY (neuropeptide Y), peptide YY, GLP (glucagon-like peptide)-1, GLP-2 and GIP (glucose-dependent insulintropic peptide; Table 1). DPIV inhibitors are in clinical trials as a new therapy for non-insulin dependent diabetes mellitus (Type II diabetes) [6,7]. Therapeutic benefit is derived from reduced inactivation of GLP-1 and GIP by DPIV-mediated cleavage, thus stimulating greater insulin production. Furthermore, on a high-fat diet, the DPIV GKO (gene knockout) mouse has reduced appetite and increased energy expenditure compared with wild-type animals [8], suggesting that DPIV-selective inhibitors may be useful as anti-obesity agents that might combat liver steatosis. Lymphocytes and endothelial and epithelial cells express DPIV (for review, see [9]). In addition to the integral membrane form, a soluble form of DPIV occurs in serum [10].

DPIV has a post-proline dipeptidyl aminopeptidase activity preferentially cleaving Xaa-Pro or Xaa-Ala dipeptides (where Xaa is any amino acid) from the N-terminus of polypeptides. The POP (prolyl oligopeptidase; EC 3.4.21.26) family, a group of aminopeptidases and

**Figure 1** Schematic presentation of the proteins of the DPIV family and of POP

The arrangement of structural domains is depicted. Approximate positions of N-glycosylation sites, cysteine residues and some residues required for enzyme activity and ADA or antibody binding are depicted. Not to scale.

endopeptidases able to hydrolyse the post-proline bond, includes the DPIV gene family. The DPIV gene family is distinguished by a pair of glutamate residues that are distant from the catalytic serine in the primary structure (Figure 1) [1], but within the catalytic pocket in the tertiary structure [11]. These glutamate residues, at positions 205 and 206 in DPIV, are essential for DP activity [12,13]. The DPIV gene family has six members, including FAP, DP8, DP9 and the two non-enzymes DPL1 and DPL2 (Table 2).

This review complements recent reviews [14–18] in discussing the structure, activities and roles of the DPIV gene family in T-cell function, chemoattraction of leucocytes, cancer, angiogenesis, fibrinolysis, haematopoiesis and energy metabolism.

IN VIVO EXPRESSION OF DPIV/CD26

DPIV is expressed in all organs, by capillary endothelial cells and activated lymphocytes and on apical surfaces of epithelial, including acinar, cells. In humans, DPIV is present in the gastrointestinal tract, biliary tract, exocrine pancreas, kidney, thymus, lymph node, uterus, placenta, prostate, adrenal, parotid, the sweat, salivary and mammary glands and endothelia of all organs examined, including liver, spleen, lungs and brain (reviewed previously, see [9,14]). DPIV is a 110 kDa glycoprotein

Table 2 Enzymes of the human DPIP family

Key: ✓, yes; ×, no; ?, not known.

Characteristic	DP8	DPIP	FAP	DP9
Hydrolysis of H-Gly-Pro	✓	✓	poor	✓
Hydrolysis of H-Ala-Pro	✓	✓	✓	✓
Hydrolysis of H-Arg-Pro	Poor	✓	✓	Poor
Cleavage of chemokines	?	✓	?	?
Gelatinase activity (collagen I)	×	×	✓	×
Dimeric form	×	✓	✓	×
Binding to adenosine deaminase	×	✓	×	×
mRNA expression in normal adult tissues	Ubiquitous	Ubiquitous	Ubiquitous	Ubiquitous
Protein expression in normal adult	?	Ubiquitous	Serum, pancreas	?
Protein expression by activated fibroblasts	?	✓	✓	?
Expression by fetal mesenchymal cells	?	✓	✓	?
Expression by activated hepatic stellate cells	?	×	✓	?
Expression by lymphocytes	✓	✓	×	✓

that is catalytically active only as a dimer. CD26 cell-surface expression on T-cells increases 5–10-fold following antigenic or mitogenic stimulation.

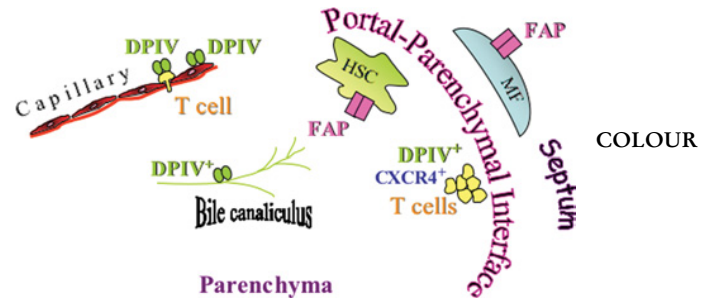
Human DPIP overexpression in mice produces fewer thymocytes and peripheral blood leucocytes from 2 months of age, more single positive CD8⁺ thymocytes and more apoptotic CD8⁺ and CD4⁺ peripheral blood lymphocytes [31].

Many epithelial tumours and cancer cell lines express DPIP, but DPIP expression is down-regulated or absent in tumour cells [1]. However, solid tumours contain DPIP in the stromal fibroblasts [1,32].

FAP

FAP has 52% amino acid identity with DPIP, but FAP and DPIP differ in expression patterns and substrate specificities (Table 2). FAP has a collagen type I-specific gelatinase activity [33,34]. In contrast, we have detected no gelatinase activity from recombinant human DPIP in zymograms of transfected CHO (Chinese-hamster ovary) cells or of purified protein [33] or in gelatinase assays of transfected monkey fibroblastic [14] or human epithelial [35] cell lines. Like DPIP, catalysis depends upon dimerization [33,36]. Interestingly, DPIP and FAP form heterodimers [37]. A soluble form of FAP has been isolated from normal bovine and human serum but, curiously, despite the abundance of serum DPIP, serum FAP is homodimeric [38,39].

Controlling gelatinases is vital for organ structure. Unlike MMPs (matrix metalloproteinases), which have a proenzyme form, the gelatinase activity of FAP is constitutive. FAP is normally restricted to a subset of glucagon: producing α -cells in pancreatic islets [32]. FAP is strongly expressed by activated HSCs (hepatic stellate cells), notably near lipid accumulation, called steatosis,

**Figure 2** Schematic representation of FAP and DPIP in cirrhotic liver

In cirrhotic nodules, FAP is expressed by myofibroblasts (mf) in the septum and by HSCs following activation in the portal–parenchymal interface. DPIP is expressed on activated T-cells, capillary endothelium and the bile canaliculus surface of hepatocytes. Not to scale.

in liver and by mesenchymal cells in other sites of tissue remodelling such as stromal fibroblasts of epithelial tumours and healing wounds and embryonic mesenchymal cells [32–34,40,41]. The FAP GKO mouse has a normal phenotype for body weight, organ weights, histological examination of major organs and haematological analysis [42].

