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Prolyl peptidases: a serine protease subfamily with high potential for drug discovery

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Much attention has recently been given to a class of proteases that cleave proteins and peptides after proline residues. This class includes dipeptidyl peptidase IV (DPP IV; also termed CD26), fibroblast activation protein α (FAP; seprase), DPP7 (DPP II; quiescent cell proline dipeptidase), DPP8, DPP9, and prolyl carboxypeptidase (PCP; angiotensinase C). More distant members include prolyl oligopeptidase (POP; post proline cleaving enzyme) and acylaminoacylpeptidase (AAP; acylpeptide hydrolase). The DPPs and related proteins contain both membrane-bound and soluble members and span a broad range of expression patterns, tissue distributions and compartmentalization. These proteins have important roles in regulation of signaling by peptide hormones, and are emerging targets for diabetes, oncology and other indications.

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Abbreviations

AAP	acylaminoacyl peptidase
DPP	dipeptidyl peptidase
FAP	fibroblast activation protein α
GCP II	glutamate carboxypeptidase II
PCP	prolyl carboxypeptidase
POP	prolyl oligopeptidase
QPP	quiescent cell proline dipeptidase
VbP	L-valinyl-L-boroproline

Introduction

Several actively pursued and potential drug targets can be found in protease clan SC (based on standard nomenclature, [1]). The enzymes from this clan that are considered in this review have serine nucleophiles and catalytic triads, ordered serine, aspartic acid then histidine in the primary sequence (see Figure 1 for a phylogeny). They are members of the α/β -hydrolase fold family. In addition to being similar in overall structure and ordering of the catalytic triad, these enzymes generally share the ability to cleave

Pro-Xaa peptide bonds (where Xaa is any amino acid). However, the placement of the scissile bond within the substrate varies, as clan SC contains dipeptidyl aminopeptidases, carboxypeptidases and endopeptidases. The term ‘DPP IV-like’ (DPP, dipeptidyl peptidase) is often used for this family of proteins, to recognize the status of DPP IV as the most widely studied member of the family and to acknowledge the loose association of these proteins that share structural or functional similarity with DPP IV. This review focuses on the potential roles of members of this class as targets for chemotherapeutic intervention.

DPP IV

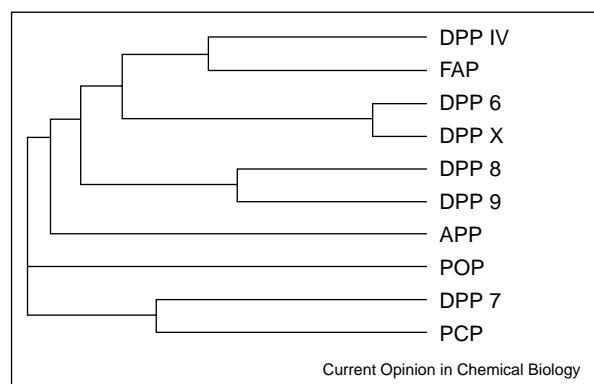
DPP IV is the most intensively studied of the enzymes in this class. It was among the first of the enzymes in the family to be identified, and has been studied in several contexts, among them as a circulating protease, a T cell signaling molecule, and an adenosine deaminase binding protein. Recent efforts have culminated in the attainment of several important goals, including the production of a DPP IV knockout mouse, the elucidation of the tertiary and quaternary structure of DPP IV, and the first human trials of DPP IV inhibitors, which show efficacy in the treatment of type 2 diabetes.

Substrates

DPP IV is an abundant, widely distributed serine protease that is able to cleave dipeptides containing proline, alanine or one of several other amino acids at the penultimate position from the amino termini of substrate proteins [2]. It is present in both soluble and membrane-bound forms. Because DPP IV can cleave dipeptides with a penultimate proline or alanine residue, it is possible that numerous proteins could be substrates for DPP IV. Importantly, a recent report demonstrated that for potential substrate proteins treated with pure DPP IV *in vitro*, the kinetics of substrate cleavage were highly variable [3•]. Among the substrates in this study, k_{cat} values for cleavage of full-length chemokines ranged over almost four orders of magnitude, whereas the k_{cat} values for cleavage of the corresponding N-terminal nonapeptides as well as for colorimetric dipeptide substrates were quite similar to one another. These results suggest that structural elements outside of the immediate primary sequence of the cleavage site are vital in determining whether a protein will be cleaved by DPP IV. As a corollary, predictions of whether a protein is a substrate for DPP IV must be verified by measuring cleavage of the proposed substrate.

