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Expanding the complexity of the human degradome: polyserases and their tandem serine protease domains

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1. ABSTRACT

The large and growing number of protease genes identified in the human genome, more than 560, reflects the complexity and relevance of these enzymes in multiple biological processes. As part of our studies on the human degradome –which is defined as the complete set of human protease genes– we have recently identified and cloned three complex polyserine proteases called polyserases. Polyserase-1 is a member of the type-II transmembrane serine protease (TTSP) family of proteolytic enzymes that undergoes a series of post-translational processing events to generate three distinct and independent serine protease domains called serase-1, -2, and -3. Polyserase-2 is a secreted enzyme that also possesses three serine protease domains, but they remain as an integral part of the initial protein product. Finally, polyserase-3 is also a secreted enzyme that contains two serine protease domains embedded in the same polypeptide chain. Despite all three human polyserases share this complex molecular design characterized by the presence of several catalytic domains in their structure, they also exhibit distinctive features including unique expression patterns and different enzymatic properties. At present, the putative functional advantages derived from the complex structural organization of polyserases remain unknown, but the widespread occurrence of these enzymes in mammalian degradomes provides additional evidence about the complexity of proteolytic systems in these organisms.

2. INTRODUCTION

The proteases comprise a large group of enzymes that share the common property of hydrolyzing peptide bonds. However, these enzymes are extremely heterogeneous in both structural and catalytic terms, which contributes to explain the broad variety of biological roles performed by proteases in all living organisms (1). The activity of proteolytic enzymes was initially associated with non-specific reactions of protein catabolism upon food digestion. This effect was first described more than 100 years ago by several authors including Ivan Pavlov. In fact, Dr. Pavlov was awarded the Nobel Prize for Physiology or Medicine in 1904 for his pioneering work demonstrating that the activation of pancreatic digestive enzymes was dependent on an additional enzymatic activity located in the upper section of the small intestine. This enzyme was first named enterokinase, but it was later renamed to enteropeptidase to emphasize its proteolytic function. Today, our view of the proteolytic world has considerably changed after the finding that proteases are involved in a wide variety of relevant biological processes including embryonic development, blood coagulation, tissue remodelling, wound healing, cell-cycle progression, angiogenesis, apoptosis, autophagy and senescence (2-8). Moreover, it is now well established that proteases participate in these key biological events through the selective and limited cleavage of specific substrates. Likewise, proteolytic

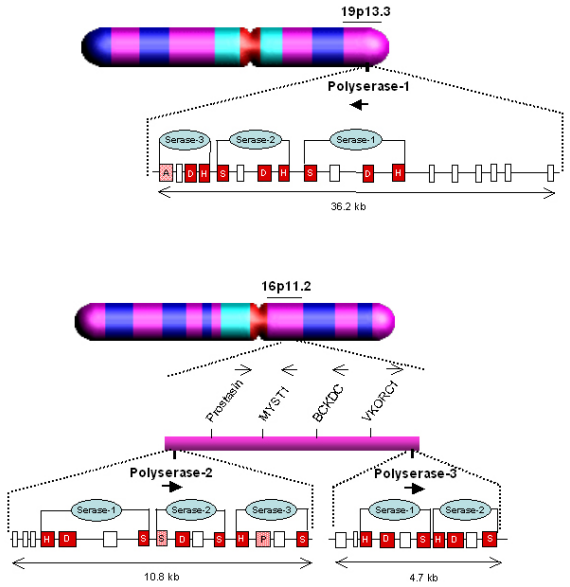

Figure 1. Chromosome location and intron-exon organization of the three human polyserases genes. The relative positions of the genes situated between the polyserase-2 and polyserase-3 genes in chromosome 16p11.2 are also included. Direction of transcription is indicated with an arrow, and the relative position of each exon with a box. Exons highlighted in dark red include the codons encoding the amino acids H, D, and S that form the catalytic triad of serine proteases. Exons highlighted in light red contain a change in the codons encoding any of these catalytic triad residues. The exons encoding the different serases of each polyserase, and the relative size of each polyserase gene are also indicated.

processes are often organized in complex cascades, in which some proteases activate others in a consecutive order. Hence, it is not difficult to understand that alterations in the structure and function of these enzymes underlie a significant number of important human diseases, including cancer, arthritis, progeroid syndromes, neurological disorders, and cardiovascular alterations (9-15).

