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Cystatins: Biochemical and structural properties, and medical relevance

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TABLE OF CONTENTS

1. ABSTRACT

The cystatin superfamily comprises a large group of the cystatin domain containing proteins, present in a wide variety of organisms, including humans. Cystatin inhibitory activity is vital for the delicate regulation of normal physiological processes by limiting the potentially highly destructive activity of their target proteases such as the papain (C1) family, including cysteine cathepsins. Some of the cystatins also inhibit the legumain (C13) family of enzymes. Failures in biological mechanisms controlling protease activities result in many diseases such as neurodegeneration, cardiovascular diseases, osteoporosis, arthritis, and cancer. Cystatins have been classified into three types: the stefins, the cystatins and the kininogens, although other cystatin-related proteins, such as CRES proteins, are emerging. The stefins are mainly intracellular proteins, whereas the cystatins and the kininogens are extracellular. The cystatins are tight binding and reversible inhibitors. The basic mechanism of interaction between cystatins and their target proteases has been established, based mainly on the crystal structures of various cathepsins, stefins and cystatins and their enzyme-inhibitor complexes. Cystatins, as rather non-selective inhibitors, discriminate only slightly between endo- and exopeptidases. They are also prone to form amyloids. The levels of some stefins and cystatins in tissue and body fluids can serve as relatively reliable markers for a variety of diseases. In this review we summarize present knowledge about cystatins and their role in some diseases.

2. INTRODUCTION

Proteolytic processing is the control mechanism by which proteases are involved in diverse vital processes in all cells of living organisms. Through evolution, proteases have adopted to the wide range of conditions in their living environment using different catalytic mechanisms for substrate hydrolysis. Consequently they are divided into five types, including cysteine-type proteases (1, 2). The first clearly recognized cysteine protease was plant papain, which represent the foundation of papain (C1) family. This family include among others lysosomal cysteine cathepsins (3) and parasitic proteases like cruzipain (4). Cysteine cathepsins participate in numerous physiological and pathological processes including antigen processing, bone remodelling, neurodegeneration, cardiovascular diseases and cancer (5-10). Potentially highly destructive activity of cysteine proteases can be regulated by their endogenous protein inhibitors (11). Considerable evidence has appeared during the first 25 years that the superfamily of cysteine protease inhibitors, the cystatins, are crucial for maintaining controlled proteolysis caused by the target cysteine proteases. Any mechanism of reducing this well regulated balance may result in substantial pathological problems. The great majority of studies were made on human cystatins in combination with human cysteine cathepsins. The aim of this review is to stress some of the most important features of cystatins, mechanism of interaction

with their target enzymes, their role in some diseases and their possible clinical relevance. However, it is impossible to include most of the references due to their enormous number. Therefore, some additional references may be found in reviews cited in this paper.

3. DISCOVERY OF THE CYSTATIN SUPERFAMILY

Studies on papain and cysteine cathepsins were unambiguously crucial for the discovery of protein inhibitors, which can be classified into several structural unrelated families, such as the cystatins, the thyropins, the propeptide-like inhibitors and others (11). The most investigated are the cystatins, which are present in a wide variety of organisms and best understood in humans. They function intracellularly and extracellularly through the formation of tight reversible complexes with their target enzymes (12). The first isolated and partially characterized protein inhibitor of cysteine proteases was from chicken egg white and was shown to inhibit papain, ficin (13, 14) and cathepsin B and C (15). Later for the same protein the name "cystatin" was proposed, indicating its function (16). The first intracellular protein inhibitor of papain, cathepsin B and H was isolated and partially characterized from pig leucocytes and spleen (17). The determined amino acid sequences of chicken cystatin (18, 19) and of human stefin (later stefin A) from the cytosol of polymorphonuclear granulocytes (20) confirmed structural differences between these two homologous proteins. At the same time, the protein inhibitor of cysteine proteases, isolated from the sera of patients suffering from autoimmune diseases (18) shared the complete identity of its N-terminal 47 residues sequence with the human γ-trace, a small basic protein without known function (21). This represents the first discovery of the functional human cystatin in contrast to γ-trace, isolated from amyloids, as biologically inactive protein (21). Based on its sequence homology to chicken cystatin (18) the name human cystatin was proposed (18, 22). Soon after, it was renamed to human cystatin C (HCC) (23). From human plasma three forms of the inhibitor, differing in molecular mass (Mr) and isoelectric point (pI) were isolated, and introduced the name "α_1-thiol proteinase inhibitor" (α_1-TPI) for the high Mr form and α_2-TPI for the other two low Mr forms (24). Using affinity chromatography on C_m-papain-Sepharose resin "α-cysteine protease inhibitor" (α-CPI) was isolated in several forms and further separated (25). Finally, when sequenced a cDNA encoding the human low- Mr α-CPI (26) it was demonstrated that the sequence show high similarity to bovine L- kininogen (27). At the same time the partial amino acid sequence of human kininogens and low-Mr α-CPI confirmed that L- kininogen and low-Mr α-CPI on one hand and H- kininogen and high -Mr α-CPI on the other hand are identical proteins (28). The accumulated data in this short period of two years and the First International Symposium on Cysteine Proteinases and their Inhibitors (Portorož, Yugoslavia - now Slovenia, September 1985, organized by V. Turk) were crucial for the nomenclature and classification of the cystatin superfamily (29). The known proteins of the cystatin superfamily comprise three distinct families of proteins: family 1 or the stefin family, family 2 or the cystatin family and family 3 or the kininogen family.

