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Research paper

Neutrophil elastase, proteinase 3 and cathepsin G: Physicochemical properties, activity and physiopathological functions

Brice Korkmaz[1], Thierry Moreau, Francis Gauthier*

INSERM U618 ''Protéases et Vectorisation Pulmonaires'', Université François Rabelais, 10 Boulevard Tonnellé, BP 3223, 37032 Tours Cedex, France

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Abstract

Polymorphonuclear neutrophils form a primary line of defense against bacterial infections using complementary oxidative and non-oxidative pathways to destroy phagocytized pathogens. The three serine proteases elastase, proteinase 3 and cathepsin G, are major components of the neutrophil primary granules that participate in the non-oxidative pathway of intracellular pathogen destruction. Neutrophil activation and degranulation results in the release of these proteases into the extracellular medium as proteolytically active enzymes, part of them remaining exposed at the cell surface. Extracellular neutrophil serine proteases also help kill bacteria and are involved in the degradation of extracellular matrix components during acute and chronic inflammation. But they are also important as specific regulators of the immune response, controlling cellular signaling through the processing of chemokines, modulating the cytokine network, and activating specific cell surface receptors. Neutrophil serine proteases are also involved in the pathogenicity of a variety of human diseases. This review focuses on the structural and functional properties of these proteases that may explain their specific biological roles, and facilitate their use as molecular targets for new therapeutic strategies.

Keywords: Serine protease; Neutrophil; Inflammation; Human diseases

1. Introduction

Human neutrophil elastase (HNE: EC 3.4.21.37), proteinase 3 (PR3: EC 3.4.21.76) and cathepsin G (CG: EC 3.4.21.20) are three serine proteases of the chymotrypsin family that are stored in the primary (azurophil) granules of polymorphonuclear neutrophils (PMNs). Their activities depend upon a catalytic triad composed of aspartate, histidine and serine residues which are widely separated in the primary sequence but are brought together at the active site of the enzymes in their tertiary structures. They were first identified as degradative enzymes responsible for eliminating intracellular pathogens and breaking down tissues at inflammatory sites [2–4], and were soon recognized as possible molecular targets for anti-inflammatory agents [5]. Circulating PMNs are the first cells to reach a site of inflammation by extravasation, thus providing a primary line of defense against bacterial infection. Neutrophil serine proteases (NSPs) act intracellularly within phagolysosomes to digest phagocytized

Abbreviations: HNE, human neutrophil elastase; PR3, proteinase 3; CG, cathepsin G; NSP, neutrophil serine protease, α1-PI, α1-protease inhibitor; ACT, α1-antichymotrypsin; PI, protease inhibitor; PMN, polymorphonuclear neutrophil; OMTKY-III, turkey ovomucoid third domain; ECM, extracellular matrix; SLPI, secretory leukocyte protease inhibitor; MNEI, monocyte neutrophil elastase inhibitor; SCCA2, squamous cell carcinoma antigen 2; NET, neutrophil extracellular traps; WG, Wegener's granulomatosis; c-ANCA, anti-neutrophil cytoplasmic antibody; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FRET, fluorescence resonance energy transfer; The nomenclature used for the individual amino acid residues (e.g. P1, P2, etc.) of a substrate and corresponding residues of the enzyme subsites (e.g. S1, S2, etc.) is that of Schechter and Berger [1].

* Corresponding author. Tel.: +33 (0)2 47 36 60 45; fax: +33 (0)2 47 36 60 46.
E-mail address: francis.gauthier@univ-tours.fr (F. Gauthier).
[1] Present address: Department of Neuroimmunology, Max-Planck-Institute of Neurobiology, D-82152 Planegg-Martinsried, Germany.

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micro-organisms in combination with microbicidal peptides and the membrane-associated NADPH-oxidase system that produces oxygen metabolites. NSPs released from cells are involved in the regulation of innate immunity, inflammation and infection [6–8], in addition to their well recognized destructive function of extracellular matrix components. This dual, apparently antinomic protective/destructive function has attracted great interest over the past decade as new specific targets were discovered [6]. It is now clear that NSPs are not only destructive proteases with broad, unspecific proteolytic activity as initially stated, but that each has specific functions in spite of their similar gene expressions, subcellular locations, 3D structures, physicochemical properties and substrate specificities [6,9]. This review focuses on the specific properties that distinguish each NSP from the others and may explain their specific biological functions.

2. Gene structure, regulation and expression

HNE, PR3 and CG are homologous proteases that have evolved from a common ancestor by gene duplication and are quite distinct from the main branches of the chymotrypsin superfamily of serine proteases [10]. The gene for HNE, *ELA2*, is located within a 50 kb segment in the terminal region of the short arm of chromosome 19p13.3 [11]. This region also contains the genes for the homologous proteins PR3 and azurocidin. The *ELA2* gene consists of five exons and four introns [11]. Each residue of the catalytic triad (H57, D102, and S195, chymotrypsinogen numbering), is located on a separate exon. High-level transcription of *ELA2* is limited to the promyelocytic stage of granulocyte development, when HNE is produced and stored in cytoplasmic azurophil granules [12], and HNE mRNA cannot be detected in mature PMNs isolated from the systemic circulation. Chromatin reorganization may be involved in the regulation of HNE transcription [13]. *ELA2* promoter region also displays binding sites for the transcription factors c-Myb, C/EBP, PU.1 and LEF-1 [14,15]. Mutations in *ELA2* have been associated with severe congenital neutropenia and cyclic neutropenia, a rare autosomal dominant disease characterized by oscillations in the production of PMNs (and other blood cells) with a 21-day periodicity [16,17]. Nearly 20 different single nucleotide polymorphisms have been deposited at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp), but no pathofunctional data on the impact of these polymorphisms are yet available. *ELA2* is transcribed in promonocyte progenitors in the bone marrow, but the protein persists in the cells through terminal differentiation to monocytes [18]. Monocytes have low concentrations of elastase [19], while the promonocyte-like cell line U-937 synthesizes large quantities of this protease [20]. The concentration of HNE in PMNs exceeds 5 mM and the total amount in a single cell has been estimated at up to 3 pg [21]. This high concentration of HNE is tightly regulated by compartmentalization in the azurophil granules. Active HNE is rapidly liberated from the granules into the extracellular space, but remains in part associated with the outer PMN plasma membrane [22,23]. Elastase is also found in mast cells and eosinophils [24].