The HSC has an important role in the pathogenesis of cirrhosis. Following liver injury, HSCs undergo activation and transdifferentiation to become myofibroblasts. Significant functional changes accompany this phenotypic change, including alterations in ECM (extracellular matrix) production and expression of various MMPs and their inhibitors. FAP is expressed by myofibroblasts and a subset of activated human HSCs at the tissue–remodelling interface, which is the PPI (portal–parenchymal interface), of cirrhotic liver [33] (Figure 2).

Table 3 Physical attributes of human DPIV-related proteins

✓, yes; ×, no; ?, not known. *The long form is a splice variant.

Attribute	DPIV	FAP	DP8	DP9	DP9 long*	DPL1	DPL2	POP
Synonyms	CD26, ADAbp	Seprase	—	—	—	DPP6, DPPX	DPP10	PEP, PREP
GenBank [®] accession	M80536	U09278	AF221634 NM_130434	AY374518 NM_139159	AF542510 AF452102	M96859 M96860	AY387785 NM_1004360	AB020018
References	[57]	[37]	[45,48]	[13,48,49]	[35]	[52]	[48,50,51]	
Gene location								
Human	2q24.2	2q24.3	15q22.32	19q13.3		7q36.2	2q14.1	6q21
Mouse	2	2	9	17		5	1	10
Number of exons	26	26	20	19	22	26	26	15
Gene size (kb)	81.8	72.8	71	47.3	48.6	911	1402	40.2
Transmembrane domain	✓	✓	×	×	×	✓	✓	×
Monomer mobility	110 kDa	95 kDa	100 kDa	110 kDa	?	97 kDa	97 kDa	80 kDa
Number of amino acids	766	760	882	863	971	865*, 803	796*	712

FAP-positive cells are present in early stages of liver injury, and FAP immunostaining intensity strongly correlates with the histological severity of fibrosis in chronic liver disease [40].

Conferring FAP expression upon a human epithelial cell line increases tumorigenicity in mice, but has the opposite effect on a melanoma cell line [43,44]. The biological importance of these observations is unknown because tumour cells do not naturally express FAP *in vivo*.

DP8 AND DP9

The discovery of DP8 and DP9, which are ubiquitously expressed enzymes with DPIV-like peptidase activity [35,45], means that previous studies using DPIV inhibitors to infer functions of DPIV will require reinterpretation where the inhibitor is found to also inhibit DP8 or DP9 [46]. However, little is known about the expression or functional significance of DP8 or DP9. DP8 and DP9 are both soluble proteins localized in the cytoplasm. Both are DPs that are active as monomers and hydrolyse *H*-Ala-Pro- and *H*-Gly-Pro-derived substrates, although less efficiently than DPIV. Neither DP8 nor DP9 exhibit gelatinase activity and no natural substrates are known.

DP8

DP8 has 26 % amino acid identity with the protein sequences of DPIV and FAP and is a dipeptidyl aminopeptidase, hydrolysing the prolyl bond after a penultimate proline [45]. However, some biochemical characteristics of DP8 are similar to the endopeptidase POP (Table 3). Like DP8, POP is a soluble cytoplasmic protein, is active as a monomer and lacks *N*-linked and *O*-linked

glycosylation sites. Like DPIV, DP8 mRNA expression is ubiquitous. DP8 mRNA levels are elevated in both activated and transformed lymphocytes. DPIV traverses the TGN (*trans*-Golgi network), which is in a secretion pathway, enters secretory vesicles then moves to the cell surface [47]. Despite finding DP8 in Golgi as well as elsewhere in cytoplasm, we have not found evidence of secretion of DP8 by transfected COS or 293T cells [35].

DP9

DP9 is the closest relative to DP8, having 61 % amino acid identity. DP9 has two forms, having open reading frames of 2589 bp and 2913 bp. A ubiquitous predominant DP9 mRNA transcript at 4.4 kb represents the short form and a less abundant 5.0 kb transcript present predominantly in muscle represents the long form (Table 3) [35,48,49]. DP9 has only two potential *N*-linked glycosylation sites. Paradoxically for a cytoplasmic protein, DP9 contains an RGD (Arg-Gly-Asp) potential cell attachment sequence, which is the best characterized integrin-binding motif, near its *N*-terminus. In contrast, the RGD motif in mouse DPIV has a different location, on propeller blade four where ADA binds to human DPIV. We obtain DP9 sizes on SDS/PAGE of 110 kDa and 95 kDa [35], whereas others report a single band at about 95–100 kDa [48,49]. DP9 has a predicted polypeptide size of 98263 Da, so we propose that intact fully glycosylated DP9 runs at 110 kDa.

Northern blot analysis on normal tissues shows DP9 mRNA expression predominantly in muscle, liver and leucocytes. However, *in silico* examination of 255 human DP9 ESTs (expressed sequence tags; UniGene Cluster Hs.237617) indicate that DP9 mRNA expression is most abundant in leucocytic cell lines and diseased and tumour-bearing tissues including melanoma [35].

Table 4 *In vivo* immunological and haematopoietic effects of DPIV inhibitors

*GKO mouse was not inhibitor treated.

Name of inhibitor	Type of inhibitor	Outcome		Reference
		Wild-type mouse	DPIV GKO mouse	
Pro-Pro Diphenyl phosphonate ester	Irreversible, competitive	Prolonged cardiac allograft survival	Not done	[63]
Ala-Pro-nitrobenzoylhydroxylamine	Irreversible, suicide	Suppression of experimental arthritis	Same as wild type	[58]
Lyz-[Z-(NO ₂)]-thiazolidide	Reversible, competitive	Suppression of experimental arthritis	Same as wild type	[58]
Isoleucyl-thiazolidide	Reversible, competitive	Exacerbates NPY + Con A-stimulated paw inflammation	Not done	[65]
Val-boro-Pro	Irreversible	Tumour Regression	Same as wild type	[66]
Val-boro-Pro	Irreversible	Accelerates recovery from neutropenia	Same as wild type	[61]
Diprotin A (Ile-Pro-Ile)	Reversible, competitive	Impaired G-CSF-induced haematopoiesis	GKO same as inhibitor*	[67]
Diprotin A or valine-pyrrolidide	Reversible, competitive	Increased bone marrow transplant efficiency	GKO same as inhibitor*	[64]

THE NON-ENZYME DPIV GENE FAMILY MEMBERS: DPL1 AND DPL2

Two enzymatically inactive proteins closely related to DPIV lack DPIV catalytic activity due to mutations of the catalytic serine residue and its neighbouring tryptophan residue, giving a surrounding sequence of Gly-Lys-Asp-Tyr-Gly-Gly instead of the motif Gly-Trp-Ser-Tyr-Gly-Gly. Since we cloned the second human DPIV paralogue that lacks the catalytic serine, we use the names DPL (DP-like) 1 and 2 for these proteins to simplify the nomenclature [1,50]. As restoring the enzyme activity of DPL1 or DPL2 would very likely require both the serine and tryptophan residues, their biological activities are probably exerted via binding interactions. It is very unlikely that provision only of a serine residue could rescue an enzyme activity in DPL2, as has been hypothesized [51].