4. EVOLUTION AND CLASSIFICATION OF THE CYSTATIN SUPERFAMILY

The first classification of the protein inhibitors of papain-like cysteine proteases, the cystatin superfamily, into three families was based on the expectancy that the members of a protein family have at least 50% sequence identity, they inhibit their target enzymes, and absence or presence of two or nine disulphide bounds (29). However, an increasing number of cystatins from various sources introduced new subdivision of the cystatins into four families (30). Whereas the first three, the stefins (single inhibitors domain), the cystatins (single inhibitors domain) and the kininogens (three domain inhibitors) are truly inhibitory (11, 31), fourth consists of non-inhibitory homologues of two cystatin-like domains, such as human α $_2$SH-glycoprotein (fetuin) and histidine-rich glycoprotein (32). The division of the members of the superfamily into four types (30) was made on the basis of the presence of one, two, or three copies of cystatin-like segments and the presence or absence of disulphide bond.

The first two proposed evolutionary dendrograms were made based on a small number of members of the cystatin superfamily (33, 34). However, the new proposed evolutionary dendrograms followed the evolution of the proteins of the cystatin superfamily along four lineages, with special attention that duplication of cystatin-like segments, has played important contribution to the understanding of the evolution of cystatins. In addition to animal cystatins, phytocystatins from plants are also related, although more distantly (35). Enormous growth of information of the complete eukaryotic and prokaryotic genomic sequences enabled increased knowledge in this field. A system wherein the inhibitor units of the protease inhibitors are assigned to 48 families on the basis of amino acid sequence similarities was proposed, Then, on the basis of three dimensional structures, 31 of the families are assigned to the clans. Consequently, the simple system of nomenclature and assignment to each clan, family and individual inhibitor is proposed (36). In this classification the cystatins are assigned to family I25 which consists of three subfamilies: I25A (the type 1 cystatins, stefins), I25B (the type 2 cystatins, cystatins), whereas I25C are mostly not inhibitors of proteases. The proposed system of families, clans and inhibitors has been implemented in the MEROPS database and can be accessed (http://merops.sanger.ac.uk) providing a mechanism for updating when new information becomes available. In addition, the analyses of all available genomic, transcriptomic and proteomic data provided a first global insight into the distribution, diversity, evolution, classification and origin of the cystatin superfamily in eukaryotes and prokaryotes (D. Kordiš and V. Turk, in preparation). In this paper we will further discuss the superfamily of cystatins grouped in three types, which is the most suitable concerning present status in the literature.

5. GENERAL PROPERTIES OF THE CYSTATIN SUPERFAMILY

5.1. Type 1 cystatins (stefins)

The protein inhibitors belonging to stefin family are single-chain proteins which lack disulphide bonds and carbohydrates and are composed of ~100 amino acid residues with Mr of ~11 kDa. They are primarily intracellular cytoplasmic proteins of many cell types, although they have been found in extracellular fluids as well (37). In mammals including human, rat, bovine, mouse and porcine, two members of the family, stefin A (cystatin A or α) and stefin B (cystatin B or β), have been identified (reviewed in 31, 38). In addition, stefin C was discovered in bovine thymus as the first tryptophan - containing stefin with a prolonged N-terminus (39), and stefin D in pigs (40). Two human stefins, stefin A and B, are potent inhibitors of papain, cathepsins L, S and H, and form tight complexes with these enzymes. The decreased activity of stefins toward exopeptidase cathepsin B for about 3-4 orders of magnitude may be explained by the steric hindrance, caused by occluding loop of cathepsin B, as seen from its crystal structure (41).