Even if a protein can be shown *in vitro* to be cleaved by DPP IV, two questions follow. First, is the protein cleaved

Figure 1



Phylogeny of human proteins related to DPP IV. Similar length regions (ca. 500 aa) including the catalytic domain of the indicated proteins were aligned using the ClustalW server at EMBL-EBI (<http://www.ebi.ac.uk/clustalw/>). Genebank accession IDs for the protein sequences used are: gi|18765694: DPP IV; gi|20140021: FAP; gi|18765696: DPP 6; gi|1169414: DPP X; gi|11095188: DPP 8; gi|17865311: DPP 9; gi|556514: AAP; gi|4506043: POP; gi|7019511: DPP 7; gi|1172047: PCP.

by DPP IV *in vivo*? Second, does the cleavage change the properties of the cleaved substrate? The DPP IV substrates that have recently received the most attention are incretins, a class of hormones involved in glucose homeostasis; neuropeptides; and chemokines, cytokines involved in lymphocyte chemotaxis. Chemokines are likely *in vivo* substrates for DPP IV because of their proposed co-localization with DPP IV, on the surface of T cells and in plasma. Also, many chemokines have either alanine or proline as their penultimate amino-terminal residue. A recent report addressed both the above questions for the CXC chemokine CXCL11/I-TAC [4]. This report is significant in its thoroughness, as the authors showed that pure DPP IV could cleave CXCL11, and that activated T cells co-express DPP IV and the CXCL11 receptor. In addition, activated T cells, but not activated T cells treated with a DPP IV inhibitor, could cleave CXCL11. Finally, the authors demonstrated that cleaved CXCL11 is biochemically and biologically distinguishable from intact CXCL11. These biological conclusions agree with those recently reported [5]. Interestingly, a more recent disclosure reports that a high concentration of the DPP IV inhibitor Lys-pyrrolidide has no effect on CXCL11/I-TAC-induced chemotaxis [6]. The apparent conflict in these sets of results highlights the necessity of correlating *in vitro* cleavage ability to *in vivo* effects. In this light, it is important to point out that only a small number of the proposed DPP IV substrates have been shown to be resistant to cleavage in animals deficient in DPP IV activity, either as a result of drug treatment or genetic mutation. This area of investigation will surely be active in the future.

Significant progress in this area has recently been reported in a disclosure of a highly sensitive mass-spectrometry-

based method for assessing kinetic parameters of substrate cleavage [7]. Using this method, the authors were able to screen libraries of potential substrates and generate individual specificity constants (k_{cat}/K_M) for each substrate from a single kinetic run. The quantitative and high-throughput nature of the assay allowed the rapid screening of over 100 potential substrates. Future developments in mass spectrometry methods and equipment should permit the rapid comparison of plasma levels of peptides derived from animals with differing DPP IV activity.

Structural studies

Another active area of recent research is the determination of high-resolution structures of DPP IV, and DPP IV inhibitor complexes [8,9^{••},10^{••}]. These papers report the structure of either recombinant human DPP IV or endogenous porcine DPP IV. The most notable difference between the structures is that the porcine DPP IV forms a tetramer in the crystal structure, whereas the human DPP IV forms only a dimer. The tetrameric structure of the endogenous porcine DPP IV, which may depend on native glycosylation, could be relevant to a role in cell adhesion for DPP IV.