DPL1 was previously called DPPX or DPP6 [52]. Expression of neuronal DPL1 increases in response to kainic acid injection into the hippocampus, suggesting possible involvement in CNS (central nervous system) plasticity [53]. DPL1 has a crucial role in the trafficking, membrane targeting and function of A-type potassium channels in somatodendritic compartments of neurons, which are important in neuronal function and in dysfunction, such as Parkinson's disease [54]. Despite the absence of DP activity, DPL1 exerts an important developmental function. The mouse *rump white* mutation, which lacks expression of the DPL1 gene, is embryonic lethal in homozygotes and causes a pigmentation defect in heterozygotes [55].

DPL2 has been cloned by others and called DPP10 [48,51,56]. Like DPL1, DPL2 is alternatively spliced. The DPL2, DPIV and FAP genes are all on chromosome 2, and DPL2 is more closely related to DPIV and FAP than is DPL1. Intronic portions of the DPL2 gene link to asthma [51]. DPL2 mRNA expression occurs in brain, adrenal gland and pancreas [48,50]. This is similar to the expression pattern of the long form of DPL1 [52,53].

Like DPL1, DPL2 associates with and modulates A-type potassium channels [56].

INHIBITOR DATA INDICATE IMPORTANCE OF DPIV-RELATED DP_s

It is likely that many DPIV inhibitors are selective for the DPIV family rather than DPIV itself [48]. Therefore some potential functions of DP8 and DP9 may be inferred from studies in which a function has been attributed to DPIV by observing diminution of that function in cells or animals treated with a DPIV inhibitor. In several paradigms, DPIV inhibitor treatment elicits similar responses in cells and animals irrespective of possession or lack of DPIV expression (Table 4). For example, DPIV inhibitors suppress collagen-induced and alkyl-diamine-induced arthritis to similar extents in DPIV-deficient German Fischer344 and wild-type rats [58]. Moreover, T-cell proliferation *in vitro* [59] and immune responses *in vivo* [58] are diminished in the presence of DPIV inhibitors; however, the DPIV-deficient rat has normal immune responses [60]. These data imply that the peptidase activities of DP8 and/or DP9 have important functions in activated lymphocytes. FAP is excluded from consideration because it is not present in leucocytes.

The DPIV/DP-II inhibitor Val-boro-Pro stimulates haematopoiesis to similar extents in DPIV GKO and wild-type mice [61], indicating that bone marrow expresses important DPIV-related enzymes. In animal models, DPIV inhibitors suppress antibody production [62] and prolong the survival of heart transplants [63]. It is possible that these phenomena also relate to functions of DP8 and/or DP9, rather than DPIV. Therapeutic DPIV inhibitors should either avoid inhibition of DP8 and DP9, or the effects of inhibiting DP8 and DP9 should be understood.

Recently, the non-selective DPIV inhibitors valine-pyrrolidide and diprotin A have been shown to greatly