The relevance and complexity of proteolytic systems has also been clearly appreciated through studies based on the bioinformatic exploration of different genomes. Thus, the human genome contains more than 560 genes encoding proteases or protease-like proteins that belong to five different catalytic classes: serine proteases, threonine proteases, aspartic proteases, cysteine proteases and metalloproteases (16). This number is even increased in the mouse and rat genomes where more than 600 genes encoding proteolytic enzymes have been identified (16, 17). Similar numbers of protease genes can also be recognized in the genomes of other model organisms such as Pan troglodytes, Drosophila melanogaster, Arabidopsis thaliana, Populus trichocarpa and Caenorhabditis elegans, which indicates that proteolysis is a fundamental mechanism for the normal development and function of all these organisms (18-22).

The increasing number of functions assigned to proteolytic enzymes has made necessary to create global concepts that could help to understand the in vivo roles of these proteins. In this regard, the term degradome defines the complete repertoire of proteolytic enzymes found in a cell, tissue or organism at any particular moment or circumstance (1). Analogously, cancer degradome would be the complete set of proteases that are involved in the initiation and/or progression of a particular neoplasia (23). From the genomic exploration of different degradomes, it can be inferred that a relevant number of genes coding for proteolytic enzymes have not yet been well characterized. In this regard, and as part of our studies focused on the characterization of mammalian degradomes, we have recently identified and cloned three complex human proteases called polyserases or polyserine proteases (24-26). These enzymes share a common structural design characterized by the presence of tandem serine protease domains. However, there are also relevant differences among them. Thus, polyserase-1 is a new member of the TTSP (type-II transmembrane serine protease) family of proteases. These enzymes are membrane-anchored proteins involved in a wide variety of crucial biological events and whose dysregulation has been associated with tumor growth and invasion processes (27-29). By contrast, polyserase-2 and -3 are secreted enzymes that share similar degree of identities with TTSPs and members of the tryptase/pancreasin family of serine proteases (30, 31). In this work, we review the available information on these three unusual proteolytic enzymes, whose functional role is still largely unknown.

3. POLYSERASE-1/TMPRSS9, A MEMBRANE-BOUND ENZYME CONTAINING THREE TANDEM SERINE PROTEASE DOMAINS

The TTSPs are a family of proteolytic enzymes that share some common structural features (27-29). Thus, all of them are membrane-anchored serine proteases containing a hydrophobic transmembrane domain at their N-terminal region and a catalytic serine protease domain at their C-terminal region. This catalytic unit possesses three conserved residues (His, Asp and Ser), that form the catalytic triad of the enzyme. Between these transmembrane and catalytic domains, a stem region containing a variable number of different modular structural domains can be identified in all TTSPs.

Pavlov´s enterokinase was the first discovered member of this family and, at present, more than twenty different TTSPs have been characterized (27-29). One of the most recently identified members of this growing family of enzymes is polyserase-1 (24). The existence of the polyserase-1 gene was first predicted through the exploration of human genome databases. Thus, different bioinformatic analysis suggested the presence of three novel genes encoding putative serine proteases in chromosome 19p13.3 (Figure 1). All these three predicted enzymes showed the highest percentage of identities with matriptase/MT-SP1 (32) and matriptase-2 (33). However, further detailed analysis of these coding regions revealed

