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Dipeptidyl peptidase 8 and 9 - guilty by association?

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1. ABSTRACT

Dipeptidyl peptidases (DP) 8 and 9 are members of the DPIV enzyme family. Other members include DPIV, fibroblast activation protein (FAP) and the non-enzymes DP6 and DP10. DPIV family members have diverse biological roles, and have been implicated in a range of diseases including diabetes, cancer, inflammatory bowel disease, multiple sclerosis (MS), arthritis and asthma. While DP8/9 biological functions are yet to be established, they have been predicted to have similar roles to the other DPs due to high sequence similarities within the active site of the enzymes. While there is mounting evidence towards the involvement of DP8 and/or DP9 in innate and acquired immunity, direct proof for the link between DP8 and DP9 and human disease is yet to be definitively shown, thus DP8 and 9 proteins remain guilty by association.

2. INTRODUCTION

DP8 and DP9 are members of the DPIV gene family (S9b), an atypical serine protease family with a reversed catalytic motif: the nucleophilic Ser residue precedes the Asp and His residues. Other S9b members include the extensively researched DPIV, fibroblast activation protein (FAP) and the non-enzymes DP6 and DP10 that lack the catalytic serine. DPIV and the other enzyme members cleave dipeptides from the N-terminus of substrates with a preference for proline in the penultimate position. The S9b proteins are characterised by a pair of conserved Glu residues in the propeller region that are critical for the dipeptidyl peptidase activity of the enzyme members (1-3).

DPIV, also called CD26 due to its role in T cell activation, is a 766-amino-acid, type-II integral membrane

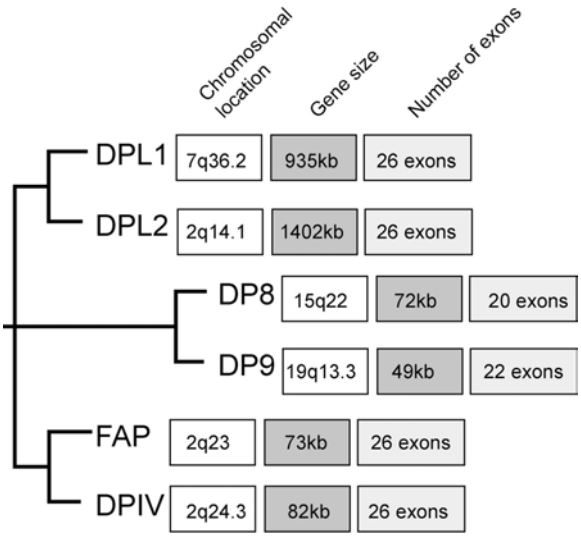

Figure 1. S9b phylogenetic representation. The S9b family is comprised of three highly homologous protein pairs: DP6/10, DP8/9, DPIV/FAP. The DPIV and FAP genes are found on the same chromosome and have similar size and exon structure. DP6 and DP10 share large gene sizes and exon structure. DP8 and DP9 share similar exon structure.

protein that cleaves dipeptides from the N-terminus of substrates, including many chemokines, neuropeptides and peptide hormones. DPIV has the ability to bind both adenosine deaminase (4) and the extracellular matrix (5, 6). Due to the broad range of DPIV functions it has been implicated in type II diabetes (7-18), arthritis (19, 20), HIV infection (21-25), cancer (26-29) and immune function (22, 30-41), as reviewed in (42, 43).

In contrast, FAP is not expressed in the adult system except at locations of tissue remodelling, such as tumours and liver disease (44, 45). FAP is also a type II integral membrane-bound glycoprotein that is unique amongst the DPIV family as in addition to exhibiting DP activity it has endopeptidase and gelatinase activity (5, 45-52). It has been suggested that in stromal cells FAP exhibits DP enzyme activity, disrupting the autocrine/paracrine loop and enhancing the growth of tumour mass, while in cancer cells FAP deploys its collagenase activity to invade the surrounding tissue (53).

In 1992, DP6 protein was first isolated from bovine and rat brains by Wada *et al* (54). Since then, it has had various names including DPPX, BSPL and DPL1 protein. Eleven years later, DP10 was identified in human brain cDNA and mapped to chromosome 2 (55, 56). Like DP6, DP10 has various names including DPPY and DPL2 protein. Despite the absence of DP activity, DP6 is essential for several processes. In studies with murine *rump white (Rw)* mice the loss or rearrangement of inversion breakpoints proximal to *DP6* disrupted the *DP6* gene. Homozygotes for the mutation were embryonic lethal and heterozygotes displayed pigmentation defects, suggesting that DP6 may have an important role in embryonic development (57). DP6 is an essential component of neuronal potassium channels (Kv) where it associates with the channels' pore-forming subunits to facilitate their trafficking and membrane targeting (58). Like DP6, DP10 is a component of the Kv4 channel complex (59). DP10 mRNA was expressed in a number of multiple sclerosis cDNA libraries (60) suggesting levels may be up-regulated in the brains of patients with this disease. Interestingly, several of the brain regions where DP10 is expressed are associated with optical reflexes and correlation centres for auditory reflexes (60). Furthermore, DP10 has been implicated in modulation of the asthmatic airway and associated inflammation in an asthma setting (61, 62).

Comparison of DP8 and DP9 with the other S9b proteins from the DNA to the protein level may provide clues as to the nature of DP8 and DP9 functions. This review will explore the current evidence at gene, protein and tissue level for this gene family in order to speculate the roles of DP8 and DP9 proteins *in vivo*.