In contrast to human stefins, at least three different stefin A variants are encoded within the mouse genome (42).Very recently it was found that two mouse stefin A variants act as fast and tight binding inhibitors of papain-like endopeptidases. Surprisingly they are significantly less potent against papain-like exopeptidases than human, porcine or bovine stefin A (43), suggesting that mice stefin A variants are involved predominantly in the regulation of endopeptidase activity of cysteine cathepsins. Some cysteine cathepsins play a crucial role in the antigen presentation process indicating that interactions between stefin A and cathepsins contribute to the species dependent diversity of the endosomal compartments which participate in immune response (43).

5.2. Type 2 cystatins (cystatins)

The members of this family were found in various organisms and are mainly extracellular, secreted proteins. Mammalian cystatins occur at relatively high concentrations in body fluids (37). They are synthesized with 20-26 residues long signal peptides. Cystatins are single-chain proteins composed of ~120 amino acid residues with Mr ~13-15 kDa, containing two intracellular disulphide bonds and are usually non-glycosylated. The exceptions are cystatin E/M (44, 45) and cystatin F (46), which are glycoproteins. Similarly to stefins, the cystatins contain the conserved QXVXG region in the central part of the molecule and the P-W pair in the C- terminal part of cystatins (12). One of two pI forms of chicken cystatin is phosphorylated (47). Chicken cystatin and HCC represent founding members of this family (12, 38). Human type cystatins include cystatin C, D, S, SA and N with about 50% or less sequence identity. More recently, human cystatin E from amniotic cells and fetal skin epithelial cells, was identified and recombinant cystatin E was isolated (44). Human cystatin M is expressed by normal mammary cells and a variety of human tissues (45). Both proteins, cystatin E and M were discovered at the same time independently and are identical and renamed as cystatin E/M (MEROPS). Recently, the expression of cystatin M/E was found to be restricted to the epidermis (48) and is most probably identical to cystatin E/M.

Cystatins are important endogenous cysteine protease inhibitors of papain-like cysteine proteases, including cysteine cathepsins, parasite proteases like cruzipain, and mammalian asparaginyl endopeptidase, legumain (4, 11, 12). Although cystatins are rather non-specific inhibitors of cysteine proteases, they are capable of discriminating between endo- and exopeptidases. The crucial step for understanding the mechanism of interaction between cystatins and papain-like enzymes was the determination of the crystal structure of chicken cystatin (49) which proposed the mechanism of interaction. Successful expression of human stefin B of cystatin superfamily (50) was crucial for its crystal structure in complex with papain (51). This crystal structure of the complex confirmed the proposed mechanism of the inhibition by the cystatins, which is fundamentally different to that for the serine proteinase inhibitors.

However, all the cystatins are competitive, reversible, tight binding inhibitors, which inhibit their target enzymes in μM to pM range (reviewed in 31). The most potent tight binding inhibitors are chicken cystatin and HCC, which inhibit papain, cathepsin L and cathepsin S in pM range (reviewed in 38, 52). HCC shows strong inhibitory capacity for rapid binding thus neutralizing protease activity in an “emergency” inhibition (11). HCC also strongly inhibits cruzipain from the protozoic parasite *Trypanosoma cruzi* suggesting a possible defensive role after infection (53).Human cystatin D, present in saliva and tears does not inhibit cathepsin B (Ki >1μM) and when compared to cystatin C and all other known cystatins is weaker inhibitor of cathepsin L but relatively stronger of cathepsin H and S with Ki values of 8.5 nM and 0.24 nM, respectively (54). Human cystatin E/M inhibits papain and cathepsin B with Ki value of 0.39 nM and 32 nM, respectively (44, 45). It was recently reported that cystatin M/E is inhibiting cathepsin L and V with Ki values of 1.78 nM and 0.47 nM, respectively (48). Interestingly, the same inhibitor also inhibit cysteine protease legumain, member of family C13 (MEROPS) with Ki value of 0.25 nM and was found that legumain and papain-like cysteine proteases were inhibited by two distinct non-overlapping sites (48, 55). Cystatin E/M and cystatin F show strong (Ki = 0.0016 nM) and relatively weak (Ki = 10 nM) inhibition of legumain, respectively (55). Cystatin F also inhibits papain and cathepsin L (Ki = 1.1 nM and 0.31 nM, respectively), but not cathepsin B (46). Stefins and other cystatins do not inhibit mammalian legumain. The crystal structure of human cystatin D provided explanation for the restricted inhibitory specificity for some papain-line proteases and its lack of inhibition for legumain (56).