The gene for PR3 (*PRTN3*) is located on chromosome 19p13.3 [25] in the same cluster as those of HNE and azurocidin [11]. The PR3 gene spans 6.57 kb pairs and consists of five exons and four introns. The organization of the gene is similar to that of other serine protease genes expressed in hematopoietic cells. The PR3 gene is larger than those for CG [26] and HNE [12,27], because its introns I and III are considerably larger. Unlike the HNE gene, the PR3 gene contains two long tracts of unusual sequence in introns I and IV [28]. The PR3 cDNA described by Bories et al. [29] differs from the PR3 cDNA reported by others [25,30], resulting in a difference of three amino acids residues in the PR3 protein sequence. PR3 mRNA is present in the early cells of the myeloid and monocytic lineage [28,31,32]. The mRNAs for PR3, HNE and CG are transcribed only in the promyelocyte and promonocyte stages of myeloid differentiation, and transcription is down-regulated upon granulocyte and monocyte maturation [11,29,33]. The 5′-flanking region of the PR3 gene contains a TATA-box [11,28,34] a PU.1 regulatory element, a CG-element, and potential binding sites for the CAAT binding protein (C/EBP) and c-Myb transcription factors. The CAAT box promoter element, which is present in the promoter regions of HNE [12] and CG [26], is not present in the PR3 promoter. The PR3 gene is expressed only in cells of the granulocyte and monocyte lineages. PR3 is present in mature monocytes and granulocytes, and is located with HNE and CG in granules of monocytes and the azurophil granules of granulocytes. PR3 can be also secreted or expressed on the cell surface of these cells [35,36]. The extent to which the enzyme is expressed on the membrane seems to be genetically determined [37]. PR3 is also found in mast cells and basophils [38]. Several reports suggest that PR3 is present in human non-hematopoietic cells, though this is still controversial [39–43].

The CG gene (*CTSG*) lies on chromosome 14q11.2, spans 2.7 kb, and, like the HNE/PR3 genes, consists of five exons and four introns [44]. Each residue of the CG catalytic triad is located on a separate exon and the position of each residue within the exon is similar to those in HNE and PR3. Scanning the 5′-flanking region and the entire coding region of the gene identified five polymorphisms [45]. The C/EBP and c-Myb sites are not present in the CG promoter, whereas the PU.1 and CG-elements are present in both the CG [26] and the HNE [12] promoter regions. Low concentrations of the mRNA that encodes CG are also detectable in monocytes and mast cells [46].

3. Physicochemical properties

The HNE gene encodes a 267-residue preproprotein that is post-translationally processed at both ends (Fig. 1A). The mature protein isolated from azurophilic granules of leukemia myeloid cells has 218 amino acid residues with two sites of *N*-glycosylation. The sequence of mature HNE was established by a combination of peptide sequencing [47] and crystallography [48]. Analysis of the cDNA sequence of HNE [12,49]

confirmed the sequence with the addition of a 20-amino-acid carboxy-terminal extension. The processing and granule targeting of the hematopoietic serine proteases has been investigated in transfected rat basophilic leukemia-1 (RBL-1) cells that produce exogenous HNE in a fashion similar to human PMNs. The N-terminus 27-residue signal peptide is cleaved by a signal peptidase during translation into the endoplasmic reticulum, yielding a proform of HNE with an amino-terminal and a carboxy-terminal propeptide. The presence of an amino-terminal prodipeptide (SE for HNE) that maintains the enzyme in an inactive conformation is a typical feature of myeloid serine proteases. The HNE propeptide is removed in the post-Golgi organelles by the cysteine protease cathepsin C (DPPI, dipeptidyl peptidase I), which also cleaves the propeptides of other proteins and peptides [50]. Removal of this amino-terminal prodipeptide allows the free amino-terminus to be inserted into the interior of the molecule, where it interacts with the aspartic acid residue adjacent to the serine of the active site. This renders the active-site pocket of the enzyme accessible to substrates and inhibitors. The main differences between the conformations of the pro- and mature forms of various serine proteases are well documented, as illustrated for thrombin [51]. The mature enzyme has three flexible surface loops and the N-terminus arranged to form the so-called activation domain. The altered conformation affects about 20% of the molecule surface. The N terminal dipropeptide is not essential for initial folding or granules targeting, but it may prevent premature activation during intracellular transport [52]. The C-terminus consists of a 20-residue tail that is normally excised and is required neither for location in granules nor enzymatic activity [53]. The enzyme responsible for cleaving off the C-terminal propeptide is not known. And deletion of the carboxy-terminal extension of HNE does not influence its targeting to granules in RBL-1 cells [53]. SDS–PAGE analysis of elastase purified from granules and elastase from sputum showed that the enzyme migrates as a major triplet of approximately 29–31–32 kDa with several isoforms (E1, E2, E3) that differ in the extent of their glycosylation [54,55]. Analysis of the minor E1 form of HNE indicated that the carbohydrate structures are complex-type bi-antenary chains that are usually associated with secretory glycoproteins. In contrast, the major E3 isoform contains truncated oligomannose-type chains [55]. The isozymes have identical kinetics constants with several substrates and inhibitors [56]. HNE is extremely cationic, having no lysine or tyrosine residues. It contains 19 arginyl residues, 18 of which form clusters on the surface giving the protein a very basic isoelectric point (p*I* 10–11). Its sequence is only moderately similar to those of PR3 (57% identity) and CG (37% identity) (Fig. 1B).

The synthesis of PR3 is regulated at the transcriptional level during granulocyte development and at the post-translational level by intracellular steps before it is stored in its enzymatically active form (Fig. 1A). Studies on the biosynthesis and processing of PR3 show that the primary product of PR3 mRNA translation is a preproenzyme. This, as in HNE, undergoes three subsequent proteolytic modifications to convert it into the mature form [57,58]. The active form of PR3 is produced by cleavage of the amino-terminal AE prodipeptide. The enzymatically active PR3 is then stored in the neutrophil azurophil granules. The final conformation of mature PR3 is stabilized by disulfide bonds and appropriate asparagine-linked glycosylation. The significance of the carboxy-terminal processing of PR3 is less clear. Cleavage next to Arg222 suggests that a trypsin-like protease is involved. While C-terminal processing does not alter the enzymatic activity, antibodies recognition or intracellular sorting of human PR3, its effect remains unknown [59]. Unlike HNE and CG, PR3 is not restricted to azurophilic granules in PMNs; it is also present at the membrane of secretory vesicles and in secondary granules [60]. The mature form of PR3 has an inactive conformation in the acidic environment of the azurophilic granules, and becomes active upon translocation to a neutral pH environment. This pH-dependent change has only been described for PR3 thus far [61]. PR3 was initially described as an elastin-degrading protease with structural and functional properties similar to those of HNE. Both proteases are highly cationic and their sequences differ mainly in the distribution of charged residues. About half of the 61 residues that differ between the two proteases are charged residues, and only 10 are found at the same position in both sequences [62] (Fig. 1B). The experimental p*I* of human PR3 ($\sim$9.5) is less basic than that of HNE [3,30]. PR3 appears as a triplet of approximately 29–32 kDa by SDS–PAGE analysis [63,64]. But only one major band of lower molecular weight is obtained after treatment with *N*-glycosidase F, which indicates that the 3 isoforms differ in their carbohydrate structures, as has been shown for HNE [65,66]. Elimination of the functional glycosylation site of PR3 had no obvious effect on granular targeting [66]. A small fraction of PR3 and related serine protease proforms escape granular targeting during synthesis, and are constitutively secreted during the myeloid differentiation of human hematopoietic progenitor cells *in vitro* [67]. A secreted proform of PR3 may be a negative feedback regulator of granulopoiesis [50,68]. The overwhelming majority of PR3 is stored in the azurophil granules as mature enzyme without the amino-terminal and carboxy-terminal propeptides. A secreted form of PR3, however, still contains the amino-terminal propeptide [58,68].