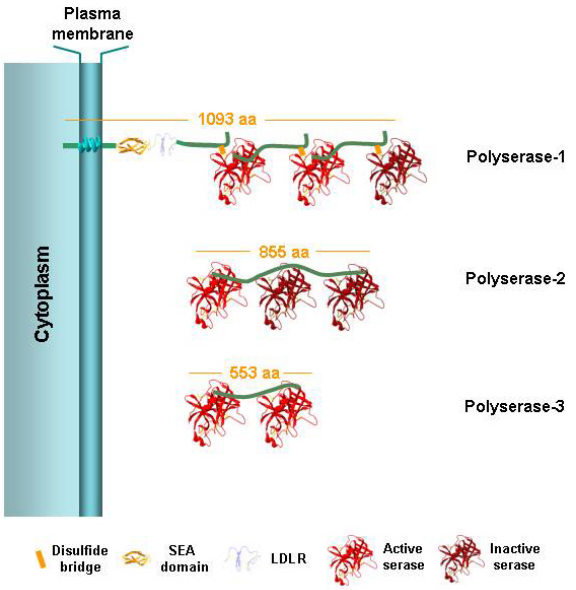

Figure 2. Domain organization of the three human polyserases. Polyserase-1 is a type II transmembrane serine protease. This model also includes the SEA domain present in the structure of the serase-1B variant of polyserase-1. Polyserase-2 and -3 are secreted enzymes. The different domains of each enzyme are indicated.

that they were located very close to each other, raising the intriguing possibility that they could be part of a single gene. By using a human liver cDNA library, a full-length cDNA for polyserase-1 was finally cloned and characterized. The conceptual translation of the isolated cDNA indicated that this gene coded for a mosaic protein of 1059 amino acids with a very complex organization. In fact, the polyserase-1 structure consists of a transmembrane domain, an LDLR (low density lipoprotein receptor) domain and three tandem serine protease domains, called serase-1, -2 and -3, respectively (Figure 2). Each serase unit is preceded by a sequence that matches the consensus motif RIVGG, which corresponds to the activation site for this class of serine proteases. This peculiar architecture is well conserved in the polyserase-1 orthologues identified in the degradome of other model organisms including *Pan troglodytes, Mus musculus, Rattus norvegicus, Bos taurus* or *Ciona savignyi*. In relation to the catalytic properties of this polyprotease, the use of synthetic fluorescent substrates has shown that the serase-1 and -2 domains are enzymatically active, whereas serase-3 is predicted to be inactive since it contains an Ala residue instead of the catalytic Ser residue conserved in all serine proteases (34). It is also remarkable that polyserase-1 undergoes a series of post-translational processing events to release its three serine protease domains, which may be finally secreted to the extracellular space (24).

Expression analysis of polyserase-1 has revealed the occurrence of a major transcript of ~5.4 kb for this enzyme in all examined human adult and fetal tissues as well as in several cancer cell lines (24). Moreover, transcripts of 3.8 and 2.4 kb can also be detected in some particular tissues, which led us to speculate about the existence of different polyserase-1 splicing forms. Consistent with this proposal, we have identified and characterized a form of this polyprotease lacking the serase-3 domain (24). Moreover, the recent identification of serase-1B containing a single serine protease domain (35), has confirmed the existence of splice variants containing one, two or three serase units adjacent to the stem region of the protein. Detailed studies by Okumura *et al.* (35) have shown that the serase-1B variant of polyserase-1 possesses an extra SEA domain (sea urchin sperm protein, enterokinase and agrin) and lacks the serase-2 and -3 units present in the full-length protein. Regarding tissue distribution of the different isoforms, it is remarkable that both polyserase-1 and serase-1B show a similar expression pattern in human tissues. However, murine serase-1B is highly expressed in testis and ovary, but it is barely detected in lung, liver or kidney, which contrasts with the widespread expression of mouse polyserase-1 in all analyzed tissues. These data indicate that different splicing events for polyserase-1 can take place in different tissues or organs. It is also worthwhile mentioning that despite its lower level of structural complexity when compared to polyserase-1, the serase-1B variant is also a catalytically active enzyme that is capable to convert pro-uPA into its active form (35). Interestingly, this proteolytic activity can be inhibited by glycosaminoglycans in a dose-dependent manner, probably through a mechanism involving the binding to the LDLR domain of polyserase-1 (35). It has also been proposed that this interaction could promote a conformational change in the enzyme or might modify the accessibility to the substrate. Furthermore, it has been reported that serase-1B can be released from the cell surface in a similar manner to that previously reported for other TTSPs like enteropeptidase (36), HAT (37), matriptase (38) or hepsin (39). However, beyond this structural and enzymatic information about polyserase-1 and their related isoforms, no information is available about the functional relevance of this polyprotease. Nevertheless, on the basis of its widespread occurrence in human and mouse tissues, it is tempting to speculate that polyserase-1 may play some role in the development or homeostasis of these organisms, as has been proposed for other members of the TTSP family of serine proteases (40-42).