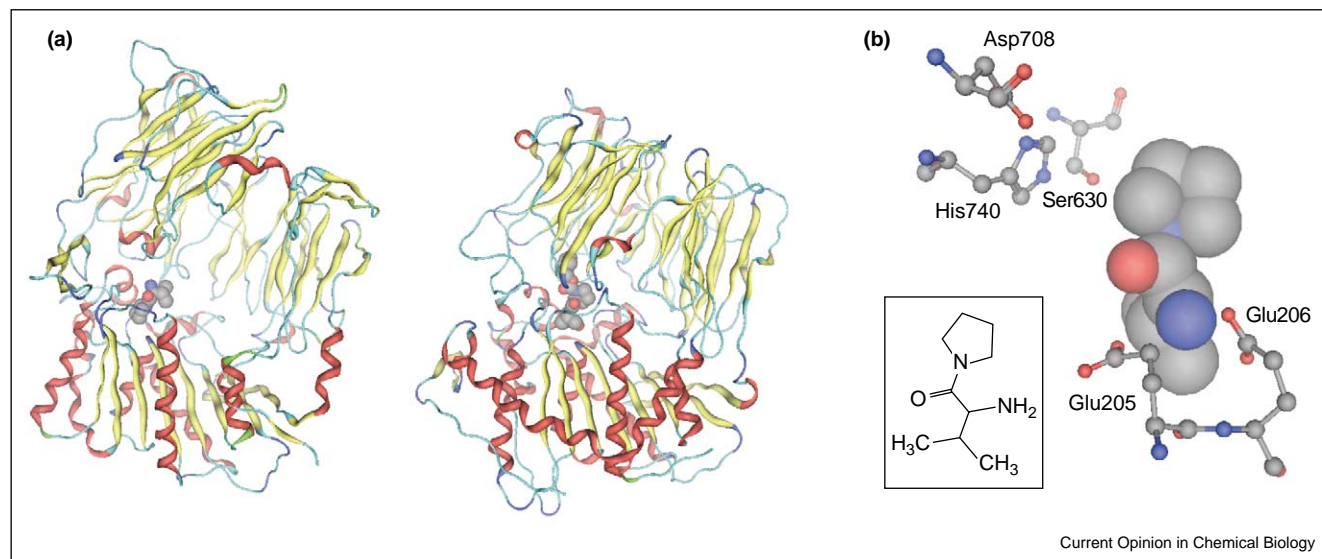
Each DPP IV monomer contains an amino-terminal eight-bladed β -propeller domain fused to a C-terminal α/β -hydrolase domain (Figure 2a). There are two paths to the active site, which is at the interface of the two domains. Substrates and products appear to be able to approach and diffuse from the active site either through the central pore of the β propeller, or through a side opening.

Several insights can be gleaned from the structures determined with bound inhibitors [9^{••},10^{••}]. First, the dipeptidyl aminopeptidase nature of DPP IV is determined by a characteristic, conserved Glu-Glu sequence (residues 205 and 206 in both the porcine and human proteins) that hydrogen bonds to the free amino terminus of the P2 residue (Figure 2b). Next, both large side chains at P2 of substrates, and large groups on the corresponding region of inhibitors are accommodated by a large cavity that makes few interactions with the inhibitors. Finally, the high potency of cyanopyrrole compounds for DPP IV can be rationalized by the structure of Engel *et al.* [10^{••}], wherein the active-site nucleophile Ser630 forms a covalent adduct with *p*-iodo-Phe-Pyr-CN. This covalent interaction is expected to be reversible [11].

Knock-out studies

Another recent landmark was the generation of mice lacking DPP IV [12^{••}]. Although a rat strain harboring a mutation in DPP IV that leads to rapid degradation of the enzyme has been long known [13], the availability of a mouse strain completely devoid of DPP IV enables genetics methods to be included in the study of DPP IV function. Overall, the DPP IV deficient mice were healthy, and although there was residual Gly-Pro-pNA

Figure 2



Structure of DPP-IV-related proteins. **(a)** Comparison of the structure of DPP IV, left, and prolyl oligopeptidase, right (PDB ID 1N1M and 1QFS, respectively, available at <http://www.rcsb.org/pdb/>). **(b)** Active-site residues in DPP IV highlighting the orientation of the Ser-His-Asp catalytic triad and the Glu-Glu motif that is necessary for amino dipeptide selection and interaction with inhibitors.