CG is synthesized as a 255-amino-acid residue protein including an 18-residue signal peptide, a two-residue activation peptide at the N-terminus and a carboxy terminal extension [69]. CG is stored in primary granules as an active protease after removal of the amino terminal prodipeptide GE (Fig. 1A). Removal of the propeptide upon proteolytic activation of CG by cathepsin C [70] gives rise to the characteristic Ile16 N-terminus of the mature enzyme. Pro-CG is activated simultaneously with or directly after its transfer to granules, but a fraction of the preform is constitutively secreted. Studies on the targeting of prodipeptide-deleted CG in a model cell system showed that the presence of the prodipeptide is not critical for sorting to granules or for constitutive secretion [71]. CG and HNE acquire complex N-linked oligosaccharide side chains, as shown by their resistance to endoglycosidase H [10,72,73]. Cleavage of the CG C-terminal extension occurs at or near Ser244–Phe245 [52,74]. As for HNE, carboxy-terminal processing of CG does not influence its targeting to granules, and the

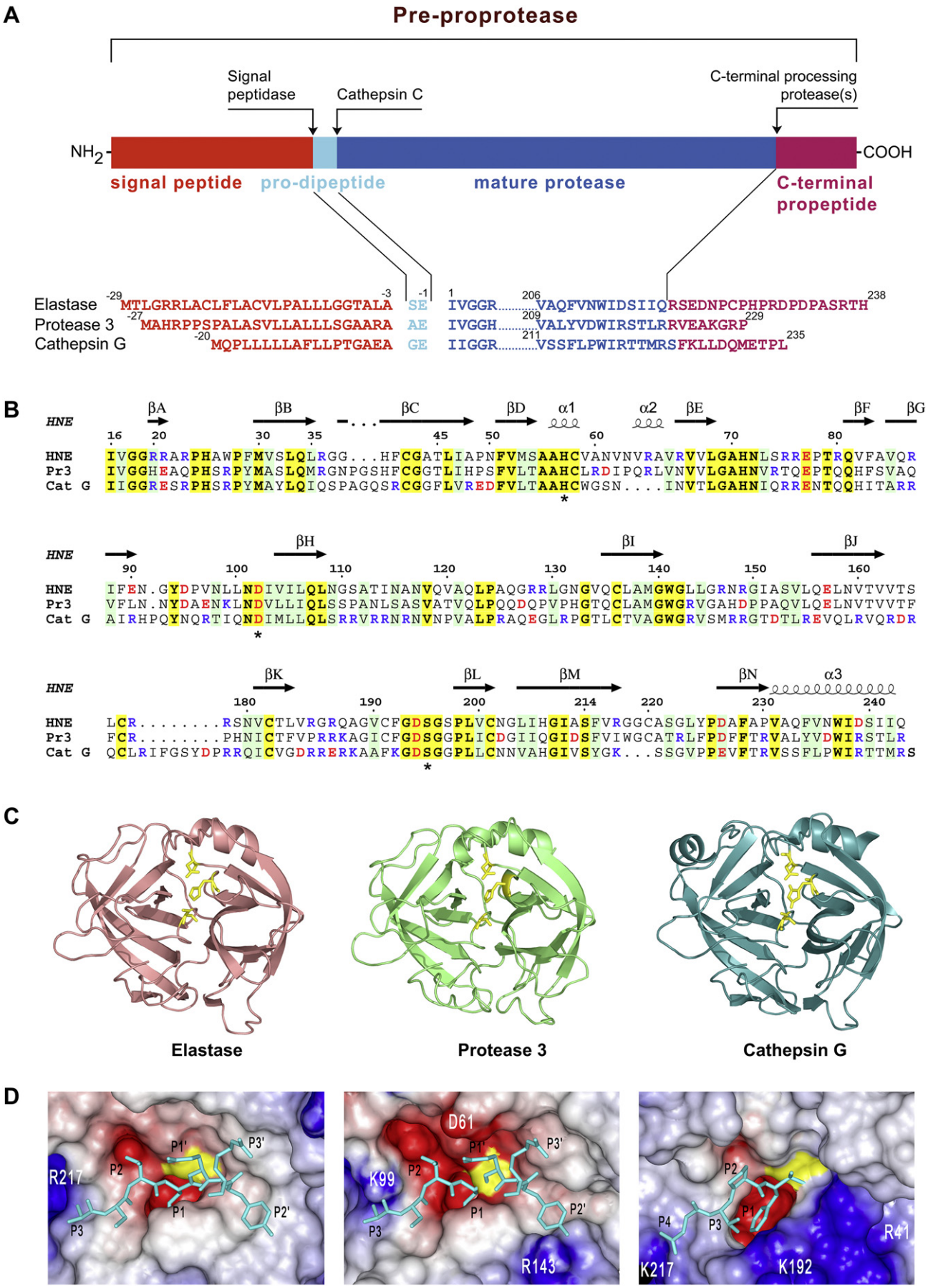

Table 1
Main characteristics of elastase, proteinase 3 and cathepsin G

	Elastase	Proteinase 3	Cathepsin G
EC number	EC 3.4.21.37	EC 3.4.21.76	EC 3.4.21.20
Gene locus and gene structure	19p13.3, 5 exons 4 introns	19p13.3, 5 exons 4 introns	14q11.2, 5 exons 4 introns
Characteristics	218 residues	222 residues	235 residues
Molecular mass (kDa)	29–33	29–32	28.5
p*I*	~10.5	~9.5	~12
No. of glycosylation sites	2	2	1
No. of disulfide bridges	4	4	3
Optimal pH for activity	8.0–8.5	~8.0	~7.5
Substrate specificity	Small hydrophobic residue at P1 position: Val, Cys, Ala, Met, Ile, Leu, Ser	Small hydrophobic residue at P1 position: Val, Cys, Ala, Met, Ser, Leu	Aromatic or positively charged residues at P1 position: Phe, Tyr, Lys, Arg
Best synthetic substrate	Abz-APEEIMRRQ-EDDnp	Abz-VADCRDRQ-EDDnp	Abz-EPFWEDQ-EDDnp
Localization in PMN	Azurophilic granules	Azurophilic granules Specific granules Secretory vesicles	Azurophilic granules
	Neutrophil cell surface after priming	On unprimed neutrophil cell surface	Neutrophil cell surface after priming
Source	Neutrophil Monocyte Mastocyte Eosinophil	Neutrophil Monocyte Basophil Mastocyte	Neutrophil Monocyte
Endogenous inhibitors	α2-Macroglobulin α1-PI/MNEI/PI9 SLPI/Elafin/pre-elafin	α2-Macroglobulin α1-PI/MNEI Elafin/pre-elafin	α2-Macroglobulin ACT/MNEI/α1-PI/PI6/SCCA2 SLPI

function(s) of the C-terminal extension remains unknown. Human CG was isolated from PMN primary granules as a mixture of three isoforms differing in their carbohydrate content (C1, C2, C3) [55]. CG is an extremely basic molecule (Fig. 1B), with 34 arginyl residues and four lysyl residues, but only 11 glutamyl and nine aspartyl residues [75]. The main characteristics of NSPs are listed in Table 1.

4. Structural characteristics and active site analysis

Serine proteases are divided into two distinct families: the chymotrypsin family, to which belong HNE, PR3 and CG, and the substilisin family, which includes bacterial enzymes that differ in their overall 3D structures but have the same active site geometry and catalytic mechanism as a consequence of convergent evolution. Amino acid substitutions within active sites give individual proteases their substrate specificity. The nomenclature for describing the interaction of a substrate with a protease was introduced in 1967 by Schechter and Berger [1]: S subsites on the protease accommodate P residues in the substrate upstream of the scissile bond, whereas S′ subsites accommodate P′ residues on the substrate C-terminal side, downstream of the cleavage site.