3. DP GENE STRUCTURE AND EXPRESSION STUDIES

Gene expression patterns and cellular localisation can give potential clues to a proteins role *in vivo*. The S9b gene family, groups into three pairs of similar proteins when phylogenetic analysis is performed. As depicted in Figure 1, DPIV and FAP, DP6 and DP10 and DP8 and DP9 form homologous pairs. DPIV and FAP share 40% amino acid (aa) identity but display different tissue expression and biological roles. DP6 and DP10 share 53% aa identity and both are predominantly expressed in the brain and both function as critical components of Kv4 channels. While, DP8 and DP9 share 58% aa identity and ubiquitous mRNA expression, their specific functions and whether they share similar *in vivo* roles is still unknown. S9b gene expression studies are further complicated by the occurrence of variant gene transcripts, in particular DP6, DP10, DP8 and DP9, all alternate use of the first exon increasing the potential for differential regulation and function within each gene.

DPIV protein is widely expressed, and as such, can have different roles depending on the cell or tissue type. DPIV is expressed in all organs by capillary endothelial cells and on activated T-cells where it is known as CD26. It's protein expression on B-cells can also be induced by pokeweed mitogen (PWM) (38). DPIV/CD26 is expressed at low levels by some resting T cells but cell surface expression increases 5 to 10 fold after stimulation with antigen or anti-CD3 plus interleukin (IL)-2 or with mitogens such as phytohemagglutinin (63, 64). Furthermore, DPIV/CD26 expression can be induced by IL-12 on T cells (65).

Conversely, FAP protein expression is restricted to sites of tissue remodelling during development, tissue-repair and carcinogenesis (45, 65-68). Despite DPIV and FAP being located adjacent to each other on chromosome 2 (Figure 1) the strict regulation of FAP reveals distinct roles for the two enzymes. However, in reactive fibroblasts of healing wounds FAP and DPIV form heterodimers (44)

displaying a combined function under particular regulatory signals. Furthermore, DPIV and FAP form complexes on endothelial cells of capillaries facilitating extracellular matrix degradation (69). The DPIV gelatin-binding domain was vital for DPIV gelatinase degradation by FAP demonstrating distinct binding and activity-dependent roles for the enzymes in the complex (69). In contrast over-expression of both wild-type and enzyme negative FAP proteins in 293T cells enhanced apoptosis and increased cell adhesion and migration demonstrating that these affects were occurring via an activity-independent mechanism (70).

While DPIV and FAP appear to have distinct expression patterns and have both independent and shared roles *in vivo*, DP6 and DP10 are mainly expressed in the brain and share common roles as auxiliary subunits in Kv4 channels (58, 59). A number of alternative transcripts exist for both DP6 and DP10 and each form is differentially expressed. Alternative splicing at the N-terminus gives rise to a number of transcript variants for DP6 (L, S, K) (71) and DP10 (a, b, c, d) (72). The DP6 long form (DP6-L) was only detected by *in situ* hybridisation in the olfactory bulb, cerebellum, and the habenula of rat brain whilst the DP6-S and K forms were more widely distributed (71). In human brain, DP6 transcript variants are expressed in similar regions (73). DP10-c and DP10-d forms were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in human brain, adrenal gland and pancreas, whereas DP10-a and DP10-b forms were only detected in the brain (72). While DP6 and DP10 share common functions, the differential expression and localisation of alternative gene transcripts suggest potential transcriptional regulation of their Kv4 channel roles.

Originally, an 882aa (AF221634) version of DP8 was cloned from a human testis cDNA library and activated peripheral blood mononuclear cell cDNA (74). Gene expression analysis by Northern blot determined DP8 like DPIV to be ubiquitously expressed with the highest expression in testis and placenta (74). During DP8 cloning other partial cDNAs for DP8 were characterised which lacked various DP8 exons including those containing residues essential for the catalytic activity of DP8 (74). More recently a longer 898aa DP8 isoform (AY354202) resulting from an alternate exon 1 was cloned from human testis (75). RT-PCR with primers that detect all variants demonstrated expression of the DP8 gene in the liver, and a number of B- and T-cell lines (74). Using a specific N-terminal primer, quantitative fluorescent-RT-PCR showed that the larger 898aa DP8 splice variant is expressed at significantly higher levels in adult testis tissue compared to foetal tissue (75). Multi-tissue PCR followed by Southern blot analysis, determined that the mRNA for the long 898aa form was predominantly expressed in the testis and pancreas, with weak expression in placenta and lung. Interestingly, in this analysis the 898aa variant was not expressed in blood leukocytes, while the 882aa variant was detected and cloned from this cell type (74, 75). The differences in mRNA tissue expression profiles for the two versions suggest distinct roles and that the two forms may be differentially regulated. Due to the high expression of the mRNA for the 898aa form of DP8 in testis it was suggested that this form plays a role in spermatogenesis and male fertility (75). Although no known targeting motifs are present in this extra 16 amino acid N-terminal region, the 898aa DP8 and DP8 proteins resulting from the non-enzyme DP8 mRNA variants described above may exhibit their novel functions via ligand-binding and not via enzyme activity.

Like DP8, DP9 was found to have two variants comprising the controversial 863aa version (AY374518) and the longer 892aa version (DQ417928) (76). There is also evidence that DP9 of a longer form greater than 960 aa (AF542510) exists but as yet the start codon for this form has not been found. An initial report suggested that the 863aa version was inactive in mammalian cells (77). However, Ajami K *et al* (2004) and Qi SY *et al* (2003) later demonstrated that the 863aa version was enzymatically active (60, 76). Recently, Bjelke J.R. *et al* (2006) again reported inactivity with their DP9 863aa expressed in Sf9 cells , while in-house DP9 863aa expressed in Sf9 cells was active but labile (M. R. Pitman unpublished results). The reason for the discrepancy remains unclear. Whilst there is controversy surrounding the activity of the short and long DP9 forms, all studies agree on the ubiquitous nature of DP9 mRNA expression. Northern blots using a 3' probe which could detect all forms determined DP9 gene expression was highest in liver, heart, spleen, blood leukocytes and skeletal muscle (76). Interestingly, a probe for the 5' DP9 (AF542510) form displayed predominant expression in skeletal muscle and liver. It was also noted that many expressed sequence tags derived from diseased tissue contained sequence present in the 960aa DP9 (76) suggesting a differential role for the long form in human disease. More studies are required to further elucidate differences between the different forms of DP9. It is yet to be discovered whether differences in the DP8 and DP9 tissue expression levels and transcript variants are indicative of distinct cellular roles. Using DPIV and FAP as an example it is also possible that DP8 and DP9 share common functions but have distinct regulation.