cleavage activity in plasma, no N-terminal degradation of GLP-1 was observed. This is significant, as GLP-1 is an incretin that is known to be a substrate of DPP IV and is involved in the glucose-dependent production of insulin [14]. As a result of the increased concentration of GLP-1 (and perhaps other incretins), the DPP IV deficient mice had enhanced oral glucose tolerance and increased levels of plasma insulin. In a promising follow-up to the initial characterization of DPP IV $-/-$ mice, Conarello *et al.* [15[•]] have explored the impact of DPP IV deletion on metabolic control. In this study, mice lacking DPP IV were shown to gain less weight on a high fat diet than wild-type mice as the result of reduced food intake and increased energy expenditure. Additionally, DPP IV $-/-$ mice demonstrated improved insulin sensitivity and resistance to high fat diet-induced hepatic lipid accumulation probably resulting from a combination of decreased lipogenesis and increased lipid oxidation. Taken together, these results suggest that DPP IV inhibitors may find utility in the control of both diabetes and obesity. Therefore, in a manner that would be difficult to accomplish with small-molecule inhibitors that may have additional targets, the DPP IV deficient mice validate DPP IV as a target for the control of blood glucose in type 2 diabetes. Importantly, the overall observations for the DPP IV deficient mice agree with those from the mutant DPP IV harboring rat strain [16,17].

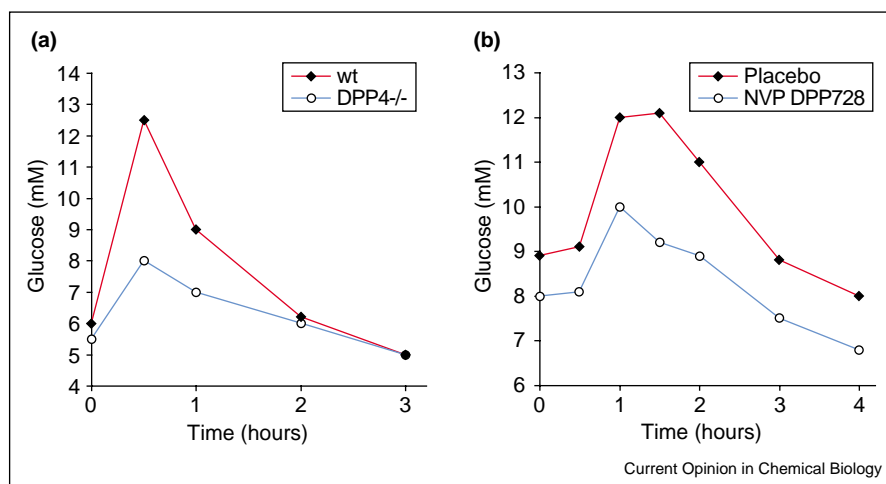
Inhibitor studies

Several DPP IV inhibitors are progressing through pre-clinical and clinical trials for the treatment of type 2

diabetes (for reviews, see [18,19]). Among these is NVP DPP728, which was recently tested in a four-week study [20^{••}]. In this study, participants were treated with either 150 mg of NVP DPP728 twice a day, 100 mg of NVP DPP728 three times a day, or placebo. Both treatment groups displayed a significant decrease in fasting plasma glucose and prandial glucose excursion as well as a decrease in the area under the 24 h glucose curve (see Figure 3 for a comparison between oral glucose tolerance tests of DPP-IV-lacking mice and diabetics treated with NVP DPP728). Interestingly, HbA_{1c} was also reduced in both treatment groups, even though the study was relatively short. NVP DPP728 was well tolerated, with the most common adverse event being pruritis, which was suggested to result from increased levels of other bioactive peptides that are DPP IV substrates.

Positive results have been seen in several recent studies of DPP IV inhibitors in rodent models of type 2 diabetes, notably those with more chronic treatment periods [21[•]-24[•]]. Taken together, these studies show that DPP IV inhibitors are likely to be clinically useful for patients at risk of developing diabetes or in the early stages of diabetes. Also of particular note is a recent study on the impact of DPP IV inhibition in rats with streptozotocin-induced diabetes [25[•]]. Long-term treatment with the DPP IV inhibitor isoleucine thiazolidide led to enhanced islet neogenesis and concomitant decrease in diabetes symptoms, suggesting that DPP IV inhibition may also be a viable therapy for type 1 and/or advanced type 2 diabetes.