Whereas cystatins inhibition of endopeptidases is rapid and tight, with Ki values of pM and even fM range, the inhibition of exopeptidases is weaker, with Ki values in μM and nM range. This can be explained by the differences in the active site regions of endo and exopeptidases (11).

The access of the inhibitors to the active site of exopeptidases is partially obstructed by occluding loops in cathepsin B (41) and cathepsin X (57) and propeptide parts in cathepsin H (58) and cathepsin C (59).

More details about properties and their interaction with cysteine proteases with special emphasis on cysteine cathepsins are reviewed (11, 12, 31, 60).

5.3. Type 3 cystatins (kininogens)

Kininogens are large multifunctional glycoproteins in mammalian plasma and secretions. For a long time have been known as the large precursor proteins of the vasodilator kinin peptides, bradykinin and calidin, involved in blood pressure regulation (61). When it was discovered that kininogens are identical with α-CPI (26, 28), the kininogens as the third family of the cystatins was established (29). Three different types of kininogens exist in mammals: H- (high-Mr) and L- (low-Mr) kininogen, both in different species, and T-kininogen known as major acute phase protein, found only in rats (62, 63). In humans H-kininogen and L-kininogen are single chain glycoproteins each composed of an N-terminal heavy chain, the kinin segment and a C-terminal light chain. The heavy chain and light chain are interconnected by disulphide bridges. The heavy chains and the kinin segments of both kininogens have identical amino acid sequences. However, the light chains of both kininogens differ (62, 64). The heavy chain is subdivided into three cystatin-like domains, designated D1-D3 (64). Only isolated domains D2 and D3 inhibit papain-like proteases and calpain (only D2), whereas domain D1 was found to lack inhibition. The D3 domain stronger inhibits papain and cathepsin L than D2 domain, both in nanomolar range. However, the intact L-kininogen inhibits papain and cathepsin L at about Ki = 0.015 nM. Although it has been shown that intact molecule and two of the three isolated kininogen domains have inhibitory potency indicating that the intact molecule have the potential for binding two molecules of cysteine proteases, they were conflicting results concerning the binding stoichiometry. Finally it has been shown that L-kininogen binds two molecules of papain, cathepsin S and L with high affinity (65). Similarly, H-kininogen binds two molecules of papain, cruzipain and cathepsin S (66). It was found that the faster binding site is also the tighter-binding site than is that of the third domain, whereas the slower binding, lower-affinity site is on the second domain.

By limited proteolysis, using trypsin, elastase and other proteases, human L- kininogen was fragmented and "proteinase sensitive regions" in the heavy chain were located (67). The presence of such fragments may also occur *in vivo* under pathological conditions, e.g. in disseminated intravascular coagulation or polytrauma. Indeed, the inhibitory fragment of 22 kDa isolated from human placenta was identical to the third domain of human kininogen. However, this kininogen domain, and HCC, were inactivated by cathepsin D, suggesting the role of cathepsin D in the regulation of cysteine cathepsins (68). It is known that *Streptococcus pyogenes*, express surface proteins with affinity for some human plasma proteins. It was found that M proteins isolated from *S. pyogenes* of different serotypes interact with both, L- and H- kininogen (69). These interactions might contribute to the host-parasite relationship in *S. pyogenes* infections.

Antibacterial peptides are important effectors of the innate immune system. Recently a novel antibacterial activity of the heparin-binding and cell-binding domain 5 (light chain) of H-kininogen was found (70). An antibacterial epitope of this domain, the peptide (His479-His498), effectively killed the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and the Gram- positive *Enterococcus faecalis*. Proteolytic degradation of H-kininogen by neutrophil-derived proteases as well as the elastase from *P. aeruginosa* resulted in fragments comprising these epitopes, indicating that kininogen-derived antibacterial peptides are released. These antibacterial peptides also interact with mammalian cells and glycosaminoglycans, thus controlling chemotaxis, apoptosis and angiogenesis (70).

It has been reported that the exogenous bradykinin (BK) induces dendritic cell maturation, driving IL-12 dependent Th1 responses through the activation of G-protein. coupled BKB-2 receptors (71). Moreover, the peripheral levels of BK were found to be controlled by toll-like receptor 2 (TLR2)/neutrophils and angiotensin converting enzyme (ACE) in a model of *Trypanosoma cruzi* infection (72), thus suggesting that it can be a modulator of innate or adaptive immunity.