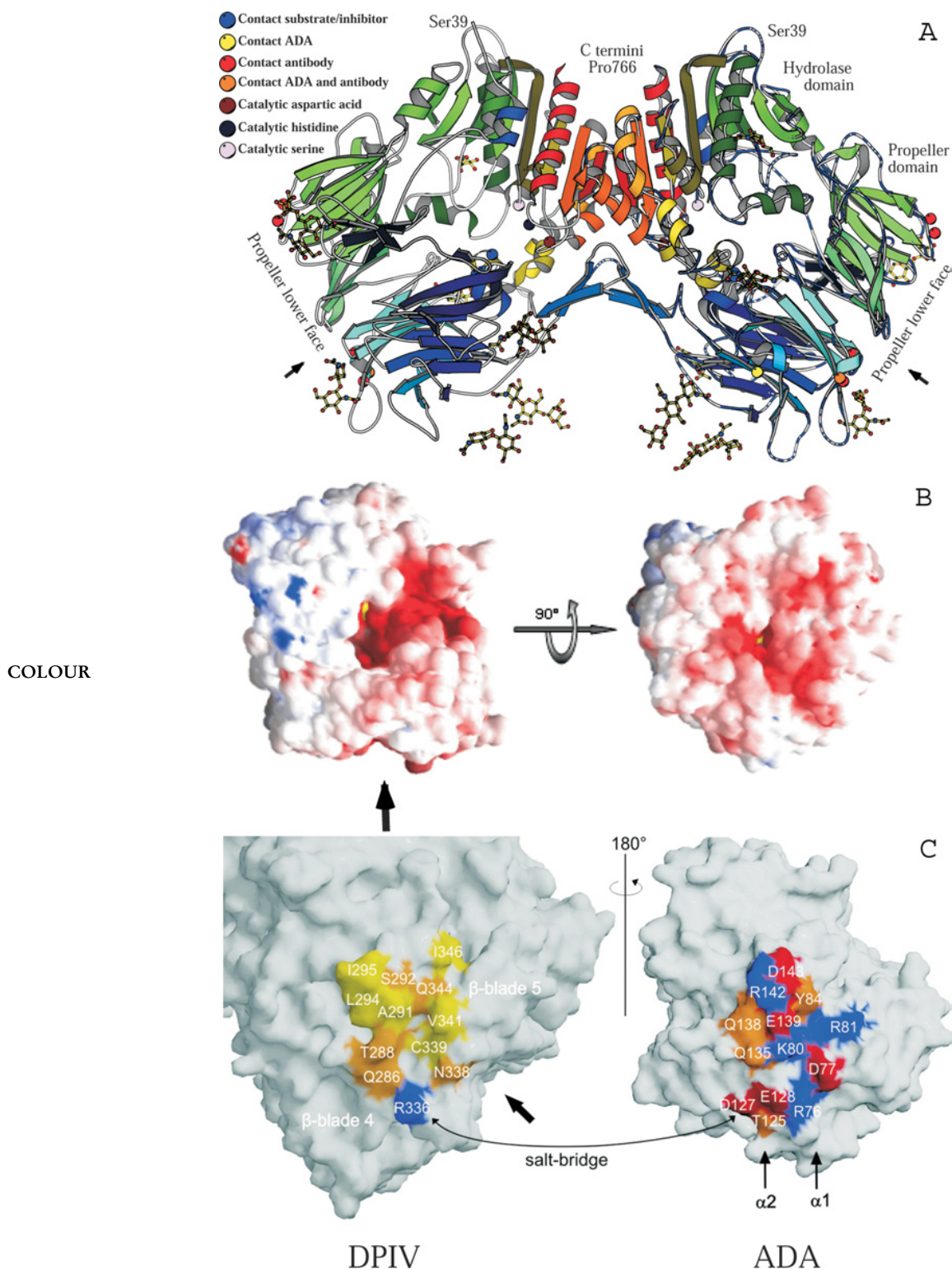


Figure 3 Secondary, tertiary and quaternary structure of DPIV

(A) The DPIV homodimer as a ribbon diagram with each monomer coloured blue to red, carbohydrates are depicted in ball-and-stick representation and positions of important amino acids as spheres. The orange and yellow residues Leu²⁹⁴ and Val³⁴¹ are essential for ADA binding, whereas the red positively charged residues Arg³⁴³ and Lys⁴⁴¹ are important in epitopes of antibodies that inhibit ADA binding [86]. Glu²⁰⁵ and Glu²⁰⁶, coloured blue, are essential for enzyme activity [12]. Both domains contribute to the dimerization interface, the β -propeller contributing two β -strands that protrude from blade 4. Substrate access to the catalytic site occurs via the side openings that face each other and are between the eight-blade β -propeller (bottom) and α/β hydrolase (top) domains. The transmembrane

enhance the efficiency of bone marrow cell transplantation [64]. The administration of DPIV inhibitor to the DPIV^{-/-} mouse in this model would be interesting.

THE THREE-DIMENSIONAL STRUCTURE OF DPIV

The seven DPIV crystal structures recently reported reflect a sudden global interest in the pharmaceutical design of DPIV inhibitors [11,72–77]. The DPIV glycoprotein is a dimer (Figure 3). Each monomer subunit consists of two domains, an α/β -hydrolase domain (residues 39–51 and 501–766) and an eight-blade β -propeller domain (residues 59–497), that enclose a large cavity of approx. 30–45 Å (1 Å = 0.1 nm) in diameter. Access to this cavity is provided by a large side opening of approx. 15 Å [76]. However, only elongated peptides, or unfolded or partly unfolded protein fragments, can reach the small pocket within this cavity that contains the active site. DPIV contains nine *N*-linked glycosylation sites that lie predominantly on the propeller domain near the dimerization interface [75] and perhaps shield this trypsin-resistant extracellular protein from proteolysis.

The active site and catalytic mechanism

The residues forming the catalytic triad are Ser⁶³⁰, Asp⁷⁰⁸ and His⁷⁴⁰. In addition, Tyr⁵⁴⁷ in the hydrolase domain is essential for catalytic activity and in the crystal structure appears to stabilize the tetrahedral oxyanion intermediate form of a substrate [78]. Two glutamate residues in the catalytic pocket, Glu²⁰⁵ and Glu²⁰⁶ (Figure 3A), align the substrate peptide by forming salt bridges to its N-terminus, leaving room for only two amino acids before the peptide reaches the active serine residue, thus explaining its dipeptide-cleaving activity. Furthermore, in the substrate second position only amino acids with smaller side chains such as proline, alanine and glycine can fit into the narrow hydrophobic pocket. Thus the crystal structures have helped to explain the substrate specificity of DPIV and the mutation data showing that Glu²⁰⁵ and Glu²⁰⁶ are essential for catalysis [12,13].

An intriguing aspect of DPIV biochemistry is the dependence of peptidase activity upon homodimerization. Dimerization requires the hydrolase domain [13] and a protrusion from the fourth blade of the β -propeller

(Figure 3). A single amino acid point mutation near the C-terminus, His⁷⁵⁰ → Glu, is sufficient to prevent dimerization [79].

The unusual propeller of DPIV

β -Propellers have four to eight blades formed by a repeated subunit containing at least 30 and generally 50 amino acids in a β -sheet of four anti-parallel strands. Propellers commonly act as scaffolding for protein–protein interactions [80,81]. The points of contact with ligand and antibodies are formed by loops contributed by adjacent propeller blades such that binding epitopes depend upon tertiary structure. DPIV has all of these characteristics.

The structure of DPIV is unique among leucocyte surface molecules. Other leucocyte surface antigens that include a β -propeller domain are CD100 [82] and integrin α -chain [83], which have seven-blade propellers. As DPIV is a type II protein, the propeller domain points its lower face towards the extracellular milieu (see Figure 3A). The eight-blade β -propeller domain of DPIV is more disordered than other propellers.

The ADA binding site on DPIV

ADA (EC 3.5.4.4) is a soluble globular 43 kDa enzyme present in all mammalian tissues. ADA catalyses the irreversible deamination of adenosine to inosine and of 2'-deoxyadenosine to 2'-deoxyinosine. ADA derived from rabbits, cattle and humans binds to human, but not mouse, DPIV. ADA binds human DPIV with a K_A of 4–20 nM. Both monomeric and dimeric DPIV bind ADA [13,77]. Localizing ADA to the cell surface by its binding to CD26 probably reduces inhibition of T-cell proliferation by extracellular adenosine.