The X-ray three-dimensional structure of HNE in complex with the turkey ovomucoid third domain (OMTKY-III), or with the synthetic inhibitors MeO-Suc-Ala-Ala-Pro-Val chloromethyl ketone/MeO-Suc-Ala-Ala-Pro-Ala chloromethyl ketone has the two six-stranded β-barrel domains typical of chymotrypsin-like serine proteases [48,76,77] (Fig. 1C). Only an intermediate segment and the carboxy-terminal segment is organized in an α-helix. The active-site residues are located in a crevice between the two β-barrels. The HNE peptide chain is stabilized by four disulfide bonds and contains two asparagine-linked carbohydrate side chains at Asn109 and Asn159. The surface distribution of the positively charged Arg residues (HNE has no Lys) is highly asymmetric. Analysis of

Fig. 1. (A) Diagram showing the structural features of the pre-proprotease form of neutrophil serine proteases. The proteins consist of a typical signal peptide followed by a prodipeptide, the protease sequence and a C-terminal extension. The mature protease as stored in the primary granules of neutrophils is generated by multiple proteolytic cleavages involving a signal peptidase, cathepsin C and a C-terminal processing enzyme at positions indicated in the sequence alignment. (B) Sequence alignments of human neutrophil elastase (UniProtKB/Swiss-Prot accession number P08246), proteinase 3 (P24158) and cathepsin G (P08311) highlighting the positively charged (blue) and negatively charged (red) residues of each sequence. The asterisk denotes the position of the amino acids of the catalytic triad (His57, Asp102, Ser195). The secondary structure elements of elastase (from PDB entry file 1PPF) are also indicated on top of sequence blocks. Numbering is based on an alignment of these proteins with the chymotrypsinogen sequence. (C) Ribbon representation of the three-dimensional structures of human neutrophil elastase (PDB entry file 1PPF), proteinase 3 (1FUJ) and cathepsin G (1CGH). The side chains of the amino acids of the catalytic triad are shown as yellow sticks. (D) Close-up of the active sites of the three neutrophil serine proteases. The molecular surface of each protease is colored according to the electrostatic potential (blue: positive regions, red: negative regions). Positively and negatively charged residues in the immediate vicinity of the active site are labeled in white. The active Ser195 is colored yellow. Each protease is shown complexed with an inhibitor to help visualize the various enzyme subsites. From left to right: human neutrophil elastase complexed with OMTKY-III (cyan sticks of the P3–P3′ region of the inhibitor), proteinase 3 superimposed on the elastase–OMTKY-III complex and cathepsin G complexed with the irreversible phosphonate inhibitor Suc-Val-Pro-PheP-(OPH)2 (cyan sticks). Atomic coordinates were extracted from PDB files as in C).

the HNE binding site in the complex with OMTKY-III shows that it makes direct contact with eight residues of the reactive loop ($_{14}$PACTLEYRP$_{22}$). The S1 pocket is hemispherical and hydrophobic due to presence of Val190, Phe192, Ala213, Val216, Phe228 and the disulfide bridge Cys191–Cys220. The bottom of the S1 pocket contains a negatively charged residue, Asp226, which is completely buried in the interior of the HNE. Its carboxylate group is hydrogen-bonded with three water molecules that help dissipate its negative charge. The S2 subsite, lined by Phe215, Leu99 and the flat side of the imidazole ring of His57 is quite hydrophobic. The inhibitor peptide chain of both chloromethyl ketone inhibitors is bound in the same conformation as that of the P4–P1 region of OMTKY-III. The S1′ subsite is relatively hydrophobic and lined with Cys42–Cys58 and Phe41 (Fig. 1D).