5.4. Other type 2 cystatins

5.4.1. CRES proteins

There are a number of other cystatins or cystatin-related proteins, expressed in different tissues and cell types in humans and other mammals. A novel cystatin-related epididymal specific (CRES) gene was found in mouse epididymis, showing substantial homology with those of well established protein inhibitors, cystatins (73). However, the CRES gene does not contain the highly conserved QXVXG region and P-W pair which are crucial for cysteine cathepsin inhibition. The CRESS gene is almost restricted to epididymis and much less expressed in testis, and no expression in any other tissue was found. Later, the same group found the same gene expressed in spermatids and the name of the protein was slightly modified to Cystatin Related Epididymal Spermatogenic (CRES) protein (74). Contrary to expectations, the recombinant CRES protein did not inhibit the cysteine proteases papain and cathepsin B but selectively inhibited serine protease prohormone convertase 2 (75). A gene was isolated from mouse fetus, related to the genes that encode cystatins, and was named testatin (76). Testatin expression is restricted to pre-Sartoli cells, and its expression was high during the early events of testis development. Two more genes from mouse Sartoli cells, named cystatin SC and cystatin TE-1, were isolated which were detected only in the testis and highly expressed in testis and epididymis, respectively (77). Expression of cystatin TE-1 in ovary and prostate was low. Several more genes were found, expressed specifically in the male reproductive tract (78 - 80), which clearly indicate the existence of a new subgroup

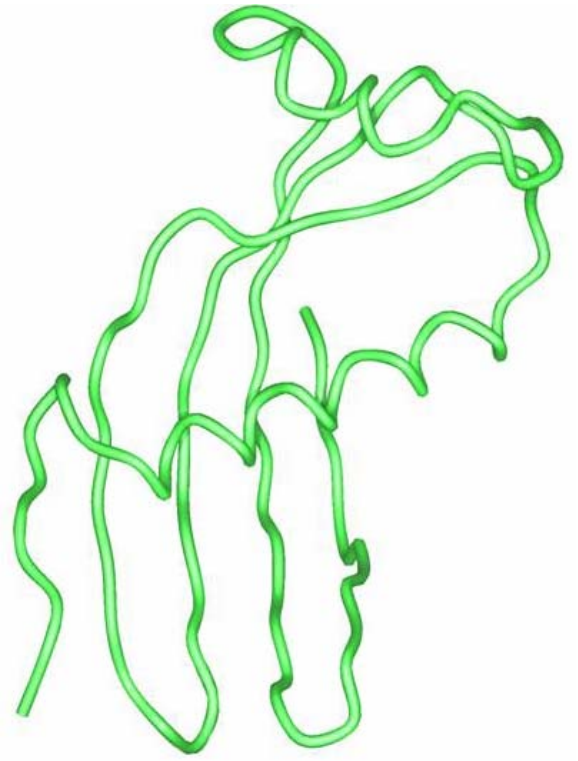

Figure 1. Fold of cystatin C (49): Cystatin C chain trace is shown in green in orientation which positiones the N-terminal "elephant trunk" and the first and the second haipin loops to the bottom from left to right.

in the type 2 cystatins (81, 82), with well conserved gene structure and, with the exception of the two hairpin loops, highly conserved in cystatins and responsible for inhibition of cysteine proteases. The role of CRESS type 2 subgroup might be important for the regulation of proteolysis in reproductive tract as well as protection against invading pathogens by inhibiting microbial proteases, as shown by cystatin 11, which shows antibacterial activity (78). Very recently it was demonstrated that CRES tends to form oligomers (83), similarly to cystatin C (84, 85) and stefin B (86, 87). Another type 2 cystatin, cystatin 10, was expressed in cartilage, localized exclusively in the cytosol of prehypertrophic and hypertrophic chondrocytes (88). Its role in the last step of the chondrocyte differentiation pathway is suggested to be as an inducer of maturation, followed by apoptosis of chondrocytes. Finally, a novel cystatin type 2 protein, named CLM, is expressed widely in normal tissue and is clearly different from CRES proteins (89). This protein could be important in hematopoietic differentiation or inflammation.

