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Human proline specific peptidases: A comprehensive analysis

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ABSTRACT

Background: Proline specific peptidases (PSPs) are a unique group of enzymes that specifically cleave bonds formed by a proline residue. The study of PSPs is important due to their role in the maturation and degradation of peptide hormones and neuropeptides. In addition, changes in the activity of PSPs can result in pathological conditions, including various types of cancer.

Scope of review: PSPs annotated from the *Homo sapiens* genome were compared and classified by their physicochemical and biochemical features and roles in vital processes. In addition to catalytic activity, we discuss non-enzymatic functions that may regulate cellular activity.

Major conclusions: PSPs apparently have multiple functions in animals. Two functions rely on the catalytic activity of the enzyme: one involved in a regulatory pathway associated with the ability of many PSPs to hydrolyze peptide hormones and neuropeptides, and the other involved in the trophic pathway associated with the proteolysis of total cellular protein or Pro-containing dietary proteins in the digestive tract. PSPs also participate in signal transduction without proteolytic activity by forming protein-protein interactions that trigger or facilitate the performance of certain functions.

General significance: PSPs are underestimated as a unique component of the normal human peptidase degradome, providing the body with free proline. A comparative analysis of PSPs can guide research to develop inhibitors that counteract the abnormalities associated with changes in PSP activity, and identify natural substrates of PSPs that will enable better understanding of the mechanisms of the action of PSPs in biological processes and disease.

1. Introduction

Proline is a structurally and functionally unique imino acid among 20 natural proteinogenic amino acids. Proline introduces tight turns into the polypeptide chain, where it dramatically changes the conformation of the polypeptide. Therefore, proline is a marker of functionally important regions of protein structures, and conformational restrictions imposed by proline motifs in the polypeptide chain indicate important structural and biological functions of this imino acid.

Proteins with a high content of proline and proline-containing peptides are among the most biologically significant in the processes of metabolism, nutrition, cell recognition and intracellular signaling [1]. Many structural and storage proteins and peptides contain proline, and the physiological need for proteins with high proline content remains throughout the entire life cycle [2]. In particular, prolamins, the main storage proteins of grain crops and cereal seeds, are important representatives of proline-rich proteins, containing up to 30% of proline residues [3,4]. Collagens are another prominent example of proteins with high content of proline and are the basis of connective tissue, providing strength and elasticity [5]. Proline and hydroxyproline residues are abundantly represented in collagens and constitute up to 25% of their sequence [6].

In nature, only a special group of peptidases, proline specific peptidases (PSPs), can hydrolyze bonds formed by a proline residue, with the exception of proteins with a N-terminal proline, which can be cleaved by leucine aminopeptidase and cytosolic non-specific dipeptidases. Previously, the most comprehensive review of PSPs was the 1997 review by Cunningham and O'Connor in this journal [7]. Since that time, the human genome sequence has been revealed [8], but information about individual PSPs most often has been obtained through the study of certain disease conditions [9–15]. The limitation is that often these analyses do not provide the overall picture of the poly-

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functionality of this highly specific group of peptidases that is required to understand their complementary specificities. We therefore summarize the current data on PSPs in human and mammals to understand the role of the complete set of PSPs found in the genome of *Homo sapiens*.

2. The set of human proline specific peptidases (PSPs)

Scientific advances in the post-genomic era have provided an opportunity to identify and characterize complexes of PSPs in various biological taxa. Since the human genome and physiology have been studied in greatest detail among higher eukaryotes, in this review we conducted a bioinformatics characterization of human PSPs as an example using NCBI [16] (GRCh38.p13) and UniProt [17] (UP000005640) databases.

Most PSPs studied to date are exopeptidases, cleaving amino acids from either end of proteins or peptides. Only one of the PSPs is an oligopeptidase, prolyl oligopeptidase (POP), also called prolyl endopeptidase, cleaving polypeptides usually less than 30 aa residues. Of the PSPs described in the comprehensive collection of exhaustively characterized peptidases, 19 types of PSPs have been found in all kingdoms of living organisms, but only 13 were found in animals [14]. All PSPs found in animals are representatives of two peptidase clans - serine and metallo peptidases, according to MEROPS classification [18] (Table 1). POP, dipeptidyl peptidases (DPP) and fibroblast activation protein (FAP) belong to the family S9 of serine peptidases: S9A (POP), S9B (DPP4, DPP8, DPP9, FAP), S9X (DPP6, DPP10). Prolyl carboxypeptidase (PRCP) and DPP2 are in the family S28 of serine peptidases. Aminopeptidases P (APP1, APP2, APP3) and prolidase (XPD) belong to the family M24 of metallo peptidases. In addition, there are two post-proline cleaving peptidases with broad substrate specificity (PPCPbs), cleaving N-terminal Pro residues that are not cleaved by any specialized PSP; these include leucine aminopeptidase (LAP) and cytosolic non-specific dipeptidase (CND), belonging to the families M17 and M20 of metallopeptidases, respectively. However, these peptidases do not belong to the PSP group and will not be considered in our review.

Many PSPs are represented by multiple isoforms resulting from alternate splicing. These splice forms [17] differ from the canonical sequences by the absence or presence of a small part of sequence somewhere in the canonical form (Table 2). Alternative splicing improves the efficiency of stored genetic information and is a key mechanism for increasing protein diversity and the implementation of a gene expression regulation system.

Concerning the physiological roles of PSPs, the available data allow us to distinguish signal, regulatory and trophic functions of PSPs of animal origin. For PSPs involved in trophic functions, we discuss the participation of PSPs in the general digestion of intracellular proteins, and specialization in the digestion of proline-rich dietary proteins inside the gastrointestinal tract.

Signal functions of PSPs are mainly a result of protein-protein interactions, but also can be associated with catalytic activity. More is known about the regulatory role of PSPs associated with the metabolism of biologically active peptides. Due to limited proteolysis, these enzymes are involved in the maturation of biologically active compounds, and in converting them into an inactive form. The trophic function of PSPs is less understood. Lysosomal localization of DPP2 and PRCP [19,20] may indicate the participation of these PSPs in the degradation of Pro-containing proteins and peptides in the normal autophagic process. PRCP, DPP4, as well as APP2, are associated with the intestinal membrane and are likely involved in digestion [21–23]. Consistent with this hypothesis, a large group of proteins with a high content of proline (more than 30%) are among the storage proteins of monocot seeds that are edible by mammals, including prolamins and glutelins [3,4], which are effectively hydrolyzed by mammalian PSPs. However, a combination of several different PSPs with complementary specificities is required to efficiently cleave proline residues from dietary proteins.

The interest in PSPs is largely supported by the fact that these peptidases in combination with other factors can participate in the development of pathological processes or serve as potential biomarkers. An analysis of the decrease or increase in the expression of PSP genes and the activity of peptidases during the course of a disease can associate these enzymes with certain types of cells, substrates or interacting partners that are included in the pathophysiology of diseases, and, ultimately, elucidate the functional role of PSPs and possible therapeutic targets. Table 3 describes in detail the properties of PSPs and identifies the most significant pathologies associated with changes in the activities of individual types of PSPs. The far from complete list of diseases presented in Table 3 illustrates the importance of the functions of PSPs *in vivo* and suggests that the study of the activities of PSPs will be useful not only for diagnosing the disease, but, to a certain extent, for predicting its development. Most importantly, understanding the involvement of PSPs in disease can lead to new treatments.

In the present review, we analyzed the complete set of PSPs annotated thus far in the human genome [17], reviewing physico-chemical and biochemical features and possible functional roles in vital biological processes, and adding the important data obtained for homologous enzymes in model mammals. In addition to catalytic function, we specifically examine the potential of non-enzymatic roles in the majority of PSPs as a result of binding to specific ligands/proteins and subsequent regulation of cellular activity.

3. Proline specific serine peptidases

Molecular masses of the serine PSP polypeptide chains from the S9 family are relatively more (80–103 kDa) than those from the S28 family (54–55 kDa). Most serine PSPs are homodimers, and all have significantly different structure from classical serine peptidases, primarily in the order of residues of the catalytic triad. Whereas classical serine peptidases are characterized by the order of Asp-His-Ser or His-Asp-Ser in the catalytic triad, all serine PSPs from the S9 and S28 families have Ser-Asp-His (Table 1).

3.1. Prolyl oligopeptidase (POP, EC 3.4.21.26)

POP is one of the most studied PSPs, belonging to the subfamily S9A. POP has 710 amino acid residues with molecular mass of about 80 kDa.

3.1.1. Structure

POP exists as a monomer consisting of two domains – one is catalytic with α/β-hydrolase fold, and the other is a noncatalytic seven-bladed β-propeller domain [24,25]. The 3D structure of POP is presented in Fig. 1A [26]. The propeller acts as a gating filter, allowing access to the active center of only short peptides [27,28]. The active center (Ser554, Asp641, His680) is located at the interface of the catalytic and β-propeller domains. The substrate binding subsite S1 of POP is between the hydrophobic side radicals of Trp595, Phe476, Val644, Val580, Tyr599 residues and side chains of Asn555 and Tyr473 (hereinafter the numbering is given by the human POP accession P48147), maintaining the Pro residue in the P1 position of the substrate. Arg643 is a key residue of the S2 subsite. The S3 binding subsite is formed by the side chains of Phe173, Met235, Cys255, Ile591, and Ala594 [28–30]. Catalytic control, including substrate entry, size selection and specificity, is ensured by a flexible loop structure at the domain interface. This loop structure comprises a loop of the propeller domain (loop A), a facing loop of the peptidase domain (loop B), and other catalytically important loops. The management of this surface loop system represents a unique way to control catalytic activity and is an attractive target for the development of inhibitors [31].

Table 1
General information about PSPs sequences and structure.