The crystal structure of recombinant PR3 expressed in insect cells has been solved by molecular replacement using the HNE structure [78]. The overall fold consists of two β-barrel domains similar to those of HNE (Fig. 1C). The PR3 structure includes a disaccharide unit covalently attached to Asn159. The PR3 substrate binding sites, S4 to S3′, are more polar than those in HNE [78]. Solvent accessible surfaces in PR3 and HNE show that their charge distributions differ significantly in the vicinity of the substrate binding region, with the Leu99, Asn61, Leu143 and Arg217 of HNE being replaced by Lys99, Asp61, Arg143 and Ile217 in PR3 [78]. The S1 pocket is hemispheric but it appears to be smaller than that of HNE due to the Val/Ile substitution at position 190. The replacement of Ala by Asp at position 213 in the S1 subsite should have little effect on specificity, as the Asp side-chain points back into the interior of the protein. Analysis of the substrate pockets in PR3 shows that subsites S2, S1′ to S3′ are in the vicinity of charged residues that are not present in HNE. For example, the S2 subsite forms a deep polar pocket of increased polarity in PR3, due to the Leu to Lys substitution at position 99. On the prime side of the binding cleft, the specificities for P1′ and P3′ should be influenced by Asp61. The loop in PR3 containing this residue is significantly displaced to bring the side-chain closer to the S1′ and S3′ sites, making them smaller and more polar. The substitutions Leu143-to-Arg and Ile151-to-Pro make the S2′ site more polar. Both proteases also have a cluster of positively charged residues remote from the active site that could favor binding to negatively charged surfaces. However, this positive cluster is disrupted by negatively charged residues in PR3 (Fig. 1D) and is therefore smaller than that of HNE [62]. PR3 also has a hydrophobic patch, formed by residues Phe166, Ile217, Trp218, Leu223 and Phe224, on its surface.

The crystal structure of human CG in complex with the peptidyl phosphonate inhibitor Suc-Val-Pro-PheP-(OPh)2 shows the characteristic fold of trypsin-like serine proteases, and is very similar to rat mast cell protease II [79]. As for HNE and PR3, the active site cleft and the catalytic residues are located along the junction of the two barrels (Fig. 1C). CG has three disulfide bridges and the side chain of Asn65 contains the single carbohydrate linkage site of the protease. Three positively charged residues (Lys217, Lys192 and Arg41) reside in the immediate vicinity of the active site and could contribute to the specificity of the protease (Fig. 1D). X-ray analysis shows that the last residue of crystallized CG purified from PMNs, which is partially defined in the electron density, is Ser244 [74]. The S1 subsite, which is larger than that of HNE and PR3, is divided into two compartments with Glu226 at the bottom. This residue unique to CG provides a structural basis for the dual specificity of CG for both aromatic and basic residues at P1. The phenyl ring of P1 Phe of the inhibitor forms favorable edge-to-edge electrostatic interactions with the negatively charged Glu226, whereas a Lys residue would be accommodated in the S1 pocket by forming hydrogen bonds and salt bridge with Glu226 [79]. The S2 subsite is demarcated by the hydrophobic Leu99 side chain and the flat side of the invariant His57 imidazole ring. The proline at P2 position of the peptidyl phosphonate inhibitor Suc-Val-Pro-PheP-(OPh)2 seems to be most favored due to its ideal contact with these two side-chains. The presence of a Lys at position 192 in the S3 pocket should favor interaction with an acidic residue at P3. On the S′ side, the Arg41 side chain is flexible and is exposed towards the active site, in contrast to most other serine proteases where the side chain of residue 41 (often Phe) is buried in the interior of the molecule. The positively charged side chain of Arg41 may favor interaction with an acidic residue at positions P2′ and P3′.

5. Enzymatic activity, specificity and inhibition

The activity of proteolytic enzymes is generally measured by the amidolytic release of a chromophore or a fluorophore covalently attached to a peptide moiety that confers specificity. But FRET substrates, whose sequences extend on both sides of the cleavage site, give more information on the specificity of proteases, especially on S′ binding sites. The first step in catalysis by serine proteases is the formation of an acyl-enzyme intermediate between the substrate and the essential serine of the active site (reviewed in [80]). This covalent intermediate is formed via a negatively charged tetrahedral transition state intermediate followed by cleavage of the peptide bond. The acyl-enzyme intermediate is hydrolyzed by a water molecule in the second deacylation, step to release the peptide and restore the Ser-hydroxyl of the enzyme. The deacylation, which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accepts the OH group of the reactive Ser.

The enzymatic activities of HNE, PR3 and CG have been investigated using protein substrates such as the oxidized insulin B chain, and synthetic chromogenic/fluorogenic peptide substrates (4-nitroanilide, thioesters, coumarylamide, FRET). HNE may have different active conformations whose occurrence depends on the environment (hydrophobicity, ionic strength, etc) [81]. It hydrolyzes the B chain of oxidized insulin at the C-terminus of small residues like Val, Ala, Ser and Cys [82]. This preference for small hydrophobic residues

was confirmed using synthetic substrates. The activity of HNE depends greatly on the length of synthetic substrates, suggesting that it has an extended substrate binding site [81]. This was confirmed by analysis of the crystal structure of its complex with OMTKY-III showing the presence of eight binding sites [48]. As a consequence of the extended site of interaction, HNE substrate specificity depends not only on the nature of the P1 amino acid, as in many serine proteases, but also on secondary interactions with S and S′ subsites. The size and hydrophobic properties of the S2 subsite explain the preference for medium-size, hydrophobic side chains, so that proline is preferred at the P2 position, while substrates with charged residue Lys, Arg and Asp at P2 are less well cleaved by HNE [62,83]. The same is true for the S3 and S4 subsites that preferentially accommodate Ala residues at P3 and P4, rather than positively charged residues [84]. The favorable effect of S′–P′ interaction on HNE is mainly due to the occupancy of S1′ [85], and further studies have confirmed that other S′ subsites beyond S1′ have no significant effect on catalysis by HNE [62,86].