5.4.2. Tick cystatins

Genes encoding cystatins have also been found in several ticks which constitute the main vector of Lyme disease in the USA and Europe. The two cystatin transcripts are encoded by two different genes in the tick *Ixodes scapularis*. Both cystatins were expressed and were characterized as sialostatin L (90) and sialostatin L2 (91). The name indicates the strong inhibition of cathepsin L. These two sialostatins, which are found in saliva, show 75% identity in their sequence, and inhibit almost equally cathepsin L, with Ki = 4.7 nM, and cathepsin V with Ki = 57 nM. No inhibition of cathepsins B, X and C was observed. Cathepsin L is a known collagenolytic enzyme and plays an important role extracellularly and intracellularly. Its collagenolytic activity is inhibited by chicken cystatin (92). Consequently, sialostatin L displays an anti-inflammatory role and inhibits proliferation of cytotoxic T-lymphocytes (90). Both tick cystatins are attractive targets for the development of anti-tick vaccines.

5.4.3. Phytocystatins

In plants, inhibitors of cysteine proteases are known as phytocystatins. They contain the QXVXG region of type 2 cystatins, but also resemble the stefins in the absence of disulphide bonds (93). There are numerous phytocystatins, expressed and characterized on the protein level from corn (94), rice (95), soybean (96), sugarcane (97), and others. Interestingly, C-terminal extended phytocystatins were found as bifunctional inhibitors of papain and legumain (98). In addition, a “multicystatin” containing two cystatin-like domains was isolated from cowpea leaves (99). Phytocystatins and other inhibitors are important for plant defence response to insect predation, may act to restrict infection by some nematodes (100), play a crucial role in response to various stress conditions (99, 101), and show great potential as tools for genetically engineered resistance of crop plants against pests (102).

6. STRUCTURE OF CYSTATINS

6.1. Basic structures and mechanism of action

Although cystatins are rather nonselective inhibitors of papain-like cysteine proteases, they are capable of slight discrimination between the endo and exo-peptidases (11, 43, 103). While the interaction constants with endopeptidases are in general in the low nM to pM range, their interaction with exopeptidases is typically weaker by several orders of magnitude.

The chicken cystatin structure has revealed a new fold, named the cystatin fold, which is based on a five stranded anti-parallel β-sheet wrapped around the central N-terminal helix (49) (Figure 1). Knowing the cystatin structure and combining it with the available biochemical data, it was possible to propose the mode of binding (49). This wedge shaped structure was proposed to bind with its sharp edge in the active site cleft of the protease. The interacting parts consist of an N-terminal trunk and two beta hairpin loops. Due to the similarity to a picture of an elephant, the model was named the elephant trunk model, which was later confirmed by the crystal structure of the stefin B-papain complex (51). The structure revealed that the highly conserved sequence of cystatins, the QVVAG region, is positioned at the end of the first β hairpin, interacting with the bottom of the active site cleft in the direct vicinity of the catalytic cysteine residue of papain. Almost a decade later this interaction was confirmed and enriched with the more intimate and tight relationship observed in the crystal structure of stefin A bound to naked