4. POLYSERASE-2/PRSS36, A SECRETED ENZYME CONTAINING THREE TANDEM SERINE PROTEASE DOMAINS

Polyserase-1 cDNA was used as query to try to localize new genes in the human genome encoding proteolytic enzymes with tandem serine protease domains in their structure. As a result of different bioinformatic searches, we identified a region in human chromosome 16p11.2 which fulfilled the requirements to encode a new polyprotease. This region was located immediately adjacent to that containing the prostasin gene, another member of the serine protease family of enzymes (43, 44) (Figure 1). Following a similar strategy as in the case of polyserase-1 and using a human liver cDNA library, we identified and cloned a full-length cDNA for a novel polyprotease called

polyserase-2 (PRSS36) (25). This new enzyme is shorter than polyserase-1 (855 *vs* 1059 amino acids) but also contains the three tandem serine protease domains detected in the first identified member of this group of polyproteases. However, further comparative analysis revealed some distinctive features between both polyserases. Thus, and in clear contrast to the above described information for polyserase-1, polyserase-2 is a secreted glycosylated enzyme whose three serase domains remain as an integral part of the same polypeptide chain. Moreover, the enzymatic activity of polyserase-2 is only due to its first serine protease domain since the second and third domains are predicted to be inactive as deduced from the occurrence of changes in at least one of the three conserved residues forming the catalytic triad of these enzymes (Figure 2). To assay the enzymatic activity of the first serine protease domain, this unit was produced independently from the rest of the molecule, and after the corresponding *in vitro* assays, its ability to hydrolyze different peptide substrates was demonstrated (25). It is also of interest the observation that only this first serine protease domain of polyserase-2 is preceded by the archetypal activation motif RIVGG, whereas the second and third domains of this polyprotease are not preceded by a sequence matching this consensus, thus making unlikely their release as independent units from the original translation product.

Polyserase-2 is expressed in fetal kidney as well as in a variety of human adult tissues, including heart, liver, muscle and placenta (25). The major transcript observed in all cases is of ~5.5 kb. However, a minor transcript of 2.4 kb and likely derived from an alternative splicing event is mainly detected in the tumor cell lines A549 (lung carcinoma) and SW480 (colon carcinoma). Furthermore, and similarly to polyserase-1, different polyserase-2 isoforms could be identified in different tissues. In this regard, it is remarkable that conceptual translation of the human expressed sequence tag BM768456 indicates that it derives from an alternative splicing event which leads to the formation of a polyprotease only containing the first and second serine protease domains. Moreover, bioinformatic analysis has revealed the occurrence of three different predicted isoforms of murine polyserase-2 (XP_995255, XP_995282 and XP_9955313), likely generated by alternative splicing and whose existence is clearly supported by expressed sequence tag evidence. Taken together, these data emphasize the relevance of post-transcriptional processing mechanisms in the generation of polyserase-2 variants.