4. POST-TRANSLATIONAL MODIFICATIONS OF DP PROTEINS

At the protein level post-translational modifications allow for variation in protein regulation. Processes such as glycosylation, phosphorylation and proteolysis can influence the structure, substrate affinity and ligand binding of proteins and alter trafficking and regulatory processes. While specific proteolytic processing of DP proteins has not been reported, non-specific DP8/9 degradation in mammalian cell extracts demonstrate protease affinity for DP8 and DP9 (M.R. Pitman, unpublished results)

DPIV is highly glycosylated (79) and traffics to the extracellular plasma membrane (80). Although no splice variants have been reported for the DPIV gene, a soluble DPIV form exists; thought to occur through proteolytic cleavage of the membrane-bound form into the circulation (81). The existence of both a membrane-bound

and soluble plasma DPIV increases the substrate repertoire available for cleavage via DPIV and the overall versatility of the enzyme (65 , 82). Similarly, FAP, DP6 and DP10 are membrane proteins, but soluble versions of these proteins have been reported (83, 62). Using tagged recombinant proteins, while DP8 and DP9 have been localised to the cytoplasm they also colocalize with wheat germ agglutinin in the Golgi apparatus of COS-7 cells (74, 76). This suggests that DP8 and DP9 may have alternative locations depending on regulation. Due to the lack of glycosylation sites on DP8 it is unlikely that the protein would be stable extracellularly.

Dubois *et al* recently published the first study purifying and identifying a natural DP9-like protein from bovine testes (85). Active DP9-like protein was purified from homogenates by a combination of ion exchange, lectin affinity, metal-chelating, hydrophobic interaction and inhibitor-affinity chromatography steps. Although DP9 has two potential glycosylation sites, during purification the DP9-like protein did not bind the lectin-affinity ConA sepharose column suggesting that natural DP9 is not glycosylated. The enriched DP9-like protein was confirmed to be DP9 by immunoblot and displayed similar size, stability and kinetic properties to recombinant DP9. An additional serine-protease was purified, displaying similar properties to recombinant DP8, however the identity could not be confirmed by anti-DP8 immunoblotting (85). The antibody may not have recognized bovine DP8. Interestingly the testis is a source rich in DP8 mRNA but DP9 was preferentially purified from this tissue. It will also be important for the N-terminus of this natural DP9 to be determined to see whether it is the 863aa or 898aa or even a longer form. Recently antibodies (Abcam, Cambridge UK) have become available for immunohistochemistry experiments (61). The development of reagents such as these is critical for determining the location, abundance and the primary sequence of native DP8 and DP9 in normal and diseased tissue.

DPIV post-translational modifications include glycosylation and phosphorylation. Insulin-dependent post-translational phosphorylation of DPIV by c-Src tyrosine kinase has been shown to occur (86). The DPIV c-Src phosphorylation was restricted to the Golgi and endosomal compartments. Therefore, cytoplasmic DP8/9 could only be processed in this manner by being first trafficked to the Golgi. However, tyrosine phosphorylated DP8 was identified in a murine WEHI-231 B cell lymphoma cell line proteomic study (87). This was the first indication of post-translational modifications for DP8, and indicates that like DPIV, DP8 may also act as a signalling molecule under some circumstances. The kinase responsible for DP8 tyrosine phosphorylation is as yet unknown but its identification would be beneficial for determining signalling cascades, which in turn may imply DP8 function. Interestingly, the phosphorylated DP8 Tyr325 is a conserved S9b residue. When comparing S9b structures (2, 88, 89), this residue is adjacent to the extended dimeric arm and may therefore be involved in altering the multimeric status of the proteins or altering binding affinity for protein-protein interactions. While DP9 phosphorylation is yet to be shown, the high homology with DP8 and shared cytoplasmic location suggest that this may be a common process. Knowledge of phosphorylation and other post-translational regulation of the DP8 and DP9 proteins will be vital to understanding their *in vivo* functions.

5. STRUCTURAL ELEMENTS OF DP8/9: COMPARISON TO DPIV AND FAP

Structural information is key to understanding protein function. To date, the crystal structures of DP8 and DP9 have not been solved. Computational modelling (90, 91) has revealed high similarity to other family members within the alpha-beta hydrolase domain. However, within the beta-propeller domain, vast differences are obvious when comparing DP8 and DP9 to other family members. The primary sequence of this domain is larger in DP8 and DP9 compared to DPIV and shares only 11-17% amino acid sequence identity compared to 36% in the alpha-beta hydrolase domain (74). DPIV uses the propeller domain to bind to other proteins (e.g. adenosine deaminase) so this diversity in the beta-propeller domain suggests that DP8 and DP9 may have autonomous roles to other DPs within the cell, interacting with other as yet unidentified cytoplasmic proteins via this domain.

The alpha-beta hydrolase domain, containing the catalytic triad is highly conserved within all DPIV family members (92). The more variable beta-propeller contains the essential glutamic acids required for orientation of substrates as depicted with NPY (neuropeptide Y) in the DPIV structure (Figure 2) (1). The substrate pockets are largely conserved and are formed by the two domains (Figure 2). The DPIV and FAP active sites are similar despite differences in substrate binding preferences (3, 88). Furthermore, the DP6 substrate pocket structure is similar to both DPIV and FAP despite the lack of catalytic function (89).