Figure 3



Oral glucose tolerance tests of DPP IV-deficient mice and diabetics treated with a DPP IV inhibitor. **(a)** Data extracted from [12**], wild-type and DPP IV $-/-$ mice were administered glucose at time 0, followed by measurement of blood glucose concentration. **(b)** Data extracted from [20**]. Type II diabetics were treated with either placebo or the DPP IV inhibitor NVP DPP728 (150 mg, twice per day) for 4 weeks. For the oral glucose tolerance test, subjects ate breakfast at time 0, followed by measurement of blood glucose concentration.

Immunological and disease associations

As DPP IV was originally identified as a protein expressed on the surface of T cells that was later shown to bind adenosine deaminase and CD45, much attention has focused on the involvement of DPP IV in immunology, oncology and other areas. These studies have been carried out in both rodent models and humans. One important issue that remains unresolved is the relevance of the rodent studies to humans, given species-specific differences in DPP IV functions. For example, murine DPP IV does not bind to adenosine deaminase [26,27], an association that has been suggested to be important for the role of DPP IV in T cell activation.

Many studies have been performed to explore the relative activity of DPP IV in patients afflicted with particular conditions compared with in controls, including rheumatoid arthritis, multiple sclerosis, cancer, AIDS and Down's syndrome (see [19] for several examples). Among recent reports, DPP IV activity has been shown to be inversely correlated to disease stage in both systemic lupus erythematosus and endometrioid adenocarcinoma [28,29]. Future studies will probably focus on determining whether there is a causative link between DPP IV and the many conditions in which its activity is either up-regulated or downregulated. Such studies will be vital for the determination of either additional indications for DPP IV inhibitors, or possible side effects to DPP IV inhibitors in clinical use for diabetes. In addition, in many cases it remains to be shown that DPP IV, and not a protein with similar activity, is responsible for the observed enzymatic activity. Likewise, experiments performed with DPP IV inhibitors should be looked on with

caution as many of these inhibit other members of the family.

FAP

Another dipeptidyl peptidase that has been widely studied is fibroblast activation protein α (FAP; also called seprase). Like DPP IV, FAP is a type II integral membrane protein able to cleave peptides with proline as the penultimate amino acid. However, FAP differs from DPP IV in that it also has gelatinase and collagenase activity [30,31] and is neither abundant nor widely expressed. FAP is found at remodeling sites in the liver and in tumors, but not in normal tissues [32]. Unexpectedly, FAP and DPP IV form a complex at invadopodia of migratory fibroblasts [33*]. Because of its expression sites and collagenase/gelatinase activity, FAP may have roles in cancer invasion and wound healing. A recent study explored the possible involvement of FAP in tumorigenesis [34**]. In this study, mice were inoculated with either FAP- or mock-transfected HEK293 cells. FAP-transfected cells elicited significantly larger tumors that grew more rapidly than those elicited by mock-transfected cells. Additionally, antibodies to FAP, but not pre-immune sera, were shown to inhibit tumor growth in this model. A humanized anti-FAP antibody has been generated, but showed no efficacy in a Phase II trial for metastatic colorectal cancer [35]. Still, given the highly restricted distribution of FAP and the lack of overt pathology in mice deficient in FAP [36], small-molecule inhibitors or antibodies to FAP may be useful therapeutics for cancer or cirrhosis.

A recent report suggests that other indications for FAP inhibitors are blood cell disorders, such as anemia and

chemotherapy-induced neutropenia [37*]. In this study, the non-specific dipeptidyl peptidase inhibitor L-valinyl-L-boroproline (VbP) was shown to stimulate the growth of hematopoietic progenitor cells, and to accelerate neutrophil and erythrocyte regeneration. Stromal cells, where the target of VbP was proposed to be localized, are known to express both FAP and DPP IV, but the neutrophil recovery in response to VbP was shown to occur in DPP IV $-/-$ mice. As a result, the biological action of VbP is mediated either through FAP or an as yet unidentified enzyme.