Three charged residues on ADA, Glu¹³⁹, Arg¹⁴² and Asp¹⁴³ [84,85], have been identified by point mutation as important for ADA–DPIV binding. The crystal structure of DPIV with ADA shows that the ADA binding is located, as predicted from the model [86], on the outer edges of the fourth and fifth blades on the lower side near the lower face of the β -propeller domain of DPIV. Only one salt bridge binds ADA to DPIV (Figure 3C). Most of the involved residues on DPIV are hydrophobic and most of the 13 involved residues on ADA, which are all polar, are charged [77]. Generally, protein–protein binding primarily involves hydrophobic surfaces with

domain (residues 7–28) is above the molecule in this view of the extracellular portion, residues 39–766, of human DPIV. The Figure was prepared using the atomic co-ordinates having Protein Data Bank code 1NIM [11], MOLSCRIPT and Canvas v. 8.06. (B) A surface representation of the DPIV monomer with the negatively charged surface coloured red and positively charged surface coloured blue. Viewed side-on (on left) and towards the propeller lower face (on right). An inhibitor is coloured yellow to indicate the position of the catalytic pocket. Reproduced from [11] with permission. (C) In these surface representations of DPIV and its ligand, ADA amino acids in the binding interface are labelled. Positively and negatively charged residues are blue and red, and non-polar and polar uncharged residues are yellow and orange respectively. The helices 1 and 2 of ADA are labelled α_1 and α_2 . The DPIV monomer is oriented similarly to the monomer at the right in (A). In each image, a large arrow points towards the central opening of the propeller lower face. Reproduced from [77] with permission. © (2004) American Society for Biochemistry and Molecular Biology.

some salt bridges that are mostly peripheral in the binding interface. Thus the amino acid composition of the ADA–DPIV binding site is unusual, perhaps due to the short evolutionary time that it has undergone selective pressure.

ADA binding to human DPIV is blocked by certain anti-DPIV MAb (monoclonal antibodies) that define a similar epitope. MAb that block ADA binding rely upon Val³⁴¹ and Thr⁴⁴⁰–Lys⁴⁴¹ of DPIV for binding [86]. The DPIV structure shows that these amino acids are on one side of the propeller and distant from both of the openings, which explains the lack of interference from either ADA or MAb with the catalytic activity of DPIV or ADA. This location of ADA binding also positions ADA away from the cell surface and perhaps increases accessibility to ligands such as A₁-adenosine receptor [87] and plasminogen-2 [85].

Protein structure in the DPIV family

Relating the structures of the DPIV family members to their substrate specificities will be valuable in designing selective inhibitors. Arg¹²⁵ in propeller blade 2 contacts inhibitors and substrates [11,72–77]. Arg¹²⁵ is conserved in DPIV in all species from bacteria to human, but is absent from DP8 and DP9. The sequence motif of the Glu²⁰⁵-containing α -helix that limits DPIV to cleavage after the second amino acid of substrates [11] is conserved in all six proteins of the DPIV gene family in all species [1]. The structures of FAP, DP8, DP9, DPL1 and DPL2 can now be predicted using DPIV as a template, but solving their structures will provide precise information concerning mechanisms of substrate specificity and protein–protein interaction in this gene family.

SOLUBLE DPIV

The naturally occurring dimeric soluble form of DPIV exists in extracellular fluids, including serum, seminal fluid, saliva and bile. The richest natural sources of soluble DPIV are seminal fluid [88,89] and kidney [75].

Altered serum DPIV levels occur in many diseases [14,90]. ADA binding assays are specific for CD26 and show that at least 90 % of serum DPIV activity on *H*-Gly-Pro is derived from CD26 [10]. The origin(s) of the remaining DPIV activity is unknown, but could include FAP [39]. The naturally occurring soluble form of CD26 starts at Ser³⁹ [10] and is derived from cell-surface CD26 [35]. The mechanism of shedding sCD26 (soluble CD26) from the cell surface is unknown, but is thought to be proteolytic [47].

The level of DPIV in serum from healthy adults is about 22 nmol *p*-nitroanilide · min^{−1} · ml^{−1}, which corresponds to approx. 7 μ g/ml. It may derive from all DPIV-expressing cells that contact blood, generally endothelial cells and lymphocytes. Serum DPIV levels generally decrease in disease unless liver injury or ex-

tensive lymphocyte proliferation is involved [14]. Serum CD26 measurement has no diagnostic value by itself. However, it may be useful in combination with other disease makers such as with α -L-fucosidase for colon cancer [91]. Perhaps the function of serum DPIV is to inactivate and thereby prevent systemic effects from bioactive peptides following their local production.

Functions of soluble DPIV

The functions of sCD26 have been studied only in relation to T-cell proliferation. Exogenous srhCD26 (recombinant human sCD26) is not itself mitogenic, but is able to enhance proliferation of activated peripheral blood lymphocytes [59,92]. Concentrations of srhCD26 greater than the optimum (0.5 μ g/ml) diminish this effect [92]. We have obtained concordant data using uninfected subjects and *Herpes simplex* virus as the recall antigen [9]. Thus sCD26 exerts regulatory effects on *in vitro* T-cell memory responses, generally decreasing strong responses and increasing weak responses.

sCD26 is taken up via binding to mannose 6-phosphate receptor by CD14⁺ monocytes, leading to an increase in their antigen-presenting activity that involves calveolin-1 binding [93]. DPIV activity probably does not contribute as the DPIV inhibitor concentrations needed to diminish T-cell proliferation *in vitro* are well above the *K_i*. Moreover, we found that srhCD26 rendered catalytically inert by point mutation produced the same effects on T-cell proliferation as wild-type srhCD26 [9].

FUNCTIONS OF DPIV

CD26/DPIV in activated and memory T-cells

Both the percentage of cells expressing CD26 and the number of molecules/cell are increased following activation of T-cells [94]. The strongest lymphocytic CD26 expression is found on cells co-expressing high densities of other activation markers such as CD25, CD71, CD45RO and CD29 [95,96]. The CD26^{bright}CD4⁺ population is the CD45RO⁺CD29⁺ memory/helper subset.

The immunological synapse contains cholesterol-rich rafts and at least some CD26 lies in these rafts [97]. The signal transduced by CD26 overlaps with the T-cell receptor/CD3 pathway, increasing tyrosine phosphorylation of p56^{lck}, p59^{lyn}, ZAP-70, phospholipase C- γ , MAPK (mitogen-activated protein kinase) and c-Cbl in that pathway (for review, see [14]).

The correlation between T-cell CD26 expression intensity and Th1-like immune responses [98–100] may involve IL (interleukin) 12-dependent CD26 up-regulation. IL12 is an inducer of Th1 responses and increases expression of CD26 on mitogen-activated T-cells [101]. DPIV potentially tilts the Th1/Th2 balance towards Th1 via both neuropeptide and chemokine cleavage.