PSPs	MEROPS classifi-cation	IUBMB	UniProt ID	NCBI ID	Length	Mw, Da	Active Center and Conserved Catalytic Residues	Signal peptide (SignalP 4.1)	Trans-membrane domain (TMHMM server)	Domain Organization	Oligomer Form (subunit organization)
POP	S9A	EC:3.4.21.26	P48147[a]	NP_002717	710	80,700	S554, D641, H680	–	–	Catalytic with α/β-hydrolase fold; non-catalytic seven-bladed β-propeller	Monomer
DPP4	S9B	EC:3.4.14.5	P27487[b]	NP_001926	766	88,279	S630, D708, H740	–	7–29	Catalytic with α/β-hydrolase fold;	Homodimer
DPP8			Q6V1X1[c]	NP_932064	898	103,358	S755, D833, H865	–	–	non-catalytic eight-bladed β-propeller	Homodimer
DPP9			Q86TI2[d]	NP_631898	863	98,263	S730, D808, H840	–	–		Homodimer
FAP			Q12884[e]	NP_004451	760	87,713	S624, D702, H734	–	7–29		Homodimer
DPP6	S9X	–	P42658[f]	NP_570629	865	97,588	Inactive homologs of DPP4	–	96–118		Homodimer
DPP10		–	Q8N608[g]	NP_065919	796	90,888		–	34-56		Homodimer
DPP2	S28	EC:3.4.14.2	Q9UHL4[h]	NP_037511	492	54,341	S162, D418, H443	1–21	–	α/β-hydrolase fold and α-helical	Homodimer
PRCP		EC:3.4.16.2	P42785[i]	NP_005031	496	55,800	S179, D430, H455	1–21	–	bundle	Homodimer
APP1	M24	EC:3.4.11.9	Q9NQW7[j]	NP_001161076	623	69,918	Mn(I): D415, D426, E537; Mn(II): D426, H489, E523, E537	–	–	Three-domain structure, C-terminal "pita-bread" fold	Homodimer
APP2			O43895[k]	NP_003390	674	75,625	Mn(I): D450, D461, E569; Mn (II): D461, H524, E555, E569	1–23	–		Homodimer/ tetramer
APP3			Q9NQH7[l]	NP_071381	507	57,034	Mn(I): D342, H424, E451, E475; Mn(II): D331, D342, E475	–	–		Homodimer
XPD		EC:3.4.13.9	P12955[j]	NP_000276	493	54,548	Mn(I): D276, D287, E452; Mn(II): D287, H370, E412, E452	–	–	Two-domain structure, C-terminal "pita-bread" fold	Homodimer

[a] Uniprot sequence version 2 (25 Nov 2008).
[b] Uniprot sequence version 2 (01 Feb 1996).
[c] Uniprot sequence version 1 (05 Jul 2004).
[d] Uniprot sequence version 3 (07 Jun 2005).
[e] Uniprot sequence version 5 (23 Mar 2010).
[f] Uniprot sequence version 2 (16 Dec 2008).
[g] Uniprot sequence version 2 (18 May 2010).
[h] Uniprot sequence version 3 (04 Nov 2008).
[i] Uniprot sequence version 1 (01 Nov 1995).
[j] Uniprot sequence version 3 (23 Jan 2007).
[k] Uniprot sequence version 3 (15 Nov 2002).
[l] Uniprot sequence version 1 (01 Oct 2000).

Table 2
Splice variants of human PSPs, obtained from UniProt[a].

PSP	UniProt ID	Isoforms
POP	P48147	Canonical
DPP4	P27487	Canonical
DPP8	Q6V1X1	Canonical
	Q6V1X1-2	723-773 missing
	Q6V1X1–3	1–16 missing
	Q6V1X1–4	1–16, 674–773 missing
	Q6V1X1–5	674–722 missing
DPP9	Q86TI2–1	Canonical (short)
	Q86TI2–2	Long: 1–1: M → MRKVKKLRLDKENTGSWRSFSLNSEGAERM
	Q86TI2–4	832–858 missing
DPP6	P42658	Canonical
	P42658-2	1-81: MASLYQRFTG…QARSDGDEED → MTTAKEPSASGKSVQQQEQ
DPP10	Q8N608–1	Canonical
	Q8N608–2	1–20: MNQTASVSHHIKCQPSKTIK → MRKVESRGEGGRE
	Q8N608–3	1–20: MNQTASVSHHIKCQPSKTIK → MTAAKQEPQPTPGARASQAQPADQ
	Q8N608–4	1–50 missing
FAP	Q12884	Canonical
	Q12884-2	1-521 missing
DPP2	Q9UHL4	Canonical
PRCP	P42785	Canonical
	P42785-2	56-56: K → KALAAGQLHICIIQLNHYKTPL
APP1	Q9NQW7	Canonical
	Q9NQW7-2	398-421 missing
	Q9NQW7–3	1–1: M → MAASRKPPRVRVNHQDFQLRNLRIIEPNEVTHSGDTGVETDGRM
APP2	O43895	Canonical
APP3	Q9NQH7	Canonical
	Q9NQH7-2	1-79 missing
	Q9NQH7–3	265–278 AFIETMFTSKAPVE → RQGFSVLSRLVSNS
		279–507: Missing.
	Q9NQH7–4	1–23 missing
	Q9NQH7–5	265–288 AFIETMFTSKAPVEEAFLYAKFEF → KSVLLARHGGSRLYSHHFGRPRLS
		289–507: Missing.
XPD	P12955	Canonical
	P12955-2	184-224: Missing.
	P12955–3	68–131: Missing.

[a] [17].

3.1.2. Specificity

POP is an endopeptidase and hydrolyzes bonds formed by the carboxyl group of proline in oligopeptides less than 30 amino acid residues. Some physico-chemical properties of POP, the most effective synthetic substrates for determining its activity, as well as inhibitors of POP activity are summarized in Table 3. Animal POP is able to degrade, in particular, peptide hormones and neuropeptides, such as oxytocin, vasopressin, substance P, neurotensin, thyrotropin-releasing hormone (TRH), but does not break down large protein molecules like denatured casein, collagen, gastrin and adrenocortico-tropin-releasing hormone [32].

3.1.3. Localization

Mammalian POP is primarily an intracellular cytoplasmic enzyme. It has no secretion signal and transmembrane region, or a lipid anchor sequence [33]. However, in some cases, POP can be released from cells. An enzyme with POP activity and a POP-like inhibitory profile is present in human plasma and in the seminal fluid, although at a significantly lower concentration than in the cell [34,35]. Tenorio-Laranga et al. [36] demonstrated the interaction of POP with the inner membrane due to the intracellular anchor. However, the mechanism of insertion and the subsequent effect of POP on extracellular substrates (neuropeptides) was unclear. One explanation is that a membrane-bound form of POP is a preparatory (stored) form to be released under certain conditions for the subsequent transport into the extracellular space. This hypothesis is supported by the data obtained by Natunen [37], where, under normal conditions, POP is a cytosolic enzyme, but under inflammatory conditions (or modeling inflammatory conditions), POP is secreted into the extracellular space. While it is unclear how this occurs, Schulz et al. [38] pointed to the possibility of tubulin-dependent cell transport of POP unrelated to the enzymatic activity of the enzyme. Using a specially synthesized substrate and a POP inhibitor, Lee et al. [39], provided a more critical approach to the presence of POP activity in plasma, where FAP activity also may be unintentionally measured. A more accurate identification of specific POP activity may require a more specific substrate [37,40], or specific antibodies.

In humans, high levels of POP activity were found in muscle, testicles, kidneys, submandibular gland, epithelium cells, fibroblasts, lymphocytes and human platelets, while relatively low levels were found in the heart and aorta [41]. Relatively high POP enzymatic activity was found in the cerebral cortex, while in other parts of the brain, low activity was observed [42]. Low POP activity was found in rat hypothalamus and hippocampus, but high levels of immunoreactive POP protein were found in human and rat hippocampi. In the hypothalamus, the immunoreactive POP protein was virtually undetectable, with the exception of the medial mammillary nucleus. In the human brain, the highest level of POP mRNA was found in the hypothalamus and prefrontal cortex, and the lowest in the cerebellum [43]. In rat brain the highest amounts of POP mRNA were found in the cerebellum and hypothalamus [44]. It should be noted that the conflicting data on the distribution of POP mRNA, immunoreactive POP protein, and the activity of POP in the brain were carried out by different scientific groups using different methods and under different conditions. Additionally, POP distribution can be affected by substances that can modify the activity of POP in the brain (for example, polyamines, oxidizing agents), the age of animals, the presence of an endogenous POP inhibitor, and a possible post-translational modification of POP. These differences may indicate strict endogenous regulation of POP, possibly through the action of an endogenous POP inhibitor, protein turnover, or its transport [43].

Table 3
General physico-chemical and enzymatic properties, localization, and tissue distribution of PSPs, with implications in pathology.