Analysis of the peptides generated from oxidized insulin B chain by PR3 shows that the major sites of cleavage resemble those by HNE, C-terminally to small hydrophobic amino acids [87]. The earliest studies with synthetic substrates also revealed the preference of PR3 for small hydrophobic amino acids like Cys, Ala, Val and norVal. Substrates with a Met at P1 are also efficiently cleaved by PR3 [88], but oxidization of P1 Met to methionine sulfoxide, as may occur physiologically, greatly reduces hydrolysis by both PR3 and HNE [88,89]. The presence of an Ile at position 190 of the PR3 sequence instead of a Val in HNE, and of an Asp at position 213 instead of an Ala reduces the size of the S1 pocket and accounts for the preference of PR3 for amino acids smaller than those accommodated in the S1 pocket of HNE that include Ile, Leu, and norLeu [83]. The deep, polar S2 subsite in PR3, due to Lys99, preferentially accommodates a negatively charged P2 residue [62]. However, replacing a negative P2 residue with a positive residue greatly decreases the specificity constant k_{cat}/K_m but does not significantly alter the K_m. This suggests that charge distribution at P2 does not impair substrate binding but helps optimizing substrate orientation within the active site [62]. Predictions of subsite positions within the PR3 active site also show that Lys99 is close to the hydrophobic S4 subsite, which is smaller in PR3 than in HNE. PR3 accommodates small hydrophobic residue at position P4 as a result of the replacement of Arg217 in HNE by an Ile residue in PR3. Residue 217, together with Trp218, probably contributes to the P4/P5 specificity [90]. A severe form of neutropenia is due to an Arg217/Gln mutation in HNE [16], although no relationship has yet been established between the disease and the catalytic activity of the protease. Most conventional peptide substrates having a chromogenic or fluorogenic group on their P1 residue are cleaved much more slowly by PR3 than by HNE. This is not the case when FRET substrates are used, which suggests that S′ subsites are important for substrate binding and/or hydrolysis. C-terminal elongation of FRET substrates greatly increases the catalytic activity of PR3, but not that of HNE [83,86]. The specificities of subsites S5 to S3′ investigated kinetically and by molecular dynamic simulations showed that subsites S2, S1′, and S2′ are the main determinants of PR3 specificity [62,90]. PR3 has a negatively charged residue at position 61 in the vicinity of the S1′ subsite, but HNE does not. As a consequence, PR3 prefers a positively charged P1′ residue, whereas HNE had no preference at that position [62]. This emphasizes the importance of the S1′ subsite for PR3 specificity, even though an electrostatic interaction at S1′ in the PR3 active site is not a prerequisite for substrate binding, explaining why PR3 can also accommodate small hydrophobic residue at position P1′. The presence of a positively charged residue at position 143 in the vicinity of the S2′ subsite of PR3, but not in HNE, is essential for discriminating between HNE and PR3 activities [62,86]. Any type of residue at P3′ is accepted by PR3 and improves the specificity constant by favoring substrate stabilization, but this is not the case for HNE. In summary, the S2, S1′, S2′, and S3′ subsites are the main determinants of the substrate specificity of PR3, and account for its difference from HNE. A consensus sequence for PR3 substrates may therefore be deduced that extends from P2 to P3′ and includes negative, small hydrophobic, positive, negative and positive residues from P2 to P3′ respectively, a sequence that is not recognized by HNE [62,90].

Unlike HNE and PR3, CG has both a chymotrypsin-like and a trypsin-like specificity, but it is much less active than homologous serine proteases like chymotrypsin and chymase on all synthetic substrates assayed so far [91,92]. CG accommodates both large, hydrophobic P1 residues (Phe, Leu, Met) and positive Lys or Arg residues due to the presence of a Glu residue at the bottom of its S1 subsite [79]. The ability of CG to accommodate different P1 residues is also due to the absence of a disulfide bond between Cys191 and Cys220, which improves flexibility and optimizes substrate binding. Like many other chymotrypsin-like enzymes, CG has a marked preference for a Pro at P2. Most of commonly used chromogenic (pNa) and/or fluorogenic (MCA) CG substrates have a Pro–Phe pair at P2–P1 [91–93]. There are no obvious binding determinants for the P3 side chain, although Lys192 has been suggested to favor the accommodation of acidic residues [79]. Like many other serine proteases, including bovine chymotrypsin [94] and porcine pancreatic elastase [95], CG does not accept a Pro residue in its S3 subsite because it would interfere with the anti-parallel β sheet structure formed between a section of the extended substrate binding site and the peptide chain of the substrate [96]. The primary specificity of CG has not been completely elucidated using synthetic substrates, but modeling studies suggest that the Ser40 in CG forms a hydrogen bond with a P1′ Ser in the substrate [92]; which provides a structural basis for the preference of this residue at P1′, as in the natural CG inhibitors, the serpins, α1-antichymotrypsin (ACT), α1-proteinase inhibitor (α1-PI or α1-antitrypsin (α1-AT)) and squamous cell carcinoma antigen 2 (SCCA2) [97]. Because of its flexibility, the side chain of Arg41 might favor the accommodation of an acidic residue at P2′ and possibly interfere with the P3′ specificity [92].

The activities of HNE, PR3 and CG are regulated by the endogenous protein inhibitors of the serpin and chelonianin families, and to a lesser extent by the polyvalent protease inhibitor α2-macroglobulin. The serpins are high molecular weight inhibitors (40 to 50 kDa: 350–500 amino acids) that fold into a conserved, metastable structure and act as suicide substrate-like inhibitors using their reactive centre loop (RCL) [98,99]. The exposed serpin reactive center loop acts as a pseudo-substrate for the target protease; it first forms a reversible, non-covalent Michaelis complex and then its RCL is cleaved and becomes inserted into the A β-sheet [99]. This conformational change results in the protease being trapped in a covalent inhibitory complex in which both molecules are inactivated. The serpins are involved in a number of fundamental biological processes, including blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, tumor suppression and hormone transport [99]. They account for approximately 2% of the total protein in human plasma, of which 70% is α1-PI, a 52 kDa secretory glycoprotein which preferentially inhibits HNE but is also able to inhibit a variety of serine proteases including PR3 and CG, thrombin, trypsin and chymotrypsin [100]. The methionyl residue in the reactive bond of the α1-PI RCL (Met_{358}–Ser_{359}) can be converted by oxidation to methionine sulfoxide in biological systems containing peroxide, hydroxyl radicals, hypochloride, chloramines and peroxynitrite liberated by activated phagocytes [101]. Oxidation of the P1 Met_{358} and that of P8 Met_{351} decrease the second order rate constant for association (k_{ass}) with HNE and CG considerably [102–104]. The main physiological inhibitor of CG is α1-antichymotrypsin [105], a 68 kDa glycoprotein produced in the liver which also inhibits chymotrypsin and chymases found in mast cells. The reactive site of human α1-antichymotrypsin has been identified at the Leu_{358}–Ser_{359} bond. HNE, PR3 and CG are also inhibited by the 42 kDa intracellular serpin monocyte/neutrophil elastase inhibitor (MNEI) [106]. MNEI present in the cytoplasm of the PMNs and macrophages displays two functional reactive sites, one at Phe_{343}–Cys_{344} allowing interaction with chymotrypsin-like protease, the other at Cys_{344}–Met_{345} that is specific for HNE, PR3, pancreatic elastase and kallikrein 3. PI9 and PI6 are two other intracellular serpins that inhibit HNE and CG respectively [107,108]. CG is also inhibited by squamous cell carcinoma antigen 2 (SCCA2), another intracellular serpin that serves as a serological marker for squamous cell tumors [97]. Secretory leucoprotease inhibitor (SLPI), an 11.7 kDa non-glycosylated protein which is expressed by epithelial cells, macrophages, PNNs, and elafin, 6 kDa non-glycosylated protein found in bronchial secretions and skin are the main chelonianin natural inhibitors of NSPs. They differ, however, by their spectrum of inhibition since elafin and its precursor trappin 2 inhibit HNE and PR3 [109,110], while SLPI inhibits HNE and CG [111]. Oxidation of methionyl residues at P1′ position in the reactive site of both SLPI (Leu_{72}–Met_{73}) and elafin (Ala_{24}–Met_{25}) by reactive oxygen species reduces their rates of association with HNE and PR3 [102,112]. Glycosaminoglycans also regulate the activity HNE and PR3: DNA partially inhibits the enzymatic activity of HNE and CG but has no effect on PR3 activity [113]. The second order rate constants for the association of NSPs with endogenous inhibitors are shown in Table 2, and a more comprehensive study on neutrophil serine protease inhibitors is available in this issue of the journal.