Differences in DP8/9 protein structure within the variable beta-propeller region can be partly attributed to large inserts present in the DP8 and DP9 sequences. The low homology loop-like regions make it difficult to predict the three-dimensional structure of this region. Additionally, the N-terminal region adjacent to the beta-propeller domain is much longer than DPIV and FAP (approximately 80 amino acids). It is likely that this region contains extra secondary structural elements like prolyl oligopeptidase/prolyl endopeptidase (PEP/POP) (90). Interestingly, this region has no homology to other structures suggesting that the region may contain a series of loops or unique structural elements. These variable loop regions within DP8 and DP9 could also contain unique ligand binding motifs or structural elements that facilitate differential regulation and processing.

Structural elements of DP8 and DP9 substrate pockets are of great interest for predicting potential substrates and inhibitors. The P1 pocket residues are conserved between family members, and are formed by residues of the alpha-beta hydrolase domain. However, non-conserved loops from the beta-propeller are predicted

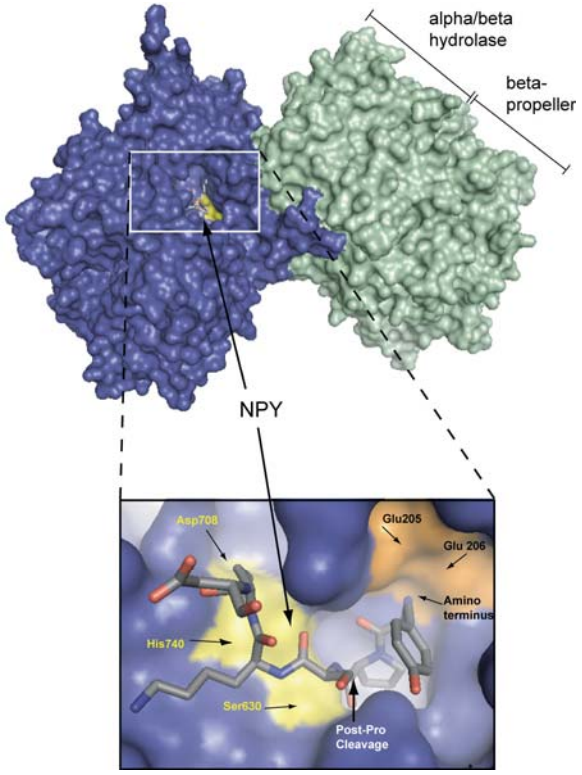

Figure 2. DPIV structure with bound NPY. Figures were made using Pymol (DeLano, 2002). The dimeric DPIV structure (PDBcode = 1R9N) is represented in surface view (blue and green, top). NPY is represented in sticks with functional oxygen groups represented in red and nitrogen in blue. The active site is comprised of the upper alpha/beta hydrolase domain and lower beta- propeller domains of each monomer. The active site (bottom) contains conserved Glu^{205}, Glu^{206} residues (orange, beta- propeller domain) required to bind the amino terminus of the substrate for catalysis by Ser^{630}, Asp^{708} and His^{740} (yellow, alpha/beta hydrolase domain). NPY is cleaved by the catalytic serine after the penultimate proline residue as indicated by the arrow.

in models to form the P2 substrate pocket of DP8 and DP9 (90, 91). Due to amino acid insertions and deletions in the less-conserved beta-propeller domain, S9b protein sequence alignments are difficult. Different arrangements of alignment gaps within this P2 loop produced alternative residues lining the P2 pocket (91). These differences would alter the electrostatic properties of the pocket. Unfortunately, the true arrangement will only be discovered by crystal structure determination. Despite this, Rummey *et al* predicted that the DP8 and DP9, P2 pockets were larger and less hydrophobic than DPIV (91). Properties such as this may be exploited for the design of selective DP8 and DP9 inhibitors, and substrate profiling. Additionally, inhibitors that select between DP8 and DP9 would be valuable tools and could be used to determine their distinct roles.

Originally, DP8 and DP9 proteins were believed to be monomeric (74, 76), however further analysis revealed that they were in fact dimeric proteins (78, 93). This difference in the quaternary structure of the proteins was most likely due to C-terminal tag interference, as it wasn't until an N-terminally tagged protein was expressed, that the dimeric properties of DP8/9 were discovered (93). Thus, DP8 and DP9 share this dimeric state with all DPIV family members (DPIV, FAP, DP6 and DP10) At high concentrations tetrameric DP8 has been observed (93) which correlates with previous observations of tetrameric human DPIV (3).

6. DISCOVERING POTENTIAL DP8/9 SUBSTRATES

Knowledge of natural substrates will shed light on DP8 and DP9 cellular roles. As yet no natural DP8/9 substrates are known, however important structural and functional information can be gleaned from *in vitro* studies with artificial substrates.

Dipeptide substrate specificity profiles of the DP8 enzyme determined that DP8 prefers proline at the P1 position, with very little cleavage observed for other residues at P1 (93). At the P2 position, DP8 prefers basic and hydrophobic residues. This differs slightly from DPIV which cleaves acidic residues at levels approximately 20-fold higher than DP8 (93). Although little cleavage was observed with alanine at the P1 position, DP8 and DP9 were shown to cleave the NH_2-His-Ala N-termini of glucagon-like peptide (GLP)-1 and GLP-2 *in vitro (*78). Interestingly, peptide YY (PYY) was cleaved by DP8, 250-fold slower than NPY despite having identical P1 and P2 residues (NH_2-Tyr-Pro). The structures of NPY and PYY (Figure 3) suggest that the folded PYY structure may have hindered DP8 binding. This highlights the importance of substrate structure for recognition and cleavage in longer substrates.