PSP	pH-optimum	Specificity[a]	Inhibitors	Localization	Distribution	PSPs in Pathological Processes	References
POP	7.0–8.5	$(Xaa)_n$-Xbb-Pro↓Xbb-$(Xaa)_n$, n = 1–13	DFP, PMSF, *Z*-Pro-prolinal	Cytoplasmic	Ubiquitous: muscles, testicles, kidneys, submandibular gland, heart, aorta, cerebral cortex	Neuropsychiatric diseases (depression, mania or schizophrenia) and neurodegenerative diseases includes Parkinson's disease, dementia with Lewy bodies, multisystem atrophy, and Hallervorden-Spatz disease	[7,17,32,33,42,55]
DPP4	8.0–9.0	Xbb-Pro(Hyp[b])↓Xbb-$(Xaa)_n$, n = 2–12	DFP, PMSF, Diprotins A, B, Vildagliptin, Sitagliptin, Alogliptin, Saxagliptin Linagliptin	Membrane-bound or soluble extracellular protein	Ubiqutous: serum, seminal fluid, saliva and bile, kidneys, gastrointestinal tract, exorcine pancreas, thymys, uterus, placenta, prostate, adrenal, parotid, sweat, salivary, mammary glands	Thyroid, ovarian, lung, skin, prostate cancers and central nervous system tumors, lung metastasis, type 2 diabetes, celiac disease	[7,9,17,79,88,108,109,122,123,201–203]
DPP8		Xbb-Pro↓Xbb-$(Xaa)_n$, n = 2–12	DFP, PMSF, Vildagliptin, Saxagliptin	Cytosolic	Ubiquitous: testis, brain,	Asthma	[17,80,86,87,93,96,122,123,136,204–206]
DPP9			DFP, PMSF, Vildagliptin, Saxagliptin	Cytosolic (short isoform), nuclear (long isoform)	Ubiquitous: testis, liver, leucocytes, diseased and tumor-bearing tissues including melanoma	Asthma, testicular tumors, osteoarthritis, idiopathic pulmonary fibrosis	
FAP		$(Xaa)_n$-Xbb-Pro↓Xbb-$(Xaa)_n$, n = 1–13; Xbb-Pro↓Xbb-$(Xaa)_n$, n = 2–12	DFP, PMSF, Linagliptin	Membrane-bound or soluble extracellular protein	Activated myofibroblasts, hepatic stellate cells in fibrosis, in stromal fibroblasts of epithelial tumors	Multiple epithelial cancers (carcinomas of the breast, colorectum, lung, stomach, pancreas and esophagus), liver fibrosis, pulmonary fibrosis, rheumatoid arthritis, osteoarthritis and atherosclerosis, chronic inflammation, liver cirrhosis	[11,17,69,74,91,122]
DPP6	Inactive homologs of DPP4			Membrane-bound	Brain	Parkinson's disease	[17,70,71]
DPP10				Membrane-bound	Brain, pancreas and adrenal gland	Parkinson's disease	
DPP2	5.5–6.3	Xbb-Pro(Hyp[b])↓Xbb-$(Xaa)_n$, n = 2–12	DFP, PMSF	Lysosomal	Kidney, brain, testis, heart, resting lymphocytes, differentiated macrophages	Neurodegenerative disorders, myopathies, hepatic cancer, gastro-intestinal disorders	[10,17,19,146,150,151]
PRCP	4.5–5.0	$(Xaa)_n$-Xbb-Pro↓Xbb, n – any number		Lysosomal	Brain, heart, placenta, lung, liver, skeletal muscle, kidney, pancreas	Obesity	[17,20,156,160,161]
APP1	7.0–8.5	Xbb↓Pro(Xaa)$_n$, n = 1–9	EDTA, Apstatin	Cytosolic	Ubiquitous: liver, pancreas	Hippocampal neurodegeneration	[17,23,178,179,207,208]
APP2				Membrane-bound	Kidney, intestine, renal proximal tubule	Premature ovarian failure, angioedema	[17,23,174,207,208]
APP3				Cytosolic/mito-chondrial	Kidneys	Cystic kidney disease	[17,23,176,177]
XPD	7.5	Xbb↓Pro(Hyp[b])	EDTA, Z-Pro	Cytosolic	Erythrocytes, leukocytes, cultured skin fibroblasts, human plasma, small intestine, kidney	Prolidase deficiency (PD), various types of cancers (breast cancer, endometrial cancer, lung cancer, pancreatic cancer, melanoma) and fibrotic processes (chronic obstructive pulmonary disease, cardiomyopathy), pancreatitis, thalassaemia	[7,17,183–186,188,190,209,210]

[a] Xaa – any amino acid, Xbb - any amino acid, except Pro.
[b] Significantly lower rate of hydrolysis.

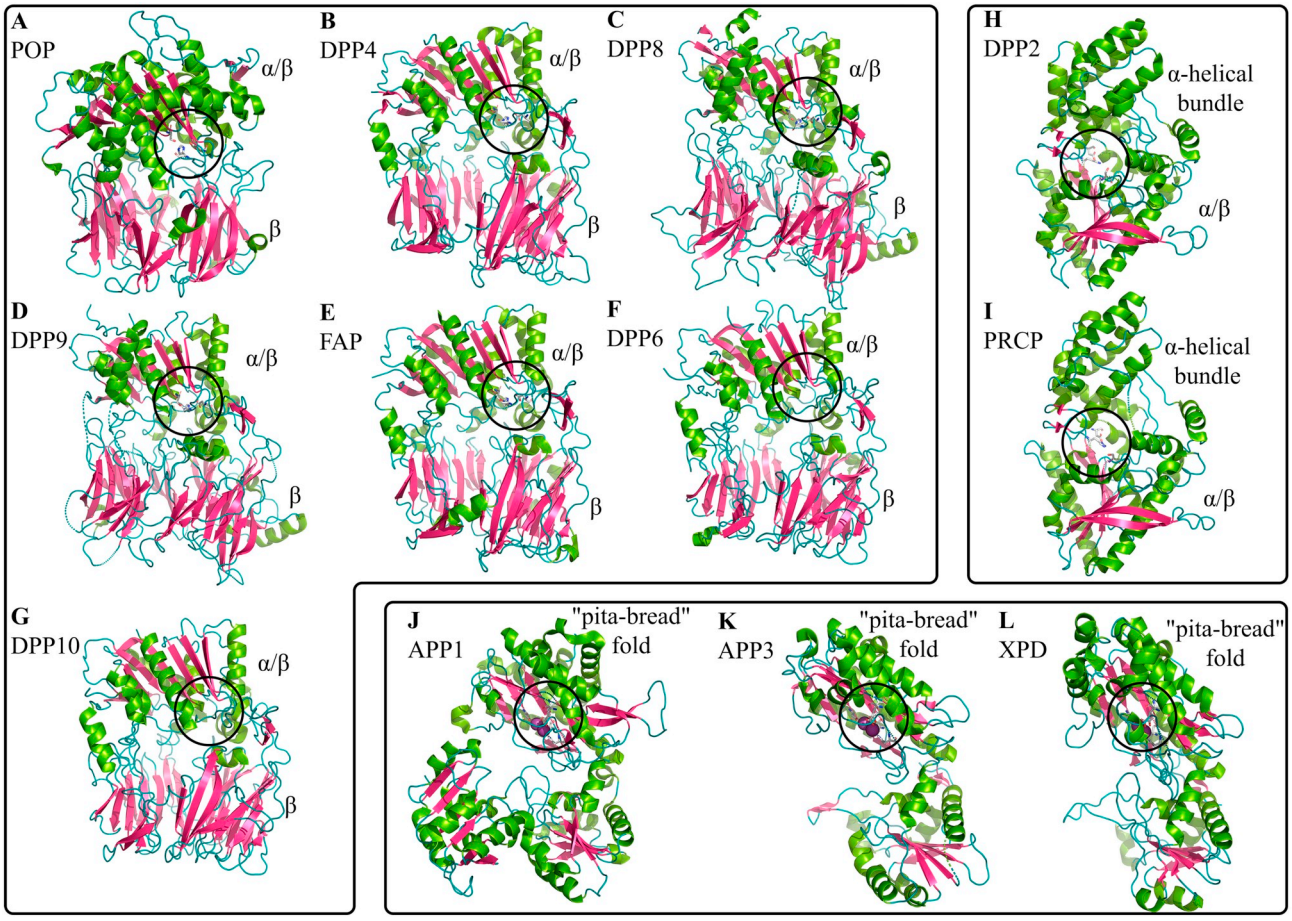

Fig. 1. Tertiary structures of S9 serine PSPs: (A) POP (PDB ID 3DDU) [25], (B) DPP4 (PDB ID 4A5S) [80], (C) DPP8 (PDB ID 6EOP) [81], (D) DPP9 (PDB ID 6EOR) [81], (E) FAP (PDB ID 1Z68) [72], (F) DPP6 (PDB ID 1XFD) [82], (G) DPP10 (PDB ID 4WJL) [71]; S28 serine PSPs: (H) DPP2 (PDB ID 4EBB) [144], (I) PRCP (PDB ID 3N2Z) [146]; M24 metallodependent PSPs: (J) APP1 (PDB ID 3CTZ) [167], (K) APP3 (PDB ID 5X49) [168], (L) XPD (PDB ID 5M4G) [184]. Colors are given by elements of secondary structures: α-helixes are green, β-sheets are purple, loops are teal. The area of the active center or metal-binding site of metallopeptidases is circled with black. Metal ions are violet. The following notations are used: in S9 serine PSPs - α/β for catalytic domain with α/β-hydrolase fold and β for β-propeller domain; in S28 serine PSPs - α/β for catalytic domain with α/β-hydrolase fold and α-helical bundle, which serves as a cap for the active center; in M24 metallodependent PSPs - the so called "pita-bread" fold for the C-terminal catalytic domain.

3.1.4. Mode of action

POP can affect biological processes in two ways: enzymatic, associated with the catalytic activity of POP, and non-enzymatic, due to protein-protein interactions. POP can enzymatically cleave neuropeptides (substance P, vasopressin, melanocyte stimulating hormone (MSH), bradykinin) and, together with matrix metallopeptidases 8 and 9, generate immunoactive peptides from collagen. One of these peptides is *N*-acetyl-prolyl-glycyl-proline, a neutrophil chemoattractant and a biomarker of an inflammatory process, and the POP may be a therapeutic target [45]. Another peptide is *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), released from thymosin β4 (Tβ4) as a result of the combined action of POP and other peptidases, which (unlike POP) can cleave large proteins [46]. Release of Ac-SDKP reduces collagen deposition in the heart and kidney of hypertensive rats by inhibiting fibroblast proliferation and decreasing macrophage infiltration. The release of Ac-SDKP was directly dependent on the presence of POP and was suppressed by the addition of a POP inhibitor. However, the decrease of the level of Tβ4 and its initial cleavage products $Tβ_{47–44}$ and $Tβ_{48–44}$ was clearly inhibited by the presence of POP, although the mechanism was not elucidated [47]. In addition to influencing fibroblast proliferation, Ac-SDKP enhanced angiogenesis, which was not observed in the presence of Tβ4, indicating a possible pro-angiogenic function of POP, carried out through the release of Ac-SDKP from Tβ4.

Using a pharmacological inhibitor of POP in blood serum and lung lysates of PCRP and ACE2 double knockout mice, the conversion of angiotensin II (Ang II) to $Ang_{1–7}$ was demonstrated to be due to POP and not angiotensin-converting enzyme 2 (ACE2) or PRCP [48]. The conversion is crucial, on the one hand, to remove Ang II and reduce its potentially harmful effect, and on the other hand, to form the $Ang_{1–7}$ peptide which has a tissue-protective effect, to oppose the undesirable chronic effect of an excess of Ang II.

Studies conducted with strong and selective POP inhibitors, as well as with inactive POP mutants, have shown that peptidase activity is not required to support certain processes that are clearly associated with POP. Protein-protein interactions between POP and binding partners (α-tubulin, neurospecific growth-associated protein-43 (GAP-43), glyceraldehyde-3-phosphate dehydrogenase and α-synuclein) likely are key in these processes, and the enzymatic activity of POP is needed only to regulate these interactions [49–52]. Thus, the role of POP in cell proliferation and growth of neuronal growth cones was not dependent on the catalytic activity of POP, but rather on protein-protein interactions. The region in the POP structure that acts as a binding site for other proteins has yet to be elucidated. One possibility is the loop structures of POP, of which loop A is probably the most important for association with the substrate, since inhibitors induce a change in the conformation that make this loop inaccessible to the substrate.