Table 5 *In vitro* studies using DPIV inhibitors

Inhibitor	Inhibitor type	Inhibited function	Reference
Lyz-[Z-(NO ₂)]-thiazolidide	Reversible, competitive	Tyrosine phosphorylation in lymphocytes	[68]
Lyz-[Z-(NO ₂)]-piperidine	Reversible, competitive	T-cell proliferation and IL2 and IL-6 production	[69]
N-Ala-Pro-O(nitrobenzoyl) hydroxylamine	Irreversible, suicide	Antibody, IL2 and IFN- γ production by lymphocytes	[70]
Lyz-[Z-(NO ₂)]-thiazolidide	Reversible, competitive	Proliferative responses to mitogens or <i>Mycobacterium tuberculosis</i> antigen: equal effect in wild type cells and DPIV-deficient Fischer344 rat cells	[58]
Diprotin A	Reversible, competitive	Inhibitor increases bone marrow cell chemotaxis	[71]

Two neuropeptides that are inactivated by DPIV, VIP (vasointestinal peptide) and PACAP (pituitary adenylate cyclase-activating peptide), increase the production by activated CD4⁺ T-cells of Th2 cytokines IL4 and IL5 and Th2 isotype IgG₁ and decrease the production of Th1 cytokines IFN (interferon) γ and IL2 and Th1 isotype IgG_{2a} [102]. The net effect of DPIV-mediated chemokine cleavage seems to favour Th1 cell chemoattraction (for review, see [14]).

The therapeutic use of DPIV inhibitors raises questions of potential effects on the immune system. Perhaps selectively inhibiting DPIV activity in humans does not impair the immune system [18]. As discussed above, non-selective DPIV inhibitors affect immune responses, suggesting that DPIV-related enzymes, rather than DPIV, are responsible for the immune effects of these compounds (Table 4).

DPIV enzyme inhibitors reduce T-cell proliferation, cytokine production and signalling *in vitro* (Table 5). However, most enzyme-negative CD26-expressing Jurkat cell clones were more easily triggered via CD26 than Jurkat cells transfected with wild-type CD26 [103]. Moreover, a Jurkat cell line that surface-expressed enzymatically inactive CD26 produced more IL2 than untransfected cells [104]. Most importantly, the co-stimulatory activity of CD26 is retained in mutants lacking most of the hydrolase domain [105]. These data indicate that proteolytic activity is not a prerequisite for the T-cell activating or co-stimulating properties of CD26 *in vitro*.

Cancer and ECM interactions with DPIV

DPIV binds the ECM component fibronectin on rat hepatocytes and breast and lung cancer cells [5,106], and this interaction is independent of its enzymatic activity. The consensus binding motif is Thr(Ile/Leu)-Thr-Gly-Leu-Xaa(Pro/Arg)-Gly(Thr/Val)-Xaa in rat fibronectin type III repeats 13, 14 and 15 [5].

The ECM interactions of DPIV may help to explain its changes in expression levels in various human cancers because cancers differ in their ability to invade neighbouring tissue. Following DPIV transfection, SKOV ovarian carcinoma cells exhibit increased cell–cell adhesion mediated by collagen or fibronectin [107]. Moreover, ovarian carcinomas with greater DPIV expression were found to

be less invasive, so perhaps the increased adhesion has an anti-invasive effect [108].

Wild-type or enzymatically-inactive DPIV overexpression in melanoma cell lines causes inhibition of tumour progression in nude mice, and inhibition of anchorage-independent growth, inhibition of cell growth and increased apoptosis *in vitro* [109]. Invasive melanoma cells lack DPIV expression [110]. Thus DPIV suppresses the malignant phenotype. An *in vitro* invasion study has shown that either wild-type or enzymatically inactive DPIV overexpression can confer reduced invasiveness upon LOX melanoma cells in Matrigel (a basement membrane matrix) [111]. Further understanding of the anti-invasion effect of DPIV might assist in the control of certain carcinomas. The *in vitro* evidence suggests that DPIV-selective inhibitors would not influence tumour invasiveness. Indeed, the non-selective inhibitor Val-boro-Pro triggers tumour regression in DPIV GKO mice as effectively as in wild-type mice [66].

DPIV, FAP and plasmin

Tissue repair involves coagulation, which results in fibrin deposition. The fibrin of a clot is usually lysed, primarily by plasmin, which is converted from its inactive form, plasminogen, by plasminogen activators. Fibrinolysis is inhibited by PAI (plasminogen activator inhibitor)-1, PAI-2 and α 2 antiplasmin, which are induced by tissue trauma. DPIV and FAP are involved in this process (Figure 4). Plasminogen-2 associates with both ADA and CD26 in a complex that enhances plasminogen-2 activation [85]. However, FAP converts α 2 antiplasmin into a more active form [39] and associates with the uPA (urokinase plasminogen activator) receptor annexin 2 [112]. Thus the actions of DPIV and FAP are opposite. As DPIV and FAP can heterodimerize, understanding the roles of DPIV and FAP in fibrinolysis will be interesting. The discovery of FAP in serum [39] was surprising because a soluble form of FAP has not been observed *in vitro*. A selective FAP inhibitor might enhance wound healing and reduce adhesions via increased fibrinolysis.

DPIV and metabolism

The peptide hormone GLP-1 is an important regulator of glucose metabolism (Figure 5). GLP-1 stimulates insulin

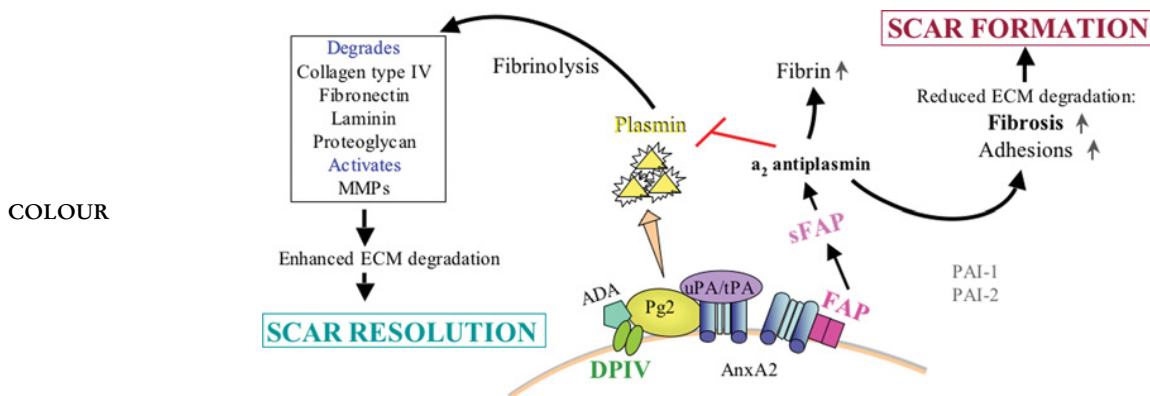


Figure 4 FAP and DPIV modulate fibrinolysis

Plasminogen-2 (Pg2) activation to plasmin increases following binding to DPIV and ADA [85]. Serum FAP (sFAP) converts α_2 antiplasmin into a more active form [39] that is better able to inhibit plasmin. FAP associates with the plasminogen receptor annexin 2 (AnxA2) [112], which is also uPA and tPA (tissue plasminogen activator) receptor.