Detailed amino acid sequence analysis revealed that each serine protease domain of polyserase-2 shows a high degree of identity (~40 %) with some TTSPs such as matriptase and matriptase-2. However, similar degrees of identities are also found with members of the tryptase/pancreasin family of serine proteases, many of which are glycosylphosphatidylinositol-anchored proteases involved in asthma (31, 45-47) or in the proteolytic processing of ion channels (30, 48). Likewise, the intron-exon organization of the first catalytic domain encoded in the polyserase-2 gene is similar to that shown by matriptase and matriptase-2. By contrast, the intron-exon organization of the second and third catalytic domains of this polyserase is more similar to that found in the genes for tryptase/pancreasin. Further phylogenetic analysis indicates that, in fact, polyserase-2 is equally related to the TTSP than to the tryptase/pancreasin families of serine proteases (25). Finally, and similar to the case of polyserase-1, no information is currently available about the biological roles performed by polyserase-2 in human tissues or about its putative functional relationship with other serine proteases sharing structural similarities with this polyprotease.

5. POLYSERASE-3, A SECRETED ENZYME CONTAINING TWO TANDEM SERINE PROTEASE DOMAINS

Polyserase-3 is the third member of the group of polyserine proteases identified in human tissues (26). The polyserase-3 gene maps to chromosome 16p11.2, in the same region where polyserase-2 is located and encodes a protein with 553 amino acids and two serine protease domains (Figure 1). Both domains are predicted to be catalytically active as they contain the three conserved residues that form the catalytic triad (Figure 2). A comparative structural analysis of this enzyme with the two previously identified human polyserases establishes a closer relationship to polyserase-2 than to polyserase-1. Thus, polyserase-3 is a secreted enzyme whose serine protease domains remain embedded in the same polypeptide chain. Phylogenetic analysis has also revealed the close relationship between polyserase-2 and -3 since all their serine protease domains form an independent branch in the dendrogram, equally distant from the TTSP and the tryptase/pancreasin families of serine proteases (Figure 3). Moreover, a detailed comparative analysis of the amino acid sequence corresponding to the two serine protease domains of polyserase-3 indicates that the highest degree of identity is found with the protease domains of polyserase-2 (~40%) as well as with those of members of the TTSPs and tryptase/pancreasin families of serine proteases (~35%). On this basis, it can be speculated that ancestors from these two families of serine protease could contribute to the formation of polyserases-2 and -3 through recombination processes or domain swapping events. However, and despite the indicated similarities between polyserase-2 and polyserase-3, two clear differences are apparent in the comparative analysis of both polyproteases. First, polyserase-2 is a secreted and glycosylated enzyme containing three serine protease domains, although only the first one is catalytically active. By contrast, polyserase-3 is a non-glycosylated enzyme that contains two catalytically active serine protease domains. Additionally, polyserase-3 is expressed in most tissues and tumor cell lines analyzed, whereas polyserase-2 shows a more restrictive expression pattern (26).

Interestingly, further structural analysis of polyserase-3 has revealed that its two serine protease domains are preceded by a region lacking the consensus activation motif RIVGG found in serine proteases. Hence, it is unlikely that this polyprotease can be activated by a trypsin-like enzyme. However, it can not be ruled out the