DPP 8/9

DPP IV and FAP are highly sequence similar and may have arisen by gene duplication. DPP 8 and DPP 9 are another pair of post-proline cleaving dipeptidases that may have arisen by gene duplication [38*,39]. Unlike FAP and DPP IV, DPP 8 and DPP 9 are soluble proteins localized in the cytoplasm. DPP 8 and DPP 9 are widely distributed in normal tissues and have not yet been associated with any particular biological process. An intriguing possibility is that some of the many functions ascribed to DPP IV and its inhibition may actually be derived from the activity and inhibition of DPP 8 and/or DPP 9.

PCP/DPP7

Another pair of post-proline cleaving enzymes is prolyl carboxypeptidase and DPP 7 (also called quiescent cell proline dipeptidase (QPP) and DPP7). Interestingly, although previously characterized in other systems, DPP 7 was 'rediscovered' as the target of a 'specific' DPP IV inhibitor in a DPP IV deficient cell line [40,41,42*,43]. This 'rediscovery' serves as a reminder that DPP-IV-like activity can arise in biological systems devoid of DPP IV and that DPP IV inhibitors can inhibit enzymes other than DPP IV.

In a search for the role of proteases as regulators of quiescent cell survival, Huber and coworkers [42*] discovered that the DPP IV inhibitor VbP triggers apoptosis in quiescent lymphocytes but not activated lymphocytes. Both CD26⁺ and CD26⁻ T cell subpopulations show equal sensitivity to VbP, ruling out DPP IV as the target of the apoptotic response. Although the observed cell death had the characteristics of apoptosis, it differed from apoptosis induced by gamma irradiation or by Fas ligation in activated lymphocytes. VbP did not induce apoptosis in activated lymphocytes and lactacystin, a proteasome inhibitor, inhibited apoptosis by VbP in quiescent lymphocytes. The results suggest a novel apoptotic pathway in these cells. The recent development of selective inhibitors for DPP II, which is identical to QPP and DPP 7 [44], should be useful for further elucidating the biological function of this protease [45].

DPP 7 (QPP) is a 58 kDa glycoprotein that is targeted to intracellular vesicles that are distinct from lysosomes [41].

The protein appears to be located within these vesicles and the active protein is secreted in an active form in response to calcium release [40]. The intracellular localization of DPP 7 along with membrane-bound proteins such as DPP IV and FAP indicates that the processing of N-terminal X-proline proteins can occur both inside and outside in a broad range of cells and cellular compartments. DPP 7 has been shown to exist as a homodimer that is essential for catalytic activity. The dimerization occurs via a leucine zipper motif, which is novel for proteases [46].

Like DPP 7, prolyl carboxypeptidase (PCP, also known as angiotensinase C) is localized to an internal organelle, the lysosome. As a result, PCP is expressed as a pre-proenzyme and is most active at low pH. Little is known about the possible biological roles of PCP, although it has been shown to be an activator of prekallikrein in endothelial cells [47].

POP/AAP

Prolyl oligopeptidase (POP; also known as prolyl endopeptidase and post-proline cleaving enzyme) is an intracellular enzyme (80 kDa) that is highly conserved in mammals and is broadly distributed with high concentrations found in the brain [48]. As mentioned previously, it is a distant relative of the other prolylpeptidases, such as DPP IV. The closest relative to POP appears to be acylaminoacyl peptidase. A 1.4 Å crystal structure has revealed that it contains an α/β -hydrolase domain and the catalytic triad is protected by the central tunnel of an unusual β -propeller [49]. The localization of POP in the brain suggested that it is involved in the maturation and degradation of peptide hormones and neuropeptides, such as substance P, oxytocin, vasopressin and angiotensins, which are substrates for the enzyme [50].

Recent studies in amnesia, depression and Alzheimer's disease have provided strong support for this hypothesis. The enhancement of cognitive function in models of amnesia by POP inhibitors has been known for some time [51,52] and antidementia effects of inhibitors appear to correlate with increased neuronal survival and neurite outgrowth [53]. Plasma concentrations of POP are elevated in mania and decreased in depression. The role of substance P in long-term potentiation has led to the speculation that substance P interaction with the neurokinin 1 receptor exerts its effect on long-term potentiation via a G-protein-mediated increase in inositol 1,4,5-triphosphate and intracellular Ca²⁺ release [54]. Thus, the putative degradation of substance P by POP would suggest that POP inhibition would elevate substance P levels and stimulate potentiation. A very recent study demonstrates convincingly that a single administration of a novel POP inhibitor causes a significant increase in substance P- and α -MSH immunoreactivity in the rat frontal cortex [55]. Because the inhibitor also abolishes degradation of

substance P and α -MSH by bacterial POP *in vitro*, the mechanistic linkage is suggestive.