POP is an attractive therapeutic target for the treatment of cognitive and neurodegenerative diseases. POP involvement in the aggregation of

α-synuclein protein, both *in vitro* and *in vivo*, is perhaps one of the most documented examples of the action of POP. In mouse and human, POP increases synuclein dimerization through binding, retaining the original full-length α-synuclein [49,53,54]. Inhibition of POP reduces synuclein aggregation and has been proposed as a new therapy for synucleinopathies, which are neurodegenerative diseases associated precisely with the accumulation and aggregation of α-synuclein in certain groups of brain cells. The accumulation of insoluble α-synuclein aggregates disrupts the function of cells and eventually leads to their death. This group of neurodegenerative diseases includes Parkinson's disease, dementia with Lewy bodies, multisystem atrophy, and Hallervorden-Spatz disease. The development of new POP inhibitors is of great interest in the treatment of synucleinopathies [55]. Scientists from the Institute for Research in Biomedicine (IRB Barcelona) have proposed a new type of POP inhibitor based on sulfonylfluoride peptidomimetics, which are irreversible, selective and have the ability to penetrate the brain. Importantly, these inhibitors are > 1000-fold selective for POP compared to two family-related peptidases (DPP4 and FAP) [56].

3.1.5. Biological functions

Identification of the physiological substrates and reaction products of POP is an important step toward understanding its biological function. *In vivo* and *in vitro* investigations of POP revealed potential biological substrates, as well as the participation of POP in regulating the activity of biologically active peptides [57,58]. In particular, POP activity has been described in children with autism and may be associated with the degradation of specific neuropeptide hormones that affect social behavior and interpersonal skills [59,60]. However, in general, the evidence that POP is directly responsible for the metabolism of neuropeptides or their precursors *in vivo* is speculative. As the main activity of POP is intracellular, it is difficult to associate this peptidase with the direct degradation of extracellular neuropeptides [61]. However, the release of POP from the cell into the extracellular space can occur, as POP has been detected in blood plasma [62] and seminal fluid, where it can degrade the fertility factor TRH [34]. POP plasma levels are decreased in depressed people and increased in patients with manic disorders and schizophrenia, indicating a possible association between changes in POP activity and neuropsychiatric diseases [63]. Changes in the level of POP in psychiatric patients with depression, mania or schizophrenia can be the result of an unregulated release of the cytosolic enzyme into the circulating blood flow and cerebrospinal fluid, because many of the polypeptides hydrolyzed by POP have a significant effect on social behavior, emotions, sensitivity, stress resistance.

The co-localization of POP and α-synuclein in the human brain also supports the hypothesis that POP is associated with the pathogenesis of neurodegenerative diseases. In addition, inhibitors of POP blocked the aggregation of α-synuclein *in vitro* and *in vivo* in a cellular and animal model for Parkinson's disease [64]. In a knockout mouse line, POP deficiency led to an increase in anxiety and motor activity [65]. The authors believe that POP deficiency causes changes in brain plasticity, which in turn affect behavior and brain development. The effect of POP on hippocampal plasticity and spatial memory formation was also demonstrated in POP knockdown mice [66]. However, the difficulty in targeting POP in medical therapeutics is that this peptidase can be involved in the regulation of many physiological processes simultaneously [28].

A quantitative determination of POP activity indicated that this activity can be used as an additional independent prognostic parameter in assessing survival of patients with colorectal cancer [67]. POP activities changed in opposite directions in the tissue and plasma of patients with colorectal cancer; low survival was characterized by low POP activity in the tissue and high in plasma, significantly higher than the level of POP activity in healthy people. The authors suggest that measuring POP activity in plasma can be a reliable, minimally invasive, and inexpensive means of determining tumor aggressiveness. POP activity in tumor tissue may be a marker of early or/and less aggressive forms of the disease.

3.2. Dipeptidyl peptidase 4 (DPP4) family (EC 3.4.14.5)

The family of DPP4 proteins includes six members: active serine peptidases DPP4, fibroblast activation protein α (FAP), DPP8 and DPP9 (subfamily S9B) and two inactive DPP4-homologs DPP6 and DPP10 (subfamily S9X) (Table3). All active DPP4 family proteins have a relatively high amino acid sequence identity, with residues of the catalytic triad in the order of Ser-Asp-His, a conserved motif GWSYGG around the catalytic residue Ser, and a pair of Glu residues in β-propeller domain - highly conserved and essential for the dipeptide cleaving activity [68].

3.2.1. Structure

DPP4 family members can be paired by the identity of amino acid sequences. DPP4 and FAP, DPP8 and DPP9, DPP6 and DPP10 have sequence identities of 52–61%; otherwise, the sequence identity between representatives of different pairs is 27–30% [69]. Among these pairs, DPP6 and DPP10 lack enzymatic activity because the catalytic Ser residue is replaced by Asp in DPP6 and by Gly in DPP10, although Asp and His residues required for the formation of the catalytic triad and the conserved Glu residues are preserved [70,71]. When the Trp residue preceding the catalytic Ser is replaced by Lys, the conserved GWSYGG motif in the α/β-hydrolase domain is transformed into GKGYGG in DPP10 and into GKDYGG in DPP6 [72,73]. The S1-subsite of DPP4, DPP8, DPP9 and FAP peptidases is formed by the residues Tyr631, Val656, Trp659, Tyr662, Tyr666 and Val711 (hereafter the numbering is given by the human DPP4 accession P27487) [74,75]. The S2-subsite includes residues Asn710, Arg125, Glu205 and Glu206. Comparing the DPP4 and FAP pair, DPP4 has an Asp residue at position 663, whereas FAP contains Ala residue in this position. In this case, FAP may have both dipeptidylpeptidase and endopeptidase activity, the latter connected with greater compactness of the Ala residue compared to Asp, which allows the substrate to pass further into the active center and to be hydrolyzed in the middle of the chain [74,76,77]. DPP4 contains nine potential N-glycosylation sites, and carbohydrates constitute about 18–25% of the total mass of the enzyme. But, despite the significant proportion of the carbohydrate moiety in the molecule, the question of the function of glycosylation in DPP4 is unclear. A mutation at one of glycosylation sites (Asn319Gln) affects enzymatic activity, dimerization ability, and transport to the plasma membrane [78]. However, point mutations of glycosylation sites (namely, the replacement of Asn to Ala) did not significantly affect the proteolytic activity, the dimerization of the enzyme and the binding of adenosine deaminase (ADA) [79].

Alternate transcriptional splice variants encoding various isoforms of DPP4 family proteins have been characterized [80]. Long and short variants of DPP9 (863 and 892 amino acids) have different localization. DPP8 and the short cytosolic form of DPP9 contain 100 amino acid residues more than DPP4. However, DPP8 and DPP9 do not have a transmembrane domain that is characteristic of other DPP4 family proteins.

Tertiary structures are solved for human DPP4 (Fig. 1B) [81,82], DPP8 (Fig. 1C) [83], DPP9 (Fig. 1D) [83], FAP (Fig. 1E) [74], DPP6 (Fig. 1F) [84] and DPP10 (Fig. 1G) [72]. All tertiary structures delineate a multi-domain protein, with an N-terminal β-propeller and a C-terminal peptidase unit (Table 1) and an active site located at the interphase of the two domains. DPP8 and the short form of DPP9 may have additional elements of a tertiary structure at the N-terminus or in a β-propeller domain containing additional protrusions compared to other members of the DPP4 family. Unlike the structure of DPP4, where ligand binding does not induce a structural reorganization, ligand binding to DPP8/9 induces significant rearrangement in the active center, indicating differences in the architecture of the active centers of DPP4 and DPP8/9 and in the mechanism of substrate binding [83]. The formation of intramolecular disulfide bonds in DPP8 and DPP9

regulates the peptidase activity of these proteins, reducing the flexibility of the tertiary structure [85]. These data present new perspectives in the development of specific inhibitors and the use of DPP8/9 as specific therapeutic targets.

The structure of the peptidase unit of DPP4 family proteins is an α/β/α sandwich, which has been described as "α/β-hydrolase" fold [74,86,87]. The α/β-hydrolase domain, containing the catalytic triad, is highly conserved throughout this family of proteins, while the β-propeller domain is variable, and may be associated with non-enzymatic functions.

3.2.2. Specificity

All active peptidases from the S9B subfamily have dipeptidyl peptidase activity specific for N-terminal Xaa-Pro sequences and hydrolyze only small oligopeptides, which is due to the steric hindrance of the catalytic site by the beta-propeller domain at the N-terminus (Table 3). DPP4 recognized either proline or hydroxyproline in substrates, but hydrolysis rate was significantly lower for substrates containing hydroxyproline [88]. In nature, peptidases from the DPP4 protein family hydrolyze growth hormone releasing factor (GRF), substance P and β-casomorphin [7]. FAP is an exception, because in addition to dipeptidyl peptidase activity, FAP also has endopeptidase activity as described above, which provides cleavage of α2-antiplasmin, gelatin and type I collagen. Mutation of the catalytic residue Ser to Ala in FAP resulted in the loss of both activities [89]. Since FAP expression is associated with wound healing, malignant tumor growth and chronic inflammation, all requiring degradation of the extracellular matrix, collagenase activity of FAP is likely important for this degradation [90]. Using degradomic and proteomic techniques, natural substrates of FAP were identified, which should provide a breakthrough in elucidating the physiological and pathological roles of this enzyme [91].

Some physico-chemical properties of the DPP4 protein family, common substrates, and inhibitors are summarized in Table 3.

3.2.3. Localization

DPP4 family proteins include both membrane-bound and soluble enzymes that exhibit a wide variety of expression patterns, tissue distributions and compartmentalization. FAP and DPP4 are integral membrane glycoproteins and require dimerization for catalytic activity [9,12] (Table 1). The majority of the protein is extracellular. The dimeric soluble form of DPP4, released from the membrane after cleavage of the transmembrane region, is found in extracellular fluids, including serum, seminal fluid, saliva and bile, and also in the kidneys. Release of the initially membrane-bound DPP4 is due to matrix metallopeptidases [9,*92*].

Inactive members of the group DPP6 and DPP10 are single-pass type II membrane-bound glycoproteins. In contrast, DPP9 and DPP8 are entirely intracellularly localized [12,93]. The dominant conformation of DPP8/9 is a homodimer, wherein at least DPP8 may be active as a monomer [94]. The N-terminal extension of the longer version of DPP9 contains a nuclear localization signal, and this form is localized in the nucleus [80,95].