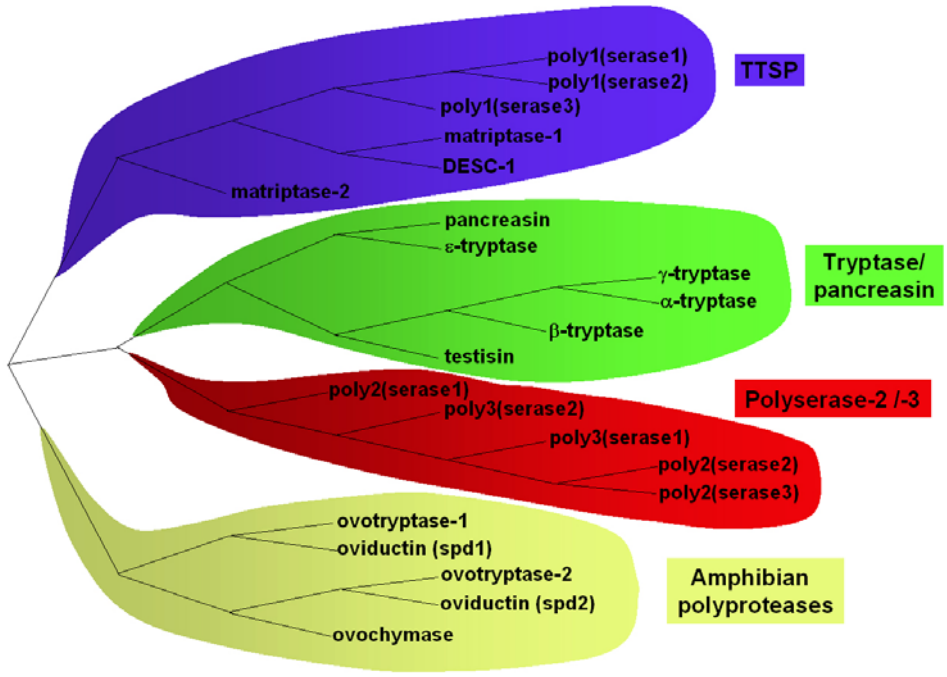

Figure 3. Phylogenetic tree of the different human and amphibian serine proteases related to human polyserases. This analysis was performed using the serine protease domains of the different enzymes analyzed, and the Basic GeneBee ClustalW 1.83 program at http://www.genebee.msu.su/clustal/basic.html. *Spd* indicates *serine protease domain.*

possibility that these polyserase-3 catalytic units could be activated through a different mechanism as previously established for α-tryptase activation (49, 50). Another characteristic that relates this polyprotease to tryptases derives from the observation that polyserase-3 may form dimers with ability to degrade the α-chain of fibrinogen (26). A polyserase-3 dimer is a equivalent structure to a β-II-tryptase tetramer, which is the active form of this serine protease able to hydrolyze the α-chain of fibrinogen (51, 52). Consistent with these observations, structural modelling of the catalytic domains of polyserase-3 has confirmed the high degree of similarity with β-II-tryptase (26). Nevertheless, it is clear that further experimental studies will be required to confirm these structural predictions as well as to evaluate the putative functional relationships between polyserase-3 and tryptases.

6. OTHER POLYPROTEASES

The occurrence of different catalytic units embedded in a single polypeptide chain, although unusual, is not an unprecedented situation. To date, two different polyserine proteases involved in fertilization processes have been identified in amphibians. The first of these polyproteases was originally discovered in *Xenopus laevis* and contains three tandem serine protease domains (53). The N-terminal and middle domains were designed as ovotryptase-1 and -2 respectively, whereas the C-terminal domain corresponds to a previously identified enzyme called ovochymase (54). This polyprotease is a secreted enzyme that undergoes a series of post-translational processing events to generate independent units, a situation that closely resembles that described for polyserase-1. The second amphibian polyserine protease identified in *Xenopus laevis* (55), *Bufo japonicus* (56) and *Bufo arenarum* (57) is called oviductin and contains two serine protease domains, albeit one of them is catalytically inactive. This polyprotease is also proteolytically processed to release its two serine protease domains.

The exploration of the different degradomes of animal models such as *Drosophila melanogaster* and *Caernorhabditis elegans*, indicates that they could contain some additional genes with several serine protease domains (22, 58). Likewise, it can not be ruled out the existence of a fourth polyprotease gene –located at human chromosome 12p11– which could correspond to the human orthologue of amphibian ovochymase (Cal *et al.*, unpublished results). Finally, it is remarkable that the human orthologue of oviductin seems to be located in chromosome 11p15, but it is predicted to contain a unique serine protease domain (Cal *et al.,* unpublished results).