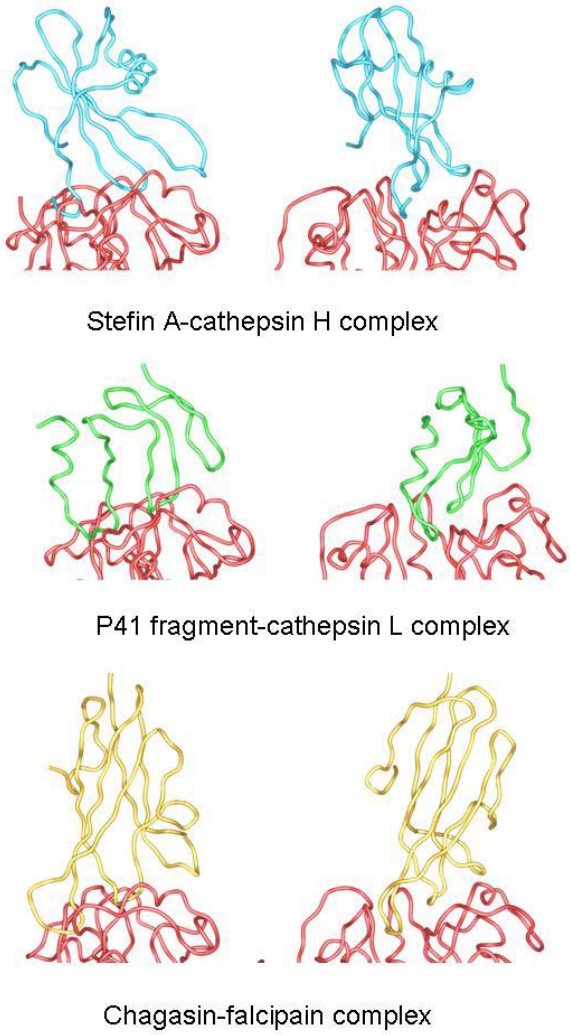

Figure 2. Binding of the "three finger tip" inhibitor. Stefin A - cathepsin H (104), p41- cathepsin L (109) and chagasin - falcipain (110) at the bottom. The chain traces of the inhibitors stefin A, p41 fragment and chagasin are shown in cyan, green and yellow colors respectively, whereas the chain traces of the underlaying enzymes below are all shown in red. The enzymes part are cut at the bottom. Each complex is shown in two views: across and along the active site cleft of the enzymes. The across and allong views are shown in left and right columns, respectively. The across views make the "three finger tips" architecture clearly visible, whereas the along views indicate that the three inhibitors fill the active site clefts along the whole length.

cathepsin H (104) (Figure 2-top). The structures are similar to the stefin B-papain complex (51), but with a few distinct differences. On binding, the N-terminal trunk of stefin A adopts the form of a hook, which pushes away the mini-chain residues unique to cathepsin H. The approach of stefin A to cathepsin H induces structural changes along the interaction surface of both molecules, whereas no such changes were observed in the stefin B-papain complex. Comparison with the structure of cathepsin H (58) showed that the rims of the cathepsin H structure are slightly displaced from their position in the free enzyme and that stefin A binds about 0.8Å deeper into the active site cleft of cathepsin H than stefin B into papain. The reason was found in the carboxymethylation of catalytic cysteine in papain (51), which reduces the interaction constant with cystatin by ~3 orders of magnitude (105). The weaker binding affinity appears to have prevented the formation of the genuine binding geometry between a papain-like enzyme and a cystatin type inhibitor.

In addition to the structurally derived data, the contribution of the individual residues within the protease binding region of cystatins was additionally investigated by mutational analysis and kinetic studies performed by several different groups (106 - 108).

When the crystal structure of the p41 fragment was determined in complex with human cathepsin L (109), it revealed that the p41 fragment and cystatin/stefin family of cysteine protease inhibitors are based on different folds but, nevertheless, exhibit similar binding topologies which appear to have converged in the way they interact with the bottom of the active site cleft of papain-like cysteine proteases. The p41 fragment binds into the V-shaped active site cleft of cathepsin L with the sharp edge of the wedge structure, similar to cystatins (Figure 2-middle). The views across the active site cleft reveal that the first binding loop of the p41 fragment overlaps with the position of the N-terminal trunk of cystatins, whereas the second and the third binding loops overlap with the position of the first and second hairpin loops of cystatins. Also chagasin, a cysteine protease inhibitor from *Trypanosoma cruzi*, has the three hairpin loops which reach down into the active site cleft of the target enzyme, blocking its activity (110, 111) (Figure 2 bottom). The β sandwich fold of chagasin is somewhat similar to that of the immunoglobulin fold (110). The three loop arrangement is reminiscent of the three fingertips which, extended, touch and recognize the binding surface. It is thus intriguing to suggest that the congruent binding of cystatin/stefin, p41 fragment and chagasin has exposed the canonical topology of inhibitors of papain-like cysteine proteases, a common solution found by convergent evolution. It should be noted though that besides these three types of inhibitors, other topologies of binding have also been discovered. The propeptide domains of various sizes sit on the primed site of the active site cleft of the protease and cover it by a long polypeptide chain which continues from the catalytic site to the N-terminus of the mature protein (112-115). In addition, staphostatin, a cysteine protease inhibitor from *Staphylococcus Aureus*, binds to its target with the side edge utilizing a beta strand which extends along the whole bottom of the active site cleft (116).

6.2. Oligomerization of cystatins: structural aspects

The structures of chicken cystatin (49, 117), human stefin B in complex with papain (51), human stefin A (118) and human stefin A in complex with cathepsin H (104) have established the new mechanism of interaction between cystatins and papain-like proteases. The new structures of oryzacystatin from rice (119) and human

cystatin D (120) confirmed the typical cystatin fold already established. The unusual crystal structure of human cystatin F was determined in its dimeric form (121). The two monomers interact through an intermolecular disulphide bridge, suggesting that reduction leads to monomer formation and inhibitory activity.