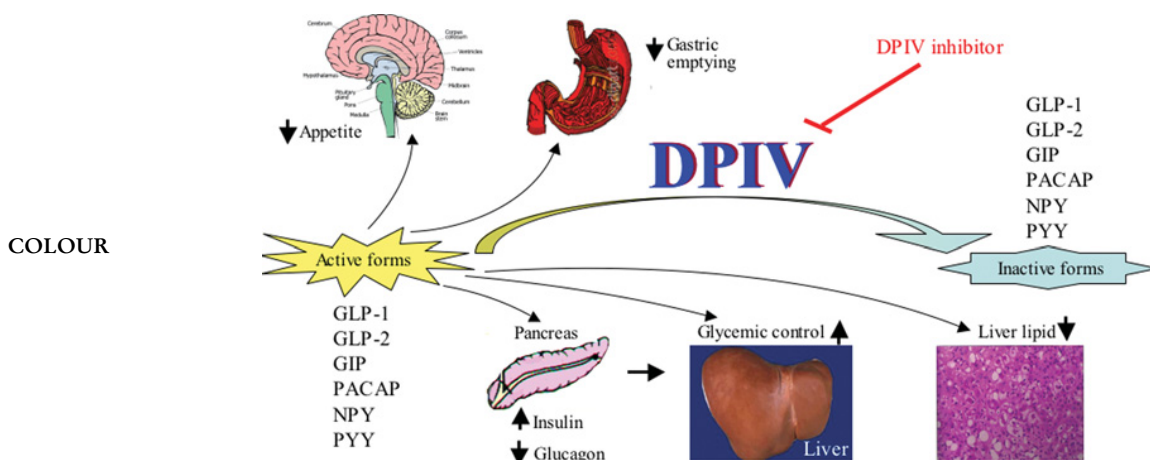


Figure 5 Metabolic benefits of DPIV inhibition

DPIV inhibitors are thought to improve glycaemic control in early stage Type II diabetes by reducing DPIV-mediated inactivation of GLP-1 [46,114]. Increased half-lives of GLP-1 and other DPIV substrates, possibly including GLP-2, GIP, GRP, NPY, peptide YY, PACAP and VIP, could reduce appetite and lipid deposition and increase energy expenditure [8].

secretion, inhibits glucagon secretion, delays gastric emptying and induces satiety. Therefore GLP-1 has a therapeutic benefit in Type II diabetes [7,113]; however, the *in vivo* half-life of GLP-1 is < 3 min. GLP-1 is inactivated by DPIV enzyme cleavage. DPIV inhibitors increase the GLP-1 half-life and probably the half-lives of other hormones that influence metabolism, such as GIP, VIP, PACAP, GRP (gastrin-releasing peptide) and GLP-2 (Table 1). The Novartis DPIV inhibitor LAF237 has been shown to improve glycaemic control and reduce glucagon levels in early stage Type II diabetes patients and prevent deterioration of glycaemic control over 52 weeks [6,114].

DPIV GKO mice display increased glucose clearance following glucose challenge [115]. Administering the DPIV inhibitor valine-pyrrolidide improves glucose

tolerance in wild-type, but not DPIV GKO, mice, showing that the improved glucose tolerance is DPIV mediated. Valine-pyrrolidide improves glucose tolerance in GLP-1-receptor-negative mice [115], indicating that this process is not entirely GLP-1 dependent. Long-term administration of DPIV inhibitors on Zucker diabetic fatty rats, a Type II diabetes model, causes an increased meal-induced insulin response as well as reduced body weight compared with controls [116,117]. Concordantly, DPIV GKO mice are protected from high-fat diet-induced obesity and associated insulin resistance and fatty liver (Table 6). This protection involves activation of PPAR α (peroxisome proliferator-activated receptor- α), involved in fatty acid oxidation, and down-regulation of SREBP-1c (sterol regulatory element-binding protein-1c), which is involved in lipid synthesis [8]. This

Table 6 Protection from obesity and insulin resistance in the DPIV-deficient mouse on a high-fat diet

Effect of high-fat diet compared with low-fat diet, with exceptions (*). *DPIV^{-/-} compared with wild-type mice when both strains fed high-fat diet. Results are summarized from the data obtained by Conarello et al. [8].

Parameter	Wild-type mice	DPIV-deficient mice
Weight	↑	
Food intake	↑	↓*
Energy expenditure		↑
Serum insulin	↑↑	↓*
Serum GLP-I		↑*
Serum leptin	↑↑	↑
Fat cell size	↑	
Hepatic lipid	↑	
SREBP in liver		↓
PPAR α in liver		↑
Pancreatic islet hypertrophy	Yes	No
Insulin resistance	Yes	No
STZ-induced hyperglycaemia and β -cell loss	Yes	No

phenomenon could involve extending the half-lives of GIP and VIP, as they influence fat metabolism. GIP has a lipolytic effect on adipocytes.

Even on a regular diet, DPIV-deficient mice and rats are lighter than wild-type by 20 weeks of age [8,118]. The reduced weight of DPIV-deficient and DPIV-inhibitor-treated rodents might be caused by increased levels of NPY, GLP-1 and peptide YY, which influence appetite [119,120] and/or the regulator of lipid and carbohydrate metabolism PACAP38 [24]. A successful pharmacological treatment for obesity probably requires simultaneously targeting the intertwined compensatory mechanisms that regulate food intake and fat storage. The multiple effects of DPIV inhibition show promise for DPIV inhibition becoming a significant part of such a treatment [46]. The possibility that DPIV inhibitors could also be useful in insulin-dependent (Type I) diabetes treatment is under investigation [8,121].

NPY

NPY is the most readily hydrolysed natural substrate of DPIV. Its primary importance is its potential role in appetite control but it is also involved in the control of energy homeostasis, blood pressure, angiogenesis, immune responses and behavioural stress responses. NPY has at least five receptors, Y1, Y2, Y4, Y5 and Y6. DPIV-truncated NPY, NPY 3–36, is unable to bind to the Y1 receptor, but is an agonist on the Y2 and Y5 receptors.