Catalytically active members of the DPP4 family, with the exception of FAP, are widely represented in various organs and tissues of mammals [96]. FAP has a limited distribution *in vivo*, and is usually absent in normal adult epithelial, neural and lymphoid cells, or in nonmalignant tumors. However, high levels of FAP are found in activated myofibroblasts and hepatic stellate cells in fibrosis and in stromal fibroblasts of epithelial tumors. Expression of FAP is induced during inflammation at sites of tissue remodeling by activated hepatic stellate cells of cirrhotic liver, and by fibroblasts of epithelial tumors and sarcomas [12]. DPP4 can also be a potential marker for a number of cancers, but the expression of DPP4 is variable among various types of cancer. For example, expression of DPP4 is upregulated in a number of aggressive types of T-cell malignancies, but progressively downregulated in endometrial adenocarcinoma [97].

DPP8 and DPP9 also are widely distributed in the human body. However, the activities of DPP8/DPP9 are higher than the activity of DPP4 only in the brain and testis [93]. High expression of DPP8 and DPP9 in pathogenesis is associated with testicular tumors, infiltrating lymphocytes in diseased liver and activated lymphocytes in inflamed lung [93]. The expression of catalytically inactive DPP6 is observed exclusively in the brain, whereas variants of the DPP10 mRNA, short and long, are found in brain, pancreas and adrenal gland [71].

3.2.4. The mode of action

The mode of action of members of the DPP4 family includes both enzymatic and non-enzymatic mechanisms. Both functions can work synergistically, in opposition, or even independently, depending on the specific protein, the microenvironment, and cell type. The enzymatic mechanism of action occurs in the hydrolysis of peptide hormones and neuropeptides, in the degradation of the extracellular matrix, and in the cleavage of Pro-rich proteins during digestive process. Using mutagenesis and a DPP9 inhibitor, activity of DPP9 was indicated in the regulation of cell behaviors and participation in cell signaling through inhibition of Akt (protein kinase B) activation, leading to increased apoptosis and suppression of cell proliferation [98]. In that study, DPP8, highly homologous to DPP9, did not affect Akt activation. The data highlighted the importance of the signaling role of DPP9 in the regulation of survival and proliferation pathways. DPP9 also is a negative regulator of tyrosine kinase Syk, a central kinase in B-cell signaling [99]. DPP9 hydrolysis of Syk produced a shortened Syk molecule with an N-terminal Ser residue that was recognized and bound by ubiquitin E3 ligase Cbl. Inhibition of DPP9 activity reduced the interaction of Cbl with Syk, indicating that pre-processing of Syk by DPP9 is required for Syk ubiquitinylation.

On the other hand, evidence of non-enzymatic functions is found in the results of studies where similar effects were observed using enzyme-inactivated DPP mutants and wild-type DPP. Cells overexpressing DPP8 and DPP9 in the presence of extracellular matrix components had different properties, such as cell adhesion, migration, and wound healing that were not associated with the enzymatic activity of peptidases, but rather with ligand binding, possibly in the β-propeller domain.

The regulatory effect of the soluble form of DPP4 on the proliferation of T-cells also did not depend on the enzymatic activity; the catalytically inert form due to a point mutation had the same effect on the proliferation of T-cells as wild-type soluble DPP4 [100]. The same was true for the effect of DPP4 and FAP on apoptosis and cell–extracellular matrix interactions, including the binding of fibronectin and collagen; collagen binds in the region of the cysteine domain between residues 238 and 495, whereas fibronectin binds to residues 469–479, [101–103]. The same is true for the interaction of DPP4 with ADA, regulating the extracellular concentration of adenosine and affecting the proliferation of T-lymphocytes [104]. In human B and T cell lines, the binding of DPP4 to recombinant soluble HIV-1 envelope glycoprotein gp120 inhibited ADA binding [105]. The results suggest that the specific function of gp120 is to inhibit the binding of ADA to DPP4, leading to severe combined immunodeficiency syndrome with significant consequences for the pathogenesis of AIDS. The formation of a complex of plasminogen with DPP4 initiated a signal transduction mechanism that regulates the expression of matrix metallopeptidase 9 by prostate cancer cells [106]. The ultimate evidence of the non-enzymatic mechanism of action is represented by DPP6 and DPP10, completely lacking an enzymatic component, but performing biological functions due to protein-protein interactions [12]. Critically missing, the identity of DPP ligand(s) are unknown [102].

3.2.5. Biological functions

Although these peptidases have similar enzymatic activities and conserved tertiary structures, different patterns of expression and localization indicate differences in their biological role. Even the same representative of this family can have opposing biological functions

(*i.e.*, anti-tumorigenic or tumorigenic effects) depending on factors such as cell type, regulation, and microenvironment. For example, DPP4 and FAP were identified both as cancer markers and potential therapeutic targets, or as peptidases with anti-tumorigenic properties [107]. The multifunctionality of the peptidases from the DPP4 family is manifested through their participation in various processes associated with normal metabolism and diseases [9,12,15]. DPP4 is represented most extensively in all these processes, and this enzyme is considered as a therapeutic target of type 2 diabetes [108,109] and as a participant in the process of lymphocyte activation. However, there was no evidence of alteration in morbidity and mortality, antigen-specific T cell proliferation and cytokine production, nor the anti-influenza antibody response in FAP knockout mice [110]. FAP, which is not associated with either normal metabolism or immune functions, is the only DPP4 family peptidase associated with fibrosis and, along with other DPP4 peptidases, can serve as a potential marker of some types of cancer.

Investigation of different model systems reveals the opposing actions of FAP on tumor growth, causing transmission of growth stimulatory or inhibitory signals by association with membrane-bound signaling molecules [111]. FAP acts in a cell-context dependent manner through a combination of peptidase activity and formation of complexes with other cell surface molecules due to protein-protein interactions. FAP expression is absent in normal adult tissues and benign tumors, unlike other members of the DPP4 family [11], but a high level of FAP is observed in multiple epithelial cancers, chronic inflammation, liver cirrhosis, and mesenchymal cells of remodeling tissue. The presence of collagenase activity in FAP, unlike DPP4, ensures the degradation of extracellular matrix proteins, which contributes to cancer progression, facilitating invasion and metastasis [11,112]. DPP4 and FAP gene loci are adjacent at human chromosome 2, 2q24.2 and 2q24.3, respectively, suggesting gene duplication, in contrast to other related genes of the DPP4 family that are on different human chromosomes [68]. When DPP4 and FAP are co-expressed in human migratory endothelial cells, FAP can form a heterodimeric membrane-bound proteolytic complex with DPP4, where both enzymes have exopeptidase activity, and additionally FAP has collagenase activity [12,113]. The functioning of the complex is due to the unique ability of the cysteine-rich region of the extracellular part of DPP4 (independent from the catalytic region with exopeptidase activity) to bind with collagen I, which facilitates local degradation of the extracellular matrix by FAP and the invasion of the endothelial cells into collagenous matrices [114,115]. In tumorigenic cells and wounds, this heterodimeric complex is assumed to play an important role in tumor invasion, spreading of metastasis, angiogenesis, and wound-healing [116]. The unique tissue distribution of FAP makes it a potential marker and therapeutic target for some human epithelial cancers [117]. At the same time, the overexpression of a catalytically inactive mutant FAP can have an antitumorogenic effect [118].

Due to the presence of soluble forms of DPP4 and FAP in the blood serum, some peptides with a Pro residue in the penultimate N-terminal position and circulating in the blood may be potential substrates for the enzymatic activity of these peptidases. Since most peptide hormones and neuropeptides contain one or more Pro residues and are small in size, the peptidases of the S9B subfamily are able to perform a regulatory role, participating in the processing and degradation of peptide hormones and neuropeptides [57]. DPP4 inactivates incretin hormones (glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)). In this regard, DPP4 inhibitors (gliptins), which prevent N-terminal degradation of endogenous incretins *in vivo* and lead to an increased concentration of intact biologically active peptides, act in plasma as antidiabetic drugs, increasing glucose-mediated insulin secretion and suppressing glucagon secretion [119,120]. A potentially wide range of competitive DPP4 inhibitors are associated with a structural feature of the enzyme, namely, the presence of an extensive cavity in the region of the active center [121], into which molecules of various size and chemical nature can integrate. Clinically significant DPP4 inhibitors can be functionally divided into two main groups: peptidomimetics imitating a dipeptide substrate, and those that are not. Among the commonly used inhibitors, peptidomimetic inhibitors include two nitrile-containing, vildagliptin and saxagliptin, as well as sitagliptin, created on the basis of β-amino acids, while alogliptin has a modified pyrimidinedione, and linagliptin is xanthine-based [122]. Sitagliptin and alogliptin are highly selective inhibitors, not affecting the activity of other representatives of the DPP4 family. Linagliptin also has an affinity for FAP, and vildagliptin and saxagliptin are able to inhibit DPP8/9 [123]. The lack of selectivity of some currently used inhibitors may be an obstacle to their application in the treatment of certain groups of patients. Therefore, the search for new highly selective inhibitors capable of forming a stable complex exclusively with a specific member of the DPP4 family is an important goal of modern medicine.

DPP4 and FAP efficiently cleave neuropeptides, such as neuropeptide Y ($NPY_{1–36}$), released during intense stress and expressed in the nervous system, endothelium, immune cells, megakaryocytes, adipose tissue and gut. NPY is involved in the regulation of energy balance and developing pleiotropic activities ranging from the control of obesity to anxiolysis and cardiovascular function. As a result of cleavage by DPP4 or FAP, $NPY_{3–36}$ loses affinity for the Y1 receptor, but increases affinity for Y2 and Y5 receptors, which leads to elimination of its vasoconstrictive properties and converts it into a vascular growth factor [124]. Further, under the action of plasma kallikrein, $NPY_{3–35}$ is formed, unable to bind to known NPY receptors [125]. DPP4 and FAP also cleave peptides containing YY motif (PYY) in the gastrointestinal tract. Binding of this PYY peptide or the cleavage product to Y receptors results in a decrease in appetite and slows gastric emptying. DPP4 and FAP also cleave substance P, which mediates multiple activities in various cell types, including cell proliferation, antiapoptotic responses, and inflammatory processes that may be limited by the proteolytic degradation of neuropeptides [124]. However, the relationship of the functional role of these peptidases with the cleavage of neurohormones requires further in-depth studies.