DP8 cleaves the DPIV substrates stromal-cell derived factor (SDF) -alpha and -beta (CXCL12) (NH_2-Lys-Pro), interferon-inducible T cell chemo-attractant (ITAC, CXCL11) (NH_2-Phe-Pro) and inflammatory protein 10 (IP10, CXCL10) (NH_2-Val-Pro) *in vitro* (94). This work confirms DP8's preference for basic, hydrophobic or aromatic residues in longer, non-chromogenic substrates. The structures of these substrates also contain highly flexible N-terminal loops (Figure 3). Twelve other chemokines with a Ser, Ala or Pro in the penultimate (P2) position were screened but neither DP8 or DPIV cleaved these. Finally four chemokine substrates were cleaved by DPIV and not by DP8. This suggests that DPIV and DP8 have different preferences for *in vivo* substrates despite having similar P1 -P2 dipeptide substrate specificity profiles. Again this could be due to P1`-P2` residues or the folded structures of the substrates (Figure 3). It is expected that DP9 will show similar preferences however DP9 cleavage of these substrates is yet to be tested.

While DP8 and DP9 have been shown *in vitro* to cleave several DPIV substrates, it is likely that many unique substrates are yet to be discovered. It is also possible due to their cytoplasmic localisation that few DP8/9 substrates will overlap with DPIV substrates.

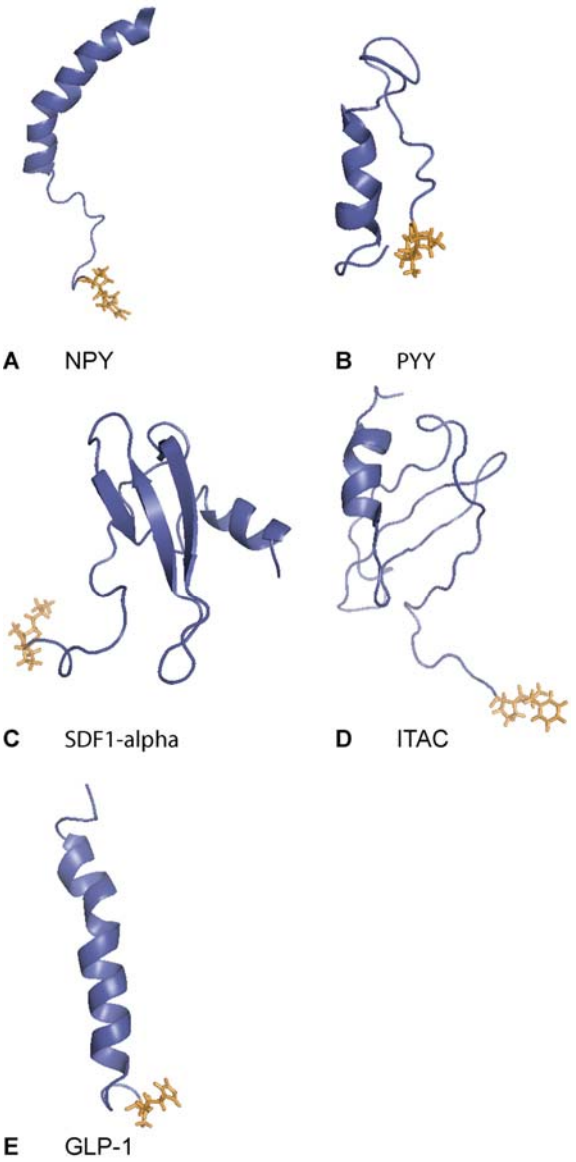

Figure 3. *In vitro* DP8 substrates. Structural representations of A NPY (PDBcode= 1RON), B PYY (PDBcode= 1QBF), C SDF1-alpha (PDBcode= 2SDF), D ITAC (PDBcode= 1RJT), and E glucagon-like peptide 1 (PDBcode= 1DOR) were made using Pymol (DeLano, 2002). Substrate structure is represented in ribbon. N-terminal dipeptide residues P1 and P2 residues are represented in orange sticks. The N-terminal regions are flexible loop regions. DP8 cleaves NPY, SDF1, ITAC and GLP-1 *in vitro* but not PYY despite matching the N-terminus of NPY. This may be due to the N-terminal region or the folded structure of PYY compared to NPY.

However, in the context of chemokine cleavage it has been proposed that DP8 could be released into the extracellular space to cleave chemokines or that these chemokines are internalised to the cytoplasm (94).