Human endothelial cells express NPY, DPIV and the NPY receptors Y1 and Y2 [122]. NPY is a potent noradrenergic sympathetic vasoconstrictor, acting via the Y1 receptor that is not triggered by NPY 3–36. Thus

DPIV may aggravate hypotension during shock states. In addition, truncated NPY triggering the Y2 and Y5 receptors could promote angiogenesis [122,123].

Y1, Y2, Y4 and Y5 in the hypothalamus and thalamus are important in regulating appetite and energy expenditure [124]. Influences of NPY on anxiety and on bone formation are mediated by the Y2 receptor in the hypothalamus [125,126].

Stimulated macrophages produce NPY and lymphocytes express NPY receptor Y1. NPY influences lymphocyte proliferation and adhesion and is chemotactic for monocytes, so NPY cleavage by DPIV may dampen immune function. Indeed, NPY and peptide YY can exacerbate ConA (concanavalin A)-induced subcutaneous inflammation in the rat and this effect increases with administration of a DPIV inhibitor [65] (Table 4).

Chemokine inactivation by DPIV

Chemokines have fundamental roles in the immune system in leucocyte trafficking regulation, maturation and homing of lymphocytes and the development of lymphoid tissues. Soluble chemokines bind to proteoglycans on endothelial cell surfaces and in ECM. DPIV inactivates or alters the specificity of many chemokines (Table 1; for reviews, see [14,17,18]). The chemokines most rapidly cleaved by DPIV are CXCL12 [SDF (stromal derived factor)-1 α and -1 β] and CCL22 [25,127]. DPIV-mediated inactivation of CXCL12 and CCL3 increases chemoattraction of monocytes. CXCL12 binds to the ECM component heparan sulphate and, while bound, it resists cleavage by DPIV [128]; however, the CXCL12 found in serum is predominantly the DPIV-cleaved form [129]. CXCL12 is a ligand of the HIV coreceptor CXCR4 and blocks infection of macrophage tropic HIV strains. DPIV and CXCR4 are co-expressed on activated T-cells in blood and liver [130,131].

The potential roles of DPIV in HIV infection relate to altering by cleavage the HIV-inhibiting capacities of the chemokines CCL5 [RANTES (regulated upon activation, normal T-cell expressed and secreted) and CXCL12 [25,132], binding gp120 of HIV [133] and binding the HIV tat protein [134]. These phenomena may contribute to the selective reduction in the numbers of CD4⁺/CD26⁺ cells in HIV infection. Whether DPIV has a net effect on HIV infection *in vivo* is unclear [135].

The hydrolysis of CXCL12 by DPIV is important in the regulation of haematopoietic stem cell production and recruitment. Unlike most chemokine-receptor pairs, CXCR4 and CXCL12 are a non-promiscuous pair. CXCL12 attracts CD34⁺ stem cells into tissues. Following DPIV-mediated cleavage, CXCL12 3–68 is a CXCR4 antagonist [27,67], so DPIV might reduce stem cell recruitment. Indeed, the DPIV inhibitor diprotin A enhances resting bone marrow cell CXCL12-mediated chemotaxis [67,71] and enhances the efficiency of bone marrow cell transplantation [64]. Surprisingly then,

G-CSF (granulocyte colony-stimulating factor)-mediated haematopoietic cell mobilization is impaired in the DPIV GKO mouse and by DPIV inhibitor treatment of wild-type mice [67,71]. In contrast, mice deficient for MMP9 or deficient for neutrophil elastase and cathepsin G, which digest both CXCL12 and CXCR4, exhibit normal neutrophil mobilization by G-CSF [136]. These experiments suggest that DPIV is important for G-CSF-induced bone marrow cell mobilization in resting mice.

In a different mouse model using cyclophosphamide-induced haematopoietic cell depletion, inhibition by Val-boro-Pro of a DPIV-related enzyme, possibly FAP, accelerates recovery from neutropenia [61]. These experiments have opened a very interesting new area of study in the DPIV family. DPIV inhibitors could prove useful for accelerating the immune system restoration that follows chemotherapy, for enhancing stem cell recruitment in tissue repair and regeneration and for enhancing the effectiveness of cord blood transplantation.

CXCL12 is crucial for stem cell recruitment to infarcted myocardium [137]. Whether DPIV inhibitors can increase stem cell recruitment to ischaemic heart requires investigation.

FUTURE DIRECTIONS

The broad range of biological systems influenced by DPIV, an enzyme activity of every animal down to the simplest archaeobacteria, makes its understanding complex and interesting. There are some of the interesting questions to address. (i) How is DPIV involved in the control of appetite? The answer will probably be complex and may involve NPY, peptide YY, PACAP38, GLP-1 and GLP-2 [24]. (ii) What are the roles of DPIV, FAP, DP8 and DP9 in tumours? What effects would enzyme inhibitors have on tumours *in vivo*? Would certain inhibitor selectivities be anti-angiogenic? (iii) Would long term administration of DPIV inhibitors interfere with leucocyte recirculation and thereby immune responses in humans? This is difficult to predict as no *in vivo* effect of DPIV on lymphocyte chemotaxis has been demonstrated. (iv) Can DPIV inhibitors enhance all CXCL12 mediated events? Perhaps DPIV inhibitors will be found to increase stem cell recruitment to all organs.

CONCLUSIONS

Potential therapeutic applications of DPIV inhibitors have fuelled interest in understanding the biological roles of DPIV. Such efforts are confounded by the ubiquitous expression of DPIV, inhibitor selectivity and the variety of identified substrates. DPIV is not essential, but is such a useful enzyme that all animal species express it. Interestingly, bacterial 'DPIV' sequences are more similar to DP8 and DP9 than to DPIV. The enzyme activity's

ancient and primary function is probably nutritional, providing more complete proteolysis of food. In mammals this is probably the role of DPIV in enterocytes, saliva and bile. Perhaps expression by various other cell types conferred additional selective advantages. The selective advantage of DPIV expression in a mammal is a net outcome derived from the benefits and drawbacks of DPIV expression by several cell types, including endothelium, lymphocytes and epithelial cells of the gut, kidney, endocrine organs and liver in a variety of nutritional, injury and disease situations. We are gradually teasing out the many biological roles that contribute to the net outcome of DPIV expression and cautiously determining the circumstances in which selectively inhibiting its peptidase activity is beneficial. The discovery and characterization of the DPIV-related peptidases should speed this process. The development of selective inhibitors of DPIV proteolytic activity and identification of ligand-binding activities in this gene family would lead to rapid advances in understanding DPIV biology.

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