The high level of DPP4 found in the intestinal brush border membranes may indicate another important peptidase function - digestive, associated with the hydrolysis and assimilation of Pro-rich proteins. For example, normal rats on a high proline (gelatin) diet had 3–6 fold increase of DPP4 levels in the intestinal brush border membranes [126], while DPP4 deficient rats lost weight when fed Pro-rich gliadins compared to deficient rats fed diets without Pro-rich proteins and control rats with normal DPP4 level [127]. During the digestion process, peptidases associated with the intestinal brush border membranes, including DPP4, hydrolyze peptides obtained during stomach digestion of gliadins and other similar cereal proteins rich in proline. In the small intestine, the exopeptidase activity of DPP4 may play a key role, contributing to a more complete hydrolysis of the peptides, which, due to the high content of Pro residues, are resistant to proteolysis by other peptidases [128–130]. Undigested gliadin peptides recognized by mucosal T-cells initiate an immune cascade in patients with celiac disease (CD), which ultimately leads to mucosal damage and other symptoms of CD [131]. The activity of intestinal DPP4 is decreased in children and adult patients with active CD [132,133]. However, statistically significant differences were not found in the activity of serum DPP4, released as a result of proteolytic cleavage of the membrane-bound enzyme, in patients with CD and the control group. Therefore, the activity of serum DPP4 cannot be used as specific noninvasive diagnostic or prognostic marker of CD disease.

Glycosylation of DPP4 also is associated with certain (patho-)physiological conditions. N-terminal sialylation of DPP4 is higher in resting T cells than in activated, and hypersialilation is associated with HIV infection, rheumatoid arthritis, systemic lupus erythematosus, and aging, while a low level of sialylation is characteristic of lung cancer [134]. The residues of mannose-6-phosphate in the carbohydrate moiety of DPP4 are essential for interaction with the receptor of insulin-like growth factor 2 (IGF2R) in activated T cells [135].

DPP8 and DPP9 are functionally similar, although DPP8 is less involved in cellular processes, such as apoptosis or antigen presentation. DPP8 and DPP9, along with other peptidases of this family, are expressed by immune cells and, together with DPP4, inactivate incretins (insulin secretion hormone stimulants) [136]. DPP8 and DPP9 enhance already induced apoptosis, while DPP9 can act as a primary inducer of apoptosis. There is some evidence that altered expression of DPP8 and DPP9 may be associated with disease pathogenesis, as increased activities are associated with asthma [137], as well as an increase in the level of DPP9 mRNA associated with testicular tumors [93].

Through siRNA silencing, DPP8, but not DPP9, was shown to be a new therapeutic target in the treatment of multiple myeloma [138]. Selective inhibition of DPP8/9 was sufficient for cytotoxic activity against acute myeloid leukemia (AML) cell lines and primary AML samples, which is an argument for the development of a strong and selective inhibitor of DPP8/9 as a therapeutic agent in the treatment of AML [139]. Chowdhury et al. [140] showed that DPP8 and DPP9 were involved in lymphocyte apoptosis, widely expressed in lymphocyte subpopulations and upregulated in activated lymphocytes in a time dependent manner. In contrast to DPP4, the participation of DPP8 and DPP9 in the proliferation of lymphocytes requires enzymatic activity [141]. DPP8 and DPP9 also were involved in the regulation of epidermal growth factor in hepatocytes, a mitogen important for hepatocyte proliferation and liver regeneration, and a change in expression levels was correlated to liver damage. In addition, within monocytes and macrophages, DPP8/9 activity suppressed the activation of inflammasomes, which are multiprotein complexes formed in response to microbe-derived pathogen-associated molecular patterns and capable of triggering the inflammatory form of programmed cell death called pyroptosis. Initiation of this process occurs through activation of procaspase-1 by the inflammasomes protein NLRP-1. Active caspase-1, in turn, cleaves and activates the inflammatory cytokines and Gsdmd protein, inducing pyroptotic cell death and stimulating a powerful immune response. DPP8/9 inhibitors suppress inhibitory activity of DPP8/9 on inflammasomes leading to activation of human NLRP-1, its homolog CARD8, and several mouse NLRP-1 alleles. The molecular details of this pathway are still not fully understood [142–144], but the data indicates the multiplicity of roles that DPP8 and DPP9 can play in the regulation of the immune system as well as in liver fibrosis.

Catalytically inactive members of the DPP4 family, the DPP6 and DPP10 proteins, are modulators of voltage-gated potassium channels in neurons, which are important in neuronal function and in dysfunction, such as Parkinson's disease. They are mainly expressed in the brain [71,145].

Thus, the DPP4 family includes six proteins with a similar tertiary structure, including α/β hydrolase and 8-blade β-propeller domains. This structure determines the common substrate specificity of the active members of the family, as well as the ability to perform both catalytic and non-catalytic functions in the cell. Different localization, physicochemical and catalytic properties of the members of this group also explain the wide representation and variety of functions attributed to representatives of the DPP4 family proteins.

3.3. Dipeptidyl peptidase 2 (DPP2, DPP7) (EC 3.4.14.2)

DPP2 is a representative of another family of serine peptidases, S28. Some physicochemical properties of DPP2, structure of substrates for determining activity, as well as inhibitors are summarized in Table 3. The amino acid sequence similarity of human and mouse DPP2 is 79.4%, and human and rat is 78.9% [10].

3.3.1. Structure

DPP2 is synthesized as a pro-enzyme and exists as a homodimer (Table 1). The 3D structure of DPP2 is presented in Fig. 1H [146]. The mature enzyme contains two main domains: α/β-hydrolase fold and α-helical bundle, which serves as a cap for the active center. Structural features of DPP2 are similar to PRCP, another PSP belonging to the same family, and the catalytic domain (α/β-hydrolase) is also similar to proteins in the DPP4 family. The amino acid similarity between human DPP2 and lysosomal PRCP is 41.2%. The catalytic site of DPP2 is located deep inside the cleft between its two domains, and substrates must reach the active center by navigating a narrow tunnel. The catalytic triad of DPP2 consists of conserved residues Ser162, Asp418 and His443. DPP2 is the first peptidase described with a leucine zipper motif, through which a functional homodimer appears to be formed [146,147]. Unlike PRCP, DPP2 contains a unique short (12 amino acid residues) insert that forms a disulfide-stabilized hairpin, sterically restricting access to the active center and, possibly, causing differences in substrate specificity between DPP2 and PRCP [148]. The non-catalytic domains of DPP2 and DPP4 are structurally different, with a β-propeller domain for DPP4 compared to spiral fold for DPP2 (the so-called SKS domain), both occupying equivalent space [146].

3.3.2. Specificity

Similar to DPP4, DPP2 releases N-terminal Xaa-Pro dipeptides but also easily hydrolyzes tripeptides, and quickly loses activity with an increase of the length of the peptide substrate. DPP2 cleaves only the fragments of substance $P_{1–4}$, bradykinin$_{1–3}$ or bradykinin$_{1–5}$, and does not act on longer peptide substrates of DPP4 [149]. Substrate specificity of DPP2 is similar to DPP4, but the best chromogenic substrate for DPP2 is Lys-Pro-pNA and the optimum pH of its activity is shifted to a more acidic region (5.5–6.3) [150,151]. The replacement of proline with hydroxyproline reduces the efficiency of hydrolysis [151,152]. In addition, DPP2 has the unique ability to cleave Pro-Xaa, Pro-Pro and Hyp-Xaa bonds. Such activity may indicate a special contribution of DPP2 to the breakdown of Pro-rich proteins, such as collagen, which consists mainly of repeating Gly-Pro-Xaa units (‘collagen triplets’).

3.3.3. Localization

The assumption of the role of DPP2 in the terminal stage of degradation of intracellular proteins is supported by the presence of a signal peptide, lysosomal localization [19] and a predominant effect on tripeptides with possible hydrolysis of some small oligopeptides. The DPP2 homodimer is also found in the lumen of intracellular vesicles other than lysosomes, suggesting that part of the total activity of DPP2 is secreted, and that secretion is regulated, apparently, by changing the flow of calcium [153].

DPP2 is expressed in all human organs and tissues examined, with high expression in kidney, brain, seminal fluid, resting lymphocytes and differentiated macrophages [10]. Quantitative distribution of DPP2 activity suggests the existence of organ and species dependencies.

3.3.4. Biological functions

DPP2 has no defined *in vivo* substrates, therefore it is difficult to determine its actual biological role. Some assumptions about possible functions can be made based on its localization and changes in activity in certain processes or pathologies. Thus, it was shown that inhibiting DPP2, either by inhibitors or small interfering RNA, causes apoptosis of quiescent G_0 lymphocytes [154]. Based on results obtained in mice with DPP2 knockdown, DPP2 was proposed as a previously unknown regulator of glucose homeostasis [155]. In addition, changes in DPP2 activity were observed in a number of pathologies, such as neurodegenerative disorders, myopathies, cancer, and gastro-intestinal disorders. The role of DPP2 has been proposed in the final stage of peptide degradation as part of the autophagic process or the degradation of endocytosed proteins [10].

3.4. Prolyl carboxypeptidase (PRCP) (EC 3.4.16.2)

PRCP belongs to the S28 family of serine peptidases. Some physicochemical properties of PRCP, structure of substrates for determining its activity, as well as inhibitors, are summarized in Table 3.

3.4.1. Structure

PRCP is synthesized as a preproenzyme with a signal and pro-peptide, and the human PRCP consists of 496 amino acids, with the active form found as a homodimer. According to experimental data, the inactive PRCP pro-enzyme can be activated by trypsin or chymotrypsin [20,156], however, analysis of the primary structure indicates that the bond after the C-terminal residue Lys of the propeptide can only be cleaved by trypsin. The S1 binding subsite of human PRCP contains Met183, Trp359, Met369 and Trp432 residues [148]. The C-terminal part of the sequence contains the so-called "serine or threonine repeat", in which Ser is repeated as the 26th residue in six out of nine repeats, with identical or similar amino acids in other positions in the repeats. In the other three repeats, Ser is replaced by Thr, Gly or Glu residues. At the sequence level, human PRCP and DPP2 are unique and not similar to other PSPs. For instance, human PRCP has 8.4% sequence identity with POP and 6.5% with DPP4 [148].