7. DESIGNING SPECIFIC DP8/9 INHIBITORS TO FURTHER ELUCIDATE THEIR FUNCTIONAL ROLES *IN VIVO*

The design and synthesis of inhibitors that are selective for DP8/9 over other DPs plus inhibitors that discriminate between DP8 and DP9 is paramount to elucidating their functional roles. Despite the lack of DP8 and DP9 crystal structure data, some DP8/9 inhibitors have recently been developed. These DP8/9 inhibitor structures are depicted in Figure 4 along with several DPIV inhibitors that are now know to target DP8/9. The inhibitor IC_{50} values for these compounds are summarised in Table 1. Like for DPIV inhibitors, amino acid analogues were used as lead compounds for inhibitor development. Proline-like ring moieties are common to all DP8/9 inhibitors as shown in Figure 4A-D. Two recent studies developed specific DP8 inhibitors with some selectivity over DPIV and DPII (95, 96). Inhibitors with the piperazine moiety were potent DP8 inhibitors with IC_{50} values ranging from 16-71 nM (95). This group concluded that the best inhibitor was 1- (4,4` -difluorobenzhydryl) piperazine (IC_{50} = 16nM) with 14-fold selectivity over DPIV (95) (Figure 4A). The second study focused on the diaryl phoshonate moiety in the P1 position. A range of amino acid residues were tested in the P1 position for DP8/9 selectivity (96), however lysine in the P2 position displayed higher selectivity for DP8/9 over DPIV. The best compound, lysine 4- acetamidophenyl, conferred >100 fold selectivity over DPIV and 200 fold over DPII (structure not shown). Remarkably, none of the inhibitors developed in these two studies were tested on DP9, but they are likely to have comparable affinities due to the high structural similarity between the two enzymes. The pharmaceutical company Point Therapeutics (recently taken over by DARA Biosciences) have developed two other DP8/9 selective inhibitors PTX1200 and PTX1210 (120, 130) (Figure 4B and 4C) which to date, display the best selectivity for DP8/9 over other DPs (Table 1). Merck have also synthesised a selective DP8/9 inhibitor ((2*S*, 3*R*)-2- (2-amino-3-methyl-1-oxopentan-1-yl)-1,3-dihydro-2*H*-isoindole hydrochloride) (12) (Figure 4D). Despite being designed to inhibit DPIV, isoleucyl thiazolidine (P32/98) (Figure 4E) and Lys (Z (NO2))-pyrrolidide (Figure 4G) display 2-10 fold selectivity for DP8/9 over DPIV and Val-Boro-Pro (Figure 4F) inhibits DP8 , DP9 and DPIV with similar affinities (Table 1) (12). However, in both rats and dogs Val-Boro-Pro or the Merck DP/9 selective inhibitor (Figure 4D) caused anaemia, thrombocytopenia, splenomegaly, multiple organ pathology, alopecia and mortality (12). For determination of the distinct role of DP8 and 9, inhibitors that differentiate between the enzymes will be required. Knowledge of both structure and natural substrates would greatly benefit inhibitor design.

8. POTENTIAL ROLES FOR DP8 AND DP9

Research into DP8 and DP9 expression in leukocytes and lungs has shown some promise in 'teasing out' potential roles for these proteins *in vivo*. Previously, cytoplasmic DP8 and DP9 specific activity was detected in leukocytes (97). This has been made more significant by the recent finding that DP8 and DP9 mRNA and enzyme

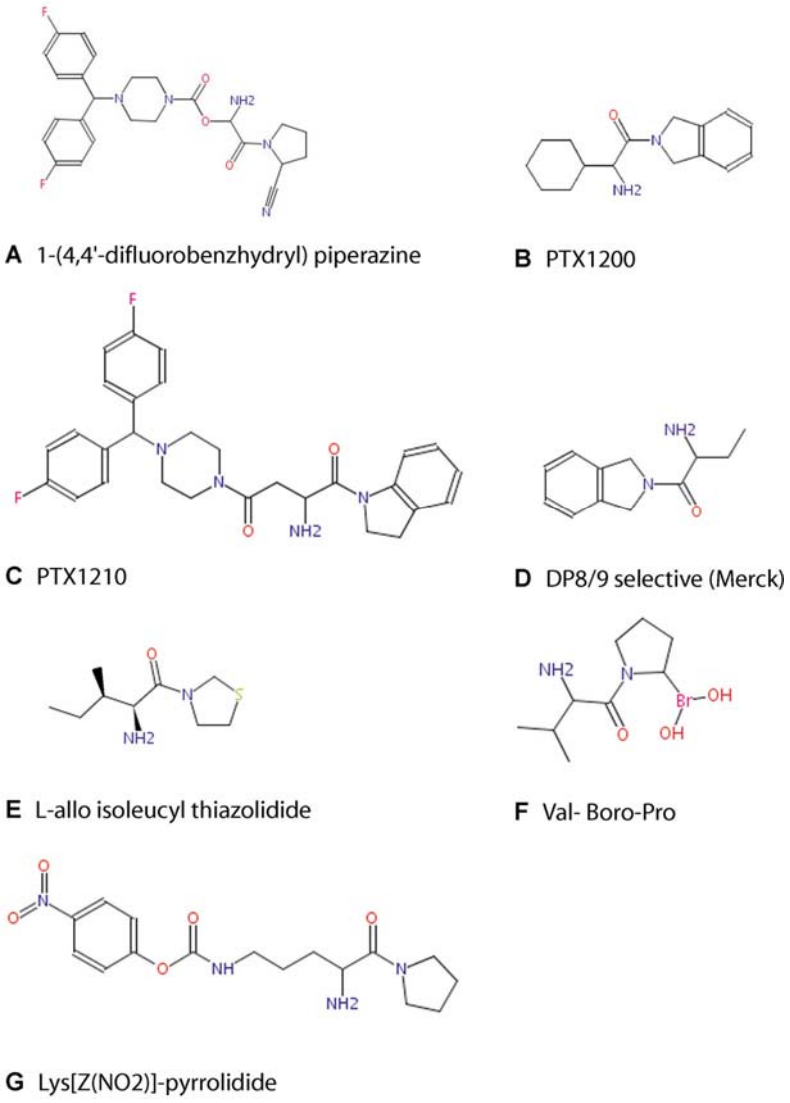

Figure 4. DP inhibitor structures. Compounds A-D are DP8/9 selective inhibitors. Compounds E-G are compounds designed for DPIV inhibition that show affinity for DP8 and 9. Inhibitor structures were produced by http://www.molinspiration.com/. Corresponding IC_{50} values are represented in Table 1.

activity were up-regulated in bronchi during induction of experimental asthma in rats (61), with some of this activity attributed to the presence of leukocytes in the lung. DPIV up-regulation was not observed, however similarly to DP8 and DP9, DP10, a potential marker for asthma, was up-regulated in the bronchial epithelium of the airways. It was suggested that differential DP10 expression was due to expression in the bronchus-associated nervous system as no expression could be detected in whole lung sections. It is possible that DP8, DP9 and DP10 up-regulation in the lung may contribute to the asthma pathology. However, further work is required to determine the specific roles of these proteins in the lung system.