Interestingly, the presence of different catalytic units within a single polypeptide chain has also been reported in two human metalloproteases: angiotensin converting enzyme (ACE) and carboxypeptidase D. ACE is a type I membrane enzyme which is involved in the physiological control of blood pressure (59-61). It contains two active domains that show different catalytic constants and interact differently with some competitive inhibitors (62, 63). Likewise, and similar to the case of polyserases,

Table 1. Comparative analysis of characteristic features of human polyserases

Characteristic Features	Polyserase-1	Polyserase-2	Polyserase-3
Number of serases	3	3	2
Number of active serases	2 (1st and 2nd)	1 (1st)	2
Localization	Membrane	Secreted	Secreted
Glycosylation	No	Yes	No
Post-translational processing	Yes	No	No
Putative activation by a trypsin-like protease	Yes (3 serases)	Yes (1 serase)	No
Tissue expression			
• Fetal tissues	Brain, Lung, Liver, Kidney	Kidney	Kidney, Liver, Lung, Brain
• Adult tissues	Heart, Brain, Placenta, Lung, Liver, Muscle, Kidney, Thymus, Pancreas, Spleen, Colon, Small Intestine, Prostate, Testis, Leucocyte	Placenta, Prostate, And Skeletal Muscle	Heart, Liver, Muscle, Placenta, Pancreas, Thymus, Prostate, Testis, Ovary, Intestine, Colon, Leukocytes
• Tumor Cell Lines	HeLa, K-563, MOLT-4, Raji, A549	SW-480, A549	HeLa, MOLT-4, SW-480

alternative splicing events have also been reported for ACE. Thus, a somatic form of this enzyme contains both metalloprotease domains, whereas the germinal form only possesses the C-terminal domain (64). Moreover, it has been reported the existence of an ileal form of ACE exclusively containing the N-terminal domain of the protein (65). Carboxypeptidase D is also a type I membrane metalloprotease but with three catalytic domains in its structure. Two of these domains are catalytically active units that exhibit different optimal pH activities, which permits this enzyme to degrade a wide range of substrates (66).

Finally, it is remarkable that a number of enzymes distinct from proteases also exhibit these complex molecular architectures characterized by the incorporation of several catalytic domains in the same polypeptide chain. These multidomain enzymes have been identified in different organisms from bacteria to human, and include kinases, luciferases, chitinases, xylanases and endoglucanases (67-71). Similar to the case of polyserases, the putative evolutionary benefits derived from these complex structural designs are still unclear in most cases. However, and hopefully, the ongoing studies on these enzymes may provide new information that could help to improve our functional knowledge of other polyproteins whose description is much more recent, as is the case of polyserases.

7. CONCLUSIONS AND PERSPECTIVES

Recent studies have allowed the identification and characterization of polyserases, a new group of serine proteases that share the common structural feature of containing several tandem serine protease domains in the same polypeptide chain. The three polyserases identified to date in human tissues are conserved in mammals and belong to two different families: polyserase-1 is a member of the TTSP group of transmembrane serine proteases, whereas polyserase-2 and -3 form a new family of serine proteases (Table 1).

At present, we can only speculate about the functional relevance of these enzymes. The resolution of the three-dimensional structures of these polyproteases could serve to better understand their mechanism of catalysis as well as to shed some light about the putative structural advantages derived from the occurrence of several catalytic units embedded in a single polypeptide chain. Another immediate challenge in the characterization of polyserases is the identification of their natural substrates. This is a key question to understand the physiological role of these enzymes as well as their possible implications in the development of some diseases. In this regard, it is noteworthy that the human polyserase-1 gene partially overlaps in antisense orientation with the translocase of mitochondrial inner membrane 13 (TIMM13) gene, which is implicated in deafness dystonia syndrome (72). Likewise, the polyserase-2 and -3 genes are closely linked to a region associated with genetic abnormalities such as autosomal dominant myxomatous mitral valve prolapse (73) or paroxysmal kinesigenic choreoathetosis (74), whose loci remain unidentified. Functional studies including those based on the generation and phenotype analysis of mutant mice lacking these enzymes will contribute to ascertain the relevance of polyserases in the context of the large and growing complexity of the human degradome.

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