6. Biological functions

NSP were first recognized as degradative enzymes that act either intracellularly to degrade ingested host pathogens or extracellularly in the breakdown of ECM components at inflammatory sites, to which PMNs are recruited [114]. Because they are all able to cleave elastin, fibronectin, laminin, collagens and proteoglycans [87,115,116], as well as a variety of plasma proteins [23,117–120], they have long been considered to be redundant, non-specific enzymes. This idea was supported by their storage as active proteases in the same PMN granules and their subsequent simultaneous release into phagocytic vacuoles and/or secretion in extracellular spaces. All three NSPs have bactericidal properties, but they act through different mechanisms that may or may not involve their proteolytic activity [121–123]. Whereas HNE cleaves the virulence factors and outer membrane proteins of Gram-negative bacteria [123], the bactericidal activity of CG is due to at least three different peptide sequences remote from the active site [121,124,125], while that of PR3 also involves a non-proteolytic mechanism similar to that of azurocidin, a neutrophil protein structurally related to NSPs but

Table 2
Second order rate constants for association of endogenous inhibitors with elastase, proteinase 3 and cathepsin G

k_{ass} (M^{-1} s^{-1})								
	Serpin				Chelonianin			
	α1-PI	ACT	MNEI	PI9	PI6	SCCA2	SLPI	Elafin
Elastase	6.5×10^{7}[a] 1.9×10^{7}[b]	NI[b]	3.4×10^{7}[d]	1.5×10^{5}[l]	NI[n]	NI[f]	1.1×10^{7}[g]	3.6×10^{6}[j]
Proteinase 3	8.1×10^{6}[i]	NI[i]	1.7×10^{7}[d]	ND	ND	ND	NI[m]	4×10^{6}[k]
Cathepsin G	4.1×10^{5}[a]	5.1×10^{7}[a] 8.1×10^{5}[b] 7.0×10^{5}[c]	2.3×10^{6}[d]	ND	6.8×10^{6}[e]	1.0×10^{5}[f]	1.5×10^{6}[h]	NI[o]

[a][102]; [b][182]; [c][183]; [d][184]; [e][108]; [f][97]; [g][185]; [h][186]; [i][87]; [j][187]; [k][188] [l][189]; [m][190]; [n][191]; [o][110].
NI, no inhibition; ND, not determined.

enzymatically inactive [30]. NSPs also help protect the body against fungal infections [30,126]. The NSPs are also microbicidal extracellularly in neutrophil extracellular traps (NETs), a complex network of actively secreted PMN chromatin, and granule proteins that trap and degrade Gram-positive and Gram-negative bacteria [127]. Extracellular HNE and CG can also cleave bacterial virulence factors such as proinflammatory flagellin [128], while PR3 processes human cathelicidin hCAP-18 to generate LL-37, a potent antibacterial 4.5 kDa peptide [129].

In addition to their degradative/bactericidal properties, NSPs are also important regulators of the local inflammatory response. They can modulate biological functions such as the control of cellular signaling through the processing of chemokines, modulating the cytokine network and activating specific cell surface receptors (reviewed in [6,8, 130]). Exposure of PMNs to cytokines and chemoattractants results in the rapid mobilization of azurophil granules to the cell surface, the release of granule proteins and the appearance of NSPs on the cell surface [131]. Like soluble proteases, membrane-bound NSPs are catalytically active against synthetic and biologically relevant substrates [86,131,132]. Unlike HNE and CG, PR3 has a bimodal distribution on the cell surface. It is not only stored in PMN secondary granules and secretory vesicles [60,133,134], but it can also be constitutively present at the neutrophil surface. The mechanism by which proteases are attached to the cell surface is not entirely clear, but it probably differs from one protease to another. HNE and CG bind to the plasma membrane of PMNs via a charge-dependent mechanism involving the negatively charged sulfate groups of heparan sulfate- and chondroitin sulfate-containing proteoglycans [135], whereas PR3 binding occurs through the GPI-anchored surface receptors FCγRIIIb [136] and/or NB1 [137].

The demonstration that NSPs, especially HNE and PR3, have different substrate specificities supports the idea that each protease has a specific role. Thus, the specific cleavage of p21 and NF-κB proteins by PR3 [138,139] that gives the protease its proapoptotic properties is explained by its preference for negatively charged residues at P2 and P2′ in the substrate. This specificity differs from that of HNE and explains its caspase 3-like properties [62]. PR3 but not HNE processes TNF-α *in vitro* [140] and activates IL-18 and IL-1β [8] whereas HNE activates the epidermal growth factor receptor [141] and induces the expression of IL-8 via Toll-like receptor-4 [142]. Activation of platelets at inflammatory sites may occur via the activation of protease-activated receptor 4 (PAR-4) [143] whereas PR3 would activate PAR-2 [144]. The main biological functions known to date for NSPs are summarized in Table 3. Because of their spatial–temporal regulation and their implication in the regulation of the biological functions of NSPs in cell signaling, innate immunity and inflammation are far from being elucidated, and the recent demonstration that HNE upregulates the expression of proteolytic enzymes from other classes will not simplify the understanding of the role of proteases during inflammation [145].