One of DPIV's most publicised roles is in glucose homeostasis which has been extensively researched and reviewed (14, 18, 98-102). The peptide hormones, GLP-1 and GLP-2 and gastric inhibitory peptide (GIP) are expressed and secreted after eating and trigger the secretion of insulin, in turn causing glucose uptake. The extracellular DPIV cleaves the N-terminus of GLP-1 and GIP, rendering them inactive (103). DPIV inhibitors preserve the circulating GLP-1 and GIP levels, improving glucose homeostasis. In diabetes mellitus (type II), where glucose homeostasis is impaired, DPIV inhibitors have been used as therapeutics. Recently, Januvia (Sitagliptin, Merck), the first commercial DPIV-inhibitor-based diabetes therapy was released onto the market (11, 98, 104). Several other compounds are in late stage development; Galvus/Vildagliptin (Novartis), Saxagliptin (Bristol-Myers-Squibb) and Alogliptin/Syr322 (Takeda) (7-10, 104-107). Although DP8 and DP9 were shown to cleave GLP-1 and 2 *in vitro (*78), their cytoplasmic location makes *in vivo* cleavage of these substrates by DP8/9 unlikely.

Table 1. IC_{50} (nM) values for DP Inhibitors

Inhibitor	DP8	DP9	DPIV	FAP	DPII	Ref
A 1- (4,4'-difluorobenzhydryl) piperazine	16	ND	227	ND	>100,000	(95)
B PTX1200	15	36	>27,000	>40,000	>30,000	(127)
C PTX1210	25	105	>50,000	>50,000	>50,000	(127)
D DP8/9 Selective	38	55	30,000	>100,000	>100,000	(12)
E L-*allo* isoleucyl thiazolidide	220	320	460	>100,000	>100,000	(12)
F Val-Boro-Pro	4	11	<4	560	310	(12)
G Lys (Z (NO_2))-pyrrolidide	154	165	1,300	51,000	1,210	(12)

ND = Not determined

Signalling via DPIV/CD26 is well known however the co-stimulatory or T-cell activating properties of CD26 *in vivo* seem to occur via an activity-independent mechanism. Association with the protein tyrosine phosphatase (PTP) CD45 through CD26's cytoplasmic domain causes aggregation of lipid rafts, and facilitates the colocalization of CD45 to the T cell receptor signaling molecules p56 (Lck), ZAP-70, and TCRzeta, thereby enhancing protein tyrosine phosphorylation of other signalling molecules and subsequent IL-2 production (108). In a similar manner, it is possible that DP8 and DP9 also have signalling roles in the cell, separate to their catalytic activity. DP8 over-expression in AD293 cells enhanced staurosporine-induced apoptosis, while DP9 increased spontaneous apoptosis (109). Interestingly, enzyme activity was not required for their impairment of wound healing, apoptosis and cell migration as similar results were observed with wild-type and enzyme negative DP8/9 proteins (109). This work suggests that DP8 and DP9, like DPIV and FAP, can influence cell-extracellular matrix interactions via protein-protein interactions; and this is an activity-independent role.

DP8 and DP9 expression on blood leukocytes and B- and T-cell lines also suggests a role for these proteins in immune processes. The majority of this work has been performed by Point Therapeutics, using their nanomolar DP inhibitor Val-Boro-Pro (PT-100, Talabostat). DP inhibition has also been investigated for the treatment of solid tumours and B-cell malignancies including chronic lymphocytic leukaemia, non-Hodgkin's lymphoma, metastatic colorectal cancer, non-small cell lung cancer and stage IV metastatic melanoma (110-116).

Due to the non-selective nature of Val-Boro-Pro, it is unknown which DP the inhibitor would be acting against in each cancer type, however preliminary work by Point Therapeutics suggests that inhibition of DP8 and DP9 can stimulate innate and adaptive immune responses which could in turn kill tumour cells. As depicted in Figure 5, DP8/9 inhibition by the inhibitor Val- Boro-Pro caused secretion of IL1-beta in macrophages via caspase-1 activation (117). IL1-beta is a pro-inflammatory cytokine implicated in various diseases including inflammatory bowel disease, diabetes mellitus and rheumatoid arthritis. The secretion of IL1-beta in human peripheral blood mononuclear cells induced paracrine signalling via chemokines and cytokines to cell types involved in both innate and acquired immunity. The inhibition dependent caspase-1 activation was confirmed with inhibitors more selective for DP8/9 (PTX1200, PTX1210) (Figure 4B-C, Table 1) (117). At present it is unclear whether inhibition of both DP8 and DP9 is required for this effect or whether the activation of caspase-1 requires only one of these proteins to be inhibited. Earlier observations of DP8-enhancement and DP9-induction of apoptosis suggest similar roles for DP8/9 in other signalling pathways (109). It is also likely that caspase-1 activation is mediated via intermediate signalling molecules. Despite this, the ability for these DP8/9 selective inhibitors to stimulate innate and adaptive immunity responses gives them potential to kill tumour cells. This exciting finding may have potential for a range of therapeutic applications, although more research needs to be done to investigate the underlying mechanism. These inhibitor-induced affects together with Yu *et al*'s study on DP8 and DP9 over-expression, suggest that both activity-dependent and potential signalling/protein binding activity-independent roles for DP8 and DP9 exist (109).