The PRCP tertiary structure (Fig. 1I) [148] is similar to DPP2 and includes a typical α/β hydrolase domain containing a catalytic triad and a novel helical bundle (SKS domain) that forms a cap over the active center. In addition, dimerization of PRCP subunits occurs, which is consistent with the biochemical properties of PRCP in solution.

3.4.2. Specificity

PRCP catalyzes cleavage of the C-terminal amino acid following a Pro residue, and the substrate *Z*-Pro-Xaa is usually used to test its activity (Table 3). The enzyme does not hydrolyze Z-Pro-Pro or Z-Pro-hydroxyproline [157,158]. The bioactive peptides angiotensin II and angiotensin III are cleaved 4 and 13 times faster, respectively, than the Z-Pro-Phe substrate corresponding to their C-terminus. In addition, the optimum pH in the breakdown of oligopeptides is broader (4.0–8.0) than in the breakdown of shorter peptide substrates (4.0–6.5) [157].

The binding of substrates by PRCP that are cleaved at the C-terminus requires access to the long substrate binding groove of PRCP. In contrast, the DPP2 molecule contains a structural insertion that creates a blocked substrate binding site, and thus DPP2 can accommodate only short dipeptidyl extensions at the N-terminus of potential substrates. These differences represent a remarkably simple evolutionary adaptation to impart the C- and N-terminal substrate specificities of PRCP and DPP2 within a conserved active-site architecture [148].

3.4.3. Localization

PRCP is a lysosomal enzyme and contains a signal peptide. It is expressed in human brain, heart, placenta, lung, liver, skeletal muscle, kidney and pancreas [20]. The lung, liver, placenta, brain and heart had the highest level of PRCP mRNA.

3.4.4. Biological function

The biological function of PRCP is related to the specificity of the enzyme to cleave the C-terminal amino acid bonded with a Pro residue in peptides, and is one of the key enzymes of the renin-angiotensin system. PRCP inactivates the vasoactive peptides angiotensin II and angiotensin III by cleaving the C-terminal Phe [157], and also a tridecapeptide α-melanocyte stimulating hormone (α-MSH), an anorexigenic neuromodulator, hydrolyzing the Pro12-Val13 bond [159,160]. Since the presence of α-MSH in the hypothalamus limits the increase of weight in mice, its inactivation by PRCP can lead to obesity. Therefore, the search and development of new PRCP inhibitors are sought as possible anti-obesity drugs. From a series of low molecular weight PRCP inhibitors that were pyrrolidinylimidazole derivatives, one was promising in reducing weight in mice *in vivo* [161]. Eight patent applications regarding the synthesis of low molecular weight PRCP inhibitors have been published [162]. However, there were mixed results with *in vivo* preclinical trials to evaluate the possibility of using various inhibitors to suppress appetite, reduce body weight and protect against obesity caused by diet. The ability of PRCP to modulate the action of peptide hormones such as angiotensin II and III, and also prekallikrein, suggests a specific role of the enzyme in hypertension, tissue proliferation and smooth-muscle growth [163]. At the same time, lysosomal localization of PRCP may indicate a role in the digestion of Pro-containing cellular proteins entering lysosomes through autophagy.

Similar to members of the DPP4 proteins family, PRCP has a function not related to enzymatic (carboxypeptidase) activity. Human plasma prokallikrein is activated by PRCP at 1:1 stoichiometric ratio. At the same time, the recognition site in prokallikrein does not correspond to the substrate specificity of PRCP. In this case, PRCP is proposed to act as an allosteric chaperone, binding and inducing a conformational change [164,165]. This protein-protein interaction of prokallikrein and PRCP can provide autocatalytic activation of the prokallikrein zymogen or allow other peptidases to convert it into an active kallikrein.

Thus, the S28 peptidase family includes two PSPs, PRCP and DPP2, similar in sequence and structural characteristics. Both enzymes are synthesized in the form of preproenzymes and have lysosomal localization and pH-optimum shifted to the acidic region. However, DPP2 is a dipeptidyl aminopeptidase, which cleaves the N-terminal dipeptides adjacent to Pro, and PRCP is a Pro-carboxypeptidase that catalyzes the cleavage of the C-terminal hydrophobic amino acid after the Pro residue. These differences in specificity may, at least partially, be associated with a single peptide insertion of a disulfide-stabilized short hairpin structure in the substrate-binding groove of DPP2.

4. Proline specific metallo peptidases

4.1. Aminopeptidases P1, P2 and P3 (APP1, APP2 and APP3) (EC 3.4.11.9)

There are three different human aminopeptidase P enzymes (APP1, APP2 and APP3), encoded by different genes located on different chromosomes. These forms are structurally related and belong to the M24 family (subfamily M24B) of metallopeptidases. Some properties of various APP are summarized in Table 1 and Table 3.

4.1.1. Structure

Amino acid sequences of APP1, APP2 and APP3 consist of 623, 674, 507 residues, respectively. Structural and metal content analysis confirmed that APP1 is a $Mn2^+$-dependent enzyme containing two $Mn2^+$ ions per subunit [166]. In APP1, one of the $Mn2^+$ ions is coordinated by Asp415, Asp426 and Glu537 residues, and the second by Asp426, Glu523, Glu537 and His489 residues (numbering is given according to human APP1 accession Q9NQW7) (Table 1). Structural analysis has shown that human APP1 exists as a dimer. Each subunit represents a unique three-domain structure, distinguishing it from other previously defined structures of *Escherichia coli* APP and prolidases, which had two-domain structures [167,168]. The 3D structures of APP1 [169] and APP3 [170] are presented in Fig. 1J, K. The C-terminal catalytic domain has a "pita-bread" fold similar to other APP, including two β-sheets surrounded by four α-helices. APP2 exists as a homodimer/tetramer. The amino acid sequences of APP1 and APP2 have 43% identity [23,171]. Based on its size and sequence similarity with APP1, APP2 likely shares the same three-domain X-prolyl aminopeptidase fold and dimerization as APP1. However, unlike APP1, APP2 binds one zinc atom to a polypeptide chain [172,173]. The similarity of the APP3 sequence was higher with aminopeptidase P from *E. coli* than with APP1 and APP2 of mammals [174].

4.1.2. Specificity

APPs are aminopeptidases that specifically cleave N-terminal amino acid residues from peptides with a Pro residue in the second position (in the P1' position relative to the cleavage bond) (Table 3). APP1 has broad substrate specificity and can cleave both Xaa-Pro dipeptides and longer peptides. APP1 hydrolyzes a number of physiological substrates, including bradykinin, substance P, corticortropin-like intermediate lobe peptide, casomorphin and [Tyr]-melanostatin. APP2 has a much

narrower substrate specificity: it cannot hydrolyze Xaa-Pro dipeptides and weakly cleaves longer peptides of the type Xaa-Pro-Yaa, where Xaa is Pro or Gly and Yaa is an amino acid with a bulky side chain [23]. Proline in the P1' position of APP2 can be replaced by 3,4-dehydroproline or homoproline, but not 4-hydroxyproline [174].

One of the best APP2 substrates is the nonapeptide hormone bradykinin with an Arg-Pro-Pro- N-terminus. Physiological substrates of APP3 are not known. APPs differ from other aminopeptidases in that they are not inhibited by bestatin, a common inhibitor of aminopeptidases.

4.1.3. Localization

APP1 is a soluble cytoplasmic ubiquitously-expressed enzyme. In humans, the highest expression level of APP1 has been found in liver and pancreas [23]. APP2 is localized on the plasma membrane of vascular endothelial cells and the brush border membranes of epithelial cells in the intestine and the renal proximal tubule. From the analysis of the APP2 structure, the enzyme has a signal peptide but lacks a transmembrane domain (Table 1). Therefore, APP2 is a type III membrane protein, which are anchored with a signal-anchor sequence, and their N-terminal domains are targeted to the ER lumen [175]. The enzyme is heavily glycosylated and contains a glycosylphosphatidylinositol (GPI) membrane anchor at the C-terminus. In humans, the highest level of expression of APP2 is found in kidneys [23]. The primary transcript of APP3 is susceptible to alternative splicing, which leads to the formation of the cytosolic or mitochondrial form of the enzyme. The mitochondrial form of APP3 has an N-terminal signal of mitochondrial localization, which is cleaved proteolytically after the import of the protein into mitochondria and is always absent in the cytosolic form. Both splicing variants are actively expressed in the kidneys [176,177].

4.1.4. Biological functions

The specific physiological substrates and molecular functions of APP1 and APP3 are not fully characterized. Despite the ability of APP1 to break down bioactive peptides *in vitro*, its cytosolic localization makes it unlikely to degrade these peptides *in vivo*. More likely, the non-specific auxiliary role of APP1 is in the intracellular degradation of peptides with a second Pro residue, possibly generated by the proteasome and resistant to the action of other peptidases [178]. Loss of APP1 leads to behavioral, cognitive and neurological deficits [179]. APP2, associated with the intestinal membrane and kidney brush borders, can perform a trophic function in the intestine or kidneys by participation in pre-absorptive degradation of both food and filterable peptides that contain Pro in the second position and a small amino acid in the third position. A very high level of APP2 in human kidney indicates the importance of this isoform in renal function. The ability of APP2 to cleave the peptide hormone bradykinin, which has a vasodilating effect, indicates a possible important physiological role of the peptidase. The APP2 inhibitor apstatin retarded the degradation of bradykinin and thereby resulted in lower blood pressure [180]. Thus, APP2 is a potential target for new cardiovascular drugs. The absence of specific natural substrates for APP3 suggests the involvement of this PSP, along with other *endo-* and exopeptidases, in total protein degradation, where it hydrolyzes Pro-containing peptides that are resistant to the action of other peptidases. However, there is evidence that mutations in the APP3 gene are associated with cystic kidney disease [176]. The physiological importance of mitochondrial localization of APP3 is not yet known. In the mitochondria, APP3 is involved in the mechanism of TNF-TNFR2 receptor-mediated signal transduction, leading to the activation of c-Jun-N-terminal protein kinases JNK1 and JNK2 [177]. The N-terminal part of mitochondrial APP3 between residues 54–79, which is absent in the cytosolic APP3 form, is responsible for binding to TNFR2. Moreover, the enzymatic activity of mitochondrial APP3 was not essential for JNK activation.