Table 3
Biological functions of elastase, proteinase 3 and cathepsin G

		Refs.
Elastase	Degradation of ECM components	[115,120,192–195]
	Degradation of plasma proteins	[23,117–119]
	Bactericidal properties	[123]
	Cleavage of inflammatory mediators	[6]
	Cleavage of receptors	[141,196,212]
	Cleavage of lung surfactant	[197]
	Activation of lymphocytes and platelets	[198]
	Activation of proteases	[199,200]
	Inactivation of endogenous inhibitors	[201,202]
	Cytokines and chemokines induction	[198,203]
	Induction of airway submucosal gland secretion	[204]
Proteinase 3	Degradation of ECM components	[87]
	Bactericidal properties	[205]
	Cleavage of inflammatory mediators	[6,140,144]
	Platelet activation	[206]
	Induction of endothelial cells, apoptosis	[207]
	Negative feedback regulation of granulopoiesis	[29]
	Target of auto-antibodies in Wegener's granulomatosis	[208]
Cathepsin G	Degradation of ECM components	[209]
	Degradation of plasma proteins	[23,120]
	Bactericidal properties	[210]
	Cleavage of receptors	[196,211,212]
	Cleavage of inflammatory mediators	[6]
	Conversion of angiotensin I to angiotensin II	[213]
	Platelet activation	[111,143]
	Induction of airway submucosal gland secretion	[204]

7. Roles of NSPs in diseases

NSPs are implicated in a variety of infectious inflammatory diseases, including lung diseases (chronic obstructive pulmonary disease, acute respiratory distress syndrome, cystic fibrosis, and ischemic-reperfusion injury (reviewed in [146–148])) and in non-infectious inflammatory processes such as glomerulonephritis, arthritis, and bullous pemphigoid [149,150]. The destructive actions of HNE have been firmly established after Laurell and Eriksson reported the link between α1-PI deficiency and air flow obstruction and emphysema [151]. This degradative potential was further demonstrated by the emphysema produced by instilling HNE into the lungs of animal models [152]. HNE-related NSPs [3] and MMPs were also subsequently shown to contribute to the matrix degradative potential that occurs in emphysema and in other lung and other tissue inflammatory diseases in which there is recruitment of blood PMNs to inflammatory sites (reviewed in [147,148,153]). The different rates at which individual proteases are controlled by their natural inhibitors, as well as the dysregulation of the extracellular protease–antiprotease balance that occurs during inflammation, lead to the activation of proinflammatory mediators. These favor the recruitment of

more PMNs via increased IL-8 production, and thus explain the chronicity of inflammatory diseases (reviewed in [7,114,154]). The secretagogue activity of all three NSPs that normally allows the binding and removal of bacteria may also explain the excessive mucus production in chronic obstructive pulmonary disease (COPD) and the plugging of the airways with mucus in cystic fibrosis [155,156]. But why secreted proteases remained enzymatically active and contribute to the chronicity of inflammation by cleaving target extracellular proteins in an environment most often replete with inhibitors remains a mystery. Quantum proteolysis in the pericellular environment [157,158], the resistance of membrane-bound proteases to high M_r protease inhibitors [159], the tight adhesion of PMNs to ECM components producing a zone inaccessible to extracellular inhibitors [160], the embedding of proteases in NETs [127], and the complexing of extracellular proteases with polyanionic partners [161] are all possible mechanisms that could help unravel this mystery. Identifying the prevailing mechanism(s) for each given pathophysiological situation will probably provide clues to the appropriate use of therapeutic protease inhibitors to control the deleterious proteolytic actions of NSPs. The recent demonstration that membrane-bound HNE is inhibited at the same rate as soluble HNE by its major physiological inhibitor α1-PI and by a lower Mr protein inhibitor [132,162] shows that additional studies are needed to elucidate how protease activities are controlled at inflammatory sites. The different binding of these proteases to PMN membranes, and their possibly different interactions with NETs and other extracellular macromolecules that may impair their activity is a serious concern for this kind of investigation.

NSPs are also involved in other pathological processes due to gene mutations, altered cellular trafficking or, in the case of PR3, an autoimmune response. PR3 is the major target antigen of the anti-neutrophil cytoplasmic autoantibodies (c-ANCA) produced in Wegener's granulomatosis (WG) [163]. The cause of WG has long remained obscure, but mounting evidence now suggests that complementary proteins play a role in the induction of autoimmunity through a complex network of idiotypic–anti-idiotypic antibodies [164,165]. The blood neutrophils from patients with active WG bear greater amounts of PR3 on their cell membranes than do neutrophils from healthy individuals because of the ongoing inflammatory process. The PR3 exposed on the cell surface is directly accessible to circulating c-ANCA, which results in complete activation of PMNs [164]. α1-PI deficiency was also shown to be positively correlated with the severity of vasculitis in WG patients thus involving the protease-inhibitor balance in the disease and further suggesting that PR3 is a biological target of α1-PI [166,167]. Inflamed oral epithelial cells also produce PR3 and are the targets of c-ANCAs during periodontitis [168]. PR3 also has a proapoptotic function through the proteolytic activation of procaspase-3 to active caspase [169], as well as the direct cleavage of NF-κB and p21 [138,139,170,171].

Mutations in the *ELA2* gene that encode HNE are the cause of human cyclic neutropenia and severe congenital neutropenia [16,17]. Nearly 50 different HNE mutations scattered throughout the promoter and coding regions have been identified, resulting in base substitutions, missense, deletion, and truncation mutations. But these mutations have no consistent effect on enzyme function [172–175]. The pathophysiology of hereditary neutropenias is an area of active investigation. The disruption of HNE intracellular processing and trafficking due to these mutations results in the accumulation of misfolded inactive protein [176]. No mutations in the genes of PR3 and CG that alter protein folding and trafficking have been described so far.

HNE would be also involved in the pathogenesis of acute promyelocytic leukemia (APL) by cleaving the PML-RAR alpha fusion protein within the nucleus, but this remains to be confirmed [177,178]. PR3 and CG are not involved in the cleavage of PML/RAR alpha fusion protein, but all three proteases are concerned by the mutation of the gene encoding cathepsin C (dipeptidyl peptidase or DPPI), a cysteine protease that processes the inactive proforms of NSPs to give the active enzymes before they are stored in PMN granules [70]. The mutations in its gene are strongly correlated with Papillon-Lefèvre syndrome and palmoplantar keratosis [179,180].

8. Concluding remarks

While proteases were first considered to be protein-degrading enzymes, they are now recognized as essential components of a variety of pathophysiological processes. Hence, they are potential targets for therapies based on inhibiting proteases. The NSPs, which are stored in huge amounts as active enzymes in specialized granules, play a central role in the biological function of PMNs, especially at inflammatory sites to which these cells are recruited and activated. All three NSPs have common degradative properties, but each has specific functions during pathological processes, as illustrated by the proteolytic regulation of the cytokine network [6]. Their other specific functions associated with inflammation, infection, innate immunity and apoptosis, remain to be clarified. A range of specific inhibitors that can efficiently target each NSP would be of great help in such studies. But only HNE has so far received the attention required to result in the development of inhibitors that target its proinflammatory and destructive actions [181]. No specific inhibitors of CG and PR3 are currently available. These inhibitors are needed both to understand the biological role of each protease, and also to combat protease-dependent tissue destruction without compromising the physiologic function of each protease.

Because NSPs are among the terminal effectors that mediate tissue destruction, elucidation of their fate and their regulation will undoubtedly provide valuable clues leading to their more efficient targeting by anti-proteases for treating chronic inflammation and other NSP-associated proteolytic processes.

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