The mechanism of caspase -1 activation is not yet fully understood. In recent years a number of caspase-1 interacting proteins have been discovered including Ipaf (118), Rip2 (119), ASC (120), PAK and Nod1 (121). These proteins bind to caspase-1 via a caspase recruitment domain (CARD). Therefore, DP8 and DP9 are unlikely to interact with caspase-1 directly as they lack CARD domains. However, the caspase-1 interacting proteins COP, Iceberg (122) and CARD-8 (123) inhibit caspase-1 activation. Therefore it is also possible that DP8/9 activate caspase-1 via loss of inhibitory proteins. It is impossible to say at this point which stage DP8 and DP9 are involved in caspase-1 activation as mediation of caspase-1 activation appears to be a complex cascade involving protein-protein interactions, phosphorylation and induced gene expression.

Earlier studies also suggest a potential role for DP8 and 9 in the immune system, as several cytokines are affected by DP8/9 inhibition. Using two DP inhibitors Lys (Z (NO_2))-pyrrolidide (Figure 4F) and Lys (Z (NO_2))-thiazolidide, IL-10, IL-12 and interferon (IFN)-gamma were shown to be down regulated in PWM stimulated T-cells (124). These inhibitors also reduce in a dose-dependent manner IFN-gamma, IL-4, and tumour necrosis factor-alpha production of myelin basic protein-stimulated T cell clones from patients with MS (125), suggesting that inhibition of DP activity may be a useful therapeutic tool in MS, or other autoimmune diseases. It is important to add that while authors of this study speculated that DPIV enzyme was responsible for the changes caused upon DP inhibition, later studies have shown that the inhibitors Lys (Z (NO_2))-pyrrolidide and Lys (Z (NO_2))-thiazolidide are actually more selective for DP8 and DP9 than for DPIV (Table 1) (12).

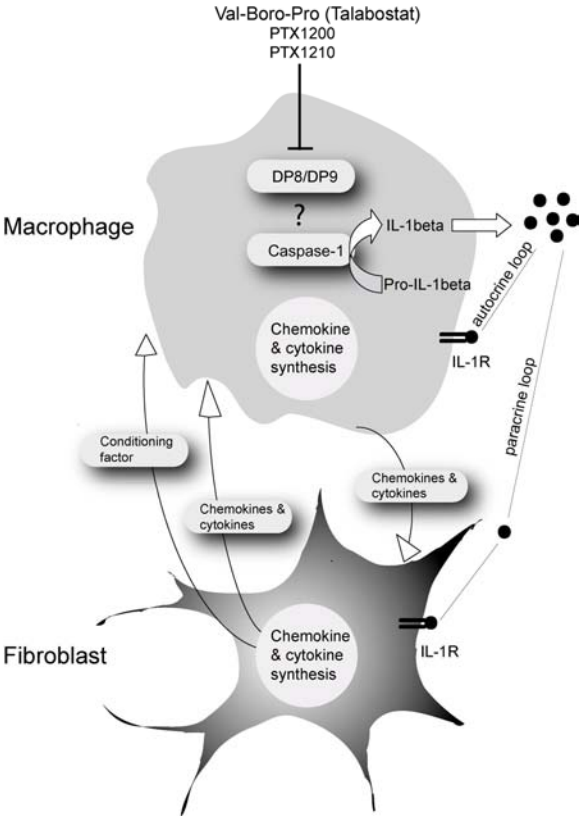

Figure 5. Caspase 1 activation and IL-1 beta production induced by Val-Boro-Pro and DP8/9 inhibitors. Adapted from (117). Inhibition of DP8/9 induces IL-1 beta production via a caspase-1 dependent pathway. The mechanism of caspase-1 activation by DP8/9 is unknown. IL1-beta causes expression of IL-1 receptor in macrophages and fibroblasts via autocrine and paracrine loop responses.

While such *in vitro* studies suggest great promise for DP8/9 inhibitors it is uncertain whether selective DP8/9 inhibitors will be a useful therapeutic tool *in vivo*, as reports of toxicity as discussed previously with selective DP8/9 inhibitors are cause for concern (12). More specifically, *in vitro* studies with the inhibitors revealed that both DP8/9 specific inhibitors and Val-Boro-Pro inhibited T-cell proliferation and IL-2 release while DPIV and DPII inhibitors had no affect (12). These side-affects highlight the importance of having true DPIV selective inhibitors for use as a diabetes therapy and highlight difficulties that DP8/9 selective inhibitors may have as therapeutics. This also implies that the functions of DP8 and DP9, although unclear, may be vital for immune signalling and function.

9. WHAT THE FUTURE HOLDS FOR DP8 AND DP9

At this stage very little is known about DP8 and DP9 *in vivo* functions. While there are definite similarities to DPIV enzyme activity, expression and structure, their longer N-termini and cytoplasmic location suggests that they may bind different substrates or ligands and therefore have unique *in vivo* roles. The effect of DP8 and DP9 inhibition on signalling in macrophages demonstrated an activity-dependent role (117). At this stage it is impossible to say whether these observations are representative of an overall role for DP8 and DP9 or are cell-specific. DPIV and FAP have demonstrated both activity-dependent and activity-independent roles, thus DP8 and DP9 have the potential to demonstrate activity-independent roles also. Using more selective inhibitors, DP8 and DP9 have now been associated with immune function, however this role is unclear. Until further evidence of direct signalling pathways or natural substrates are determined, DP8 and DP9 remain guilty by association.

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Abbreviations: aa: amino acid, DP: dipeptidyl peptidase, DP6-L: DP 6 long form, FAP: fibroblast activation protein, GLP: glucagon like peptide, GIP: gastric inhibitory polypeptide, IL: interleukin, ITAC: interferon-inducible T cell chemo-attractant, Kv4: potassium channel, MS: multiple sclerosis, NPY: neuropeptide Y, PYY: peptide YY, PWM: pokeweed mitogen, RT-PCR: reverse transcriptase -polymerase chain reaction, SDF: stromal-cell derived factor

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