4.2. Prolidase (Xaa-Pro dipeptidase, XPD) (EC 3.4.13.9)

XPD is a member of the M24 family of metallopeptidases, requiring the binding of two bivalent manganese ions for catalysis. However, a partial substitution of Mn/Zn has been reported [181]. The two forms of prolidase, XPD I and XPD II, differ in molecular weights (56 kDa and 95 kDa, respectively), the requirement for metal ion, temperature stability, and substrate specificity [182–184]. Both XPD I and XPD II activities have been found in humans [137]. However, only the gene encoding XPD I has been identified in the human genome (EMBL-EBI: AC008744.6), for which three protein isoforms are known, two of which have shorter transcript variants yielding shorter proteins than the major isoform I (UniProt: P12955). XPD I from human and rat share 86% sequence similarity [183].

4.2.1. Structure

The first crystal structure of prolidase was resolved from the hyperthermophilic archaeon *Pyrococcus furiosus* [168]. The structure of human prolidase was modeled based on sequence homology with methionine aminopeptidase and aminopeptidase P of *E. coli* [185]. In 2017, the crystal structure of human prolidase (XPD I) (Fig. 1 L) confirmed that it is a homodimer consisting of two 493 amino acid long chains (Mw = 54,548 Da) oriented C2 symmetrically relative to one another [186]. Each subunit has two domains: the N-terminal domain (residues 1–184), and the C-terminal domain (residues 185–493) containing the active site of the enzyme. The C-terminal domains contain the so-called pita-bread fold. In human prolidase, one of the Mn^{2+} ions is coordinated by Asp276, Asp287 and Glu452 residues, and the second by Asp287, His370, Glu412 and Glu452 residues (numbering is given according to human prolidase accession P12955) (Table 1).

4.2.2. Specificity

Prolidase is a multifunctional enzyme that has the unique ability to hydrolyze imidodipeptides, with C-terminus Pro or Hyp residues, although the presence of Hyp in this position leads to a sharp drop in the rate of hydrolysis (Table 3). This peptidase exhibits a very narrow substrate specificity, cleaving structurally unique bonds between the hydrophobic amino acid residue at the N-terminus, and the Pro residue in *trans* conformation at the C-terminus [7,13,187]. In humans, XPD I is most active on a Gly-Pro substrate, whereas XPD II has high Met-Pro cleavage activity and low Gly-Pro cleavage activity. Two forms of prolidase also are found in rats, where XPD I is more active with Ser-Pro and Ala-Pro than with Gly-Pro, and XPD II is most active with Met-Pro [183].

4.2.3. Localization

Both forms, XPD I and XPD II, are cytosolic proteins and are active as homodimers. XPD I is most active in the small intestine and kidney, whereas the activity of XPD II was not variable in the tissues studied; only XPD I was found in human plasma [188]. Both forms of XPD were isolated from normal human erythrocytes, leukocytes, and cultured skin fibroblasts [182,183].

4.2.4. Biological functions

Prolidase is an important enzyme in the recycling of proline from imidodipeptides at the final stage of protein degradation in the catabolic pathway of exogenous (food) and endogenous (such as extracellular collagen) proteins, providing the organism with the necessary amount of free proline for resynthesis of proline-containing proteins [5]. Since free proline is required for collagen biosynthesis, which is essential for maintaining the structure and integrity of tissue [13], prolidase may act as a limiting factor in the formation of new collagen [5]. Collagen degradation, observed under metabolic stress, is triggered by the activation of matrix metallopeptidases. During the final stage, the emerging imidodipeptides enter the cell through specific peptide transporters [189], where they encounter prolidase. Thus, prolidases

perform an important function in the terminal stage of the turnover of the extracellular matrix.

The increasing interest in the functions of prolidase is due to the fact that mutations in a prolidase gene cause a rare recessive disease - a prolidase deficiency, or PD, which is characterized by severe skin lesions, recurrent respiratory infections, and mental retardation [187,190]. PD patients have a decrease or absence of XPD I activity in erythrocytes, leukocytes and cultured fibroblasts, with concomitant accumulation of uncleaved imidodipeptides in the urine [191]. At the same time, XPD II remains a normally functioning form in patients with PD, but the decrease or disappearance of the activity of XPD I leads to abnormalities in absorbing proline in the digestion of dietary proteins in the small intestine, as well as in reabsorbing proline generated from endogenous protein in the kidney [192].

The mechanism of XPD deficiency in the development of painful symptoms in humans remains poorly understood, as attempts to correct the loss of the enzyme activity of XPD have been largely ineffective [193]. In fact, the development of the disease in patients with PD may not be associated with catalytic activity, but with the protein-protein interaction between XPD and epidermal growth factor receptor (EGFR) [194]. Prolidase directly binds and activates EGFR, leading to the stimulation of signaling proteins involved in a variety of cellular responses. Furthermore, the dipeptidase activity of XPD is not required for the activation of EGFR, and XPD activates EGFR only when present in the extracellular space. Although XPD is a cytosolic protein, its release into the extracellular space from damaged cells and tissues has been detected and subsequently activates EGFR. XPD differs from all other known EGFR ligands, since does not have an EGF motif and is not synthesized as a transmembrane protein. Thus, the activation of EGFR by prolidase contrasts with other XPD activities, including stimulation of hypoxia inducible factor 1α, and also transformation of growth factor β1 [195], since these XPD activities were associated with the metabolism of imidodipeptides and required the intracellular presence of XPD. Perhaps the key to PD treatment should not be directed toward enzymatic activity, but instead related to the stimulation of EGFR signaling induced by the interaction of XPD with EGFR.

Changes in prolidase activity are found in many disease states, such as various types of cancers and fibrotic processes [13], and in most cases may serve as a convenient marker for early detection and monitoring of these diseases. Due to the high substrate specificity and differential expression in cancer cells, the use of prolidase as a potential target has been proposed to activate specific anti-cancer proline-containing dipeptide-like prodrugs [196,197]. Prodrugs, or drugs that are inactive when administered and are activated after metabolization, are a very promising form of specific anti-cancer therapy with limited effect on surrounding cells.

Recombinant human prolidase, along with the prolidases of various bacteria and archaea, hydrolyze toxic organophosphorous compounds and render them harmless [198,199]. The purified enzyme is able to hydrolyze sarin, cyclosarin and soman with different rates of hydrolysis. The use of prolidase encapsulated in stable liposomes and circulating in the blood for long periods can help to protect military personnel from dangerous contact with nerve gas [200].

Thus, PSPs belonging to the M24 family of metallopeptidases include two groups of enzymes – APPs and XPD. All members of this family are characterized by a classic “pita-bread” fold, they are active as homodimers and most of them have several forms. They have different localization (cytoplasmic, mitochondrial, membrane-bound) and play an important role in the final stages of proteolysis of proline-containing proteins and oligopeptides.

5. Conclusion

In this review, we have summarized that human PSPs and similar PSPs in model mammals belong to two different classes of proteolytic enzymes, to different clans, families, and subfamilies, but have a number of common properties. All active enzymes specifically hydrolyze peptide bonds formed by a Pro residue, which are resistant to proteolysis by peptidases with broad substrate specificity. All serine PSPs contain the α/β hydrolase domain, and metallopeptidases have the “pita-bread” domain. The majority function as homodimers and perform biological functions associated with both the peptidase catalytic activity and the ligand-binding ability of the enzyme.

We have discussed different functional roles for PSPs in biological systems. The catalytic activity of PSPs in regulatory pathways is associated with the ability of many PSPs to hydrolyze peptide hormones, neuropeptides, prodrugs. Catalytic activity of PSPs can also be directly associated with the proteolysis of total cellular protein or Pro-rich dietary protein in the digestive tract. PSPs can also participate in signal transduction, which does not require catalytic activity and is associated with the formation of protein-protein contacts that trigger or facilitate the performance of certain specific functions.

There is insufficient data on the balance between the enzymatic and non-enzymatic functions of PSPs and their total contribution to biological processes. Yet, we should not underestimate the role of PSPs set as a unique part of the normal human peptidase degradome providing the body free proline. In this case, any exogenous or endogenous proteins are substrates of PSPs, and the important role of the PSP complex is to provide proline residues necessary for new protein synthesis. The increase in the level of activity of PSPs also has been associated with some diseases, and in many cases true natural substrates or ligands of PSPs required to identify their specific biological functions have not been described. Of particular relevance to human disease is the search and development of new PSP inhibitors that normalize the abnormalities associated with changes in PSP activity. Most PSPs are members of the serine peptidase clan and have a number of chemical and functional properties common to other members of this clan, so that application of inhibitors as therapeutics may be limited by cross-interaction with other serine peptidases *in vivo*. Another consideration is that PSPs can participate in the regulation of many physiological processes, also complicating the use of inhibitors [28]. Members of the DPP4 family have overlapping specificities. However, the physiological functions of DPP8 and DPP9 still are not understood. The intracellular localization of these enzymes and the low rate of hormones cleavage diminishes the possibility of DPP8 and DPP9 participation in the hydrolysis of these peptides *in vivo*. At the same time, they may be intracellular enzymes that process unidentified natural substrates. There is no direct evidence of the participation of these enzymes in the processes of cancer, fibrosis and other tissue-remodeling processes due to the lack of specific substrates and inhibitors. Therefore, the structural and biochemical analysis of representatives of this PSP group can identify their true substrates, which will be a key step in understanding their exact functions and mechanisms of their involvement in biological processes and diseases. These studies also will facilitate the structure-based drug design of pharmaceutically important inhibitors. A selection of very specific synthetic substrates and inhibitors is needed to elucidate the *in vivo* role of each representative of the group.

The question of targeting PSPs from the S28 family as potential pharmaceuticals also remains debatable. The physiological functions of DPP2 are largely unknown due to the lack of well-defined natural substrates. The anti-inflammatory and cardioprotective potentials of PRCP are known. However, for the treatment of hypertension, there are already many commercial drugs with different mechanisms of action, and therefore the requirements for alternative drugs are very high, and noticeable additional benefits can be a decisive factor in gaining the attention of pharmaceutical companies [163]. So far, determining the structures of PSPs has provided the basis for a rational design of selective inhibitors of these enzymes, with the goal of minimizing cross-reactive drugs that can help in the treatment of cardiovascular and metabolic diseases, such as obesity and type 2 diabetes. Furthermore, detailed elucidation of the structure of PSPs will help to shed light on the evolutionary relationships of these peptidases through the analysis

of the architectural organization of their domains.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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