A recent study has provided intriguing evidence that three diverse mood-stabilizing drugs, lithium, carbamazepine and valproic acid, all exert their effects through this common POP-modulated mechanism [56••]. The drugs were found to inhibit the collapse of neuron growth cones and increase growth cone area. The effects were reversed by inositol, suggesting that inositol depletion is involved in the mechanism. Both lithium and valproic acid were found to be inhibitory to the development of the slime mold *Dictyostelium* and deletion of the gene that codes for POP in this organism conferred resistance to both drugs. Finally, two specific inhibitors of POP were found to abolish the effects of all three drugs on neuron cone collapse and area. The finding suggests a fundamental link between POP and mood-stabilizing drugs. Clearly, more work will be required to understand the utility of POP inhibition and activation in specific mood disorders.

Acylaminoacyl peptidase (AAP, also known as acylpeptide hydrolase), like POP, is a cytosolic enzyme, but is unique in the family for its substrate preference. Known AAP substrates are short peptides with blocked N-termini. After cleavage by AAP, the acylated amino terminal residue is removed, leaving a peptide with a free N-terminus shortened by a single amino acid. Little is known about the biological role of AAP, but evidence is emerging that it is a more sensitive target of organophosphorus compounds than acetylcholinesterase [57–59]. As a result, it could be the case that the target of organophosphorus compounds that have shown promise in enhancing cognition may be AAP, rather than acetylcholinesterase as originally thought [60].

Other proteins

Several other proteins are often mentioned as being members of the DPP IV family. DPP6 and its homologues (in this case, DPP stands for dipeptidyl peptidase-like) are highly sequence similar to DPP IV, with the notable exception of the serine nucleophile [61]. As a result of a serine to glycine substitution, these proteins are not active as proteases. However, DPP6 and its homologues do have biological roles that may be related to additional roles manifested by active proteases in the family. In this light, it is notable that a mutation in DPP6 is lethal in homozygote mice [62], and that the DPP6 homolog DPPX is an essential component of neuronal potassium channels [63•]. Another 'outlier' protein is attractin, a serum protein that copurifies with DPP-IV-type enzyme activity [64]. It is unlikely that attractin itself is a protease, as it bears no significant sequence similarity to active-site residues of any known protease (a stretch of amino acids that is not conserved between the human and mouse proteins was suggested to be similar to known proteases, but was later shown to reside in the long signal sequence

of attractin, and therefore not a part of the mature protein [65]). Additionally, recombinant attractin has very little activity, and purification of DPP IV-type enzyme activity of human serum yielded only DPP IV [66•]. This point is, however, controversial (see [65,67]). Because attractin mutant mice show severe neuronal phenotypes [68], attractin's DPP IV-type activity and inhibition by DPP IV inhibitors warrant further study. Another protein that has been suggested to have DPP-IV-type activity is glutamate carboxypeptidase II (GCP II; also known as *N*-acetylated- α -linked acidic dipeptidase or NAALADase). In this case, crude and partially purified extracts of GCP II-containing cells were shown to have low levels of Gly-Pro-AMC cleaving activity [69]. However, later experiments with pure preparations of the extracellular domain of GCP II showed that this domain was devoid of Gly-Pro-AMC cleaving activity, although active for *N*-acetyl-aspartyl-glutamate hydrolysis [70]. These later results suggest that DPP-IV-type activity is not an intrinsic property of GCP II/NAALADase.

Conclusions

The breadth of functions of the DPPs suggests that this serine protease subfamily plays a major role in the regulation of a variety of biological functions through the processing of critical peptide hormones. As the biology of the DPPs is elucidated, we can expect that several therapeutic indications will emerge from this biology that will suggest the possibility of small-molecule intervention in the treatment of important human diseases.

Acknowledgements

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