The determination of the crystal structure of the native HCC in its dimeric form (122) was an important contribution to understanding the formation of amyloid, the abnormal fibrillar protein aggregates of γ-trace protein, found in Icelandic patients suffering from hereditary cerebral haemorrhage with amyloidosis (HCHA) (21), now known as hereditary cystatin C amyloid angiopathy (HCCAA). Our discovery that human γ-trace is in fact a protein inhibitor of cysteine cathepsins was of crucial importance for further understanding of this disease (18, 22). A variant of HCC (the L68Q mutant) forms massive amyloid deposits in brain arteries of young adults. The crystal structure of HCC reveals that the dimerization occurs through a three-dimensional domain swapping mechanism to form oligomeric proteins, and suggests the mechanism of its aggregation in the brain arteries of HCCAA patients (122). Oligomerization is greatly accelerated with a naturally occurring L68Q mutant and its N-terminally truncated form, by the first ten amino acid residues of the native HCC, found in the patients. However, the structure of this truncated form of HCC also reveals dimerization with domain swapping, forming the new crystal structure of four independent HCC dimers (123). It was found recently that purified oligomers of HCC fibrillize faster than the monomers (124). The authors suggest that the oligomers and fibrils are formed by propagated domain swapping. The fibril formation occurs in other cystatins such as chicken cystatin (125) and human stefin B under *in vitro* conditions (86, 126). It was shown that the simple domain swapping model proposed for amyloid formation is not compatible with the observed data for human stefin B, in which proline (Pro) isomerization is important in preventing steric clashing (127). Indeed, it had already been reported that the formation of tetramers of stefin B involves a previously unidentified process of intermolecular contacts, termed “hand shaking”, which occurs concurrently with *trans to cis* isomerization of Pro74 (87). This Pro is widely conserved throughout the cystatins, including HCC, a highly amyloidogenic protein, indicating that isomerization of Pro residue can play a crucial role in amyloidogenesis (87).

7. PATHOPHYSIOLOGY

7.1. Cancer

Cysteine cathepsins are involved in the degradation of extracellular matrix, facilitating the growth, invasion and metastasis of tumour cells, and also in tumour angiogenesis (reviewed in 10, 128, 129 - 131). An association of increased cysteine cathepsin activity with angiogenic vasculature and invasive fronts of carcinoma has recently been demostrated (132). Functional down regulation of cathepsin activity was found to correlate with increased expression of the cystatins. Cystatin C (133), cystatin M (45, 134) and stefin A (135) are over expressed in epithelial and mesenchymal tumour cells. In contrast, no cystatin M protein was detected in metastatic mammary epithelial tumour cells (45). The loss of expression of cystatin M may be associated with the progression of a primary tumour to a metastatic phenotype. Higher cathepsins B and L activities were associated with lowered stefin A inhibitory activity in patients with breast carcinoma (136). Similar increases of cathepsin B, H and L activities were detected in sera from patients with breast carcinoma (137). In the cytosol of tumour patients suffering from head and neck carcinoma, the activities of cathepsins B and L correlated significantly with those for stefins A and B (138). The levels of stefins A and B and cystatin C from patients with colorectal cancer correlate with high levels of extracellular inhibitors and with short survival of the patients, thus supporting previous studies suggesting a contributory role of protease inhibitors in the progression of cancer (139). It was proposed that, in patients with malignant diseases, serum cystatin C correlates with the progression of the disease, and not just with glomerular filtration rate (140).

7.2. Neurodegeneration

It is well known that cysteine proteases are implicated in various pathologies of the brain. Alzheimer’s disease and many other neurodegenerative disorders are associated with the accumulation of abnormal protein assemblies in the central nervous system. In contrast there are not many data concerning the role of cystatins in these processes. Only two genetic diseases are known in which mutations in human cystatin C and human stefin B are associated with disease status.

7.2. 1. Hereditary cystatin C amyloid angiopathy (HCCAA)

This is an autosomal dominant disorder characterized by deposition of amyloid, primarily in the central nervous system, although amyloid deposits have also been detected in other organs. This is the first human disorder known to be caused by deposition of cystatin C amyloid fibrils in the walls of the brain arteries, leading to single or multiple strokes with fatal outcome (142). The amyloid deposited is composed mainly of the Leu68Gln variant of cystatin C (143, 144) and is associated with mutation in the cystatin C gene (145). The recommended review is in (146). Very recently, in two consecutive papers published by the same group (147, 148), normal human cystatin C is shown to bind soluble amyloid-β peptide and to inhibit cerebral amyloid deposition in amyloid-β precursor protein (APP) in transgenic mice, thus substantially diminishing amyloid-β deposition. This indicates a protective role for cystatin C in the pathogenesis of Alzheimer’s disease.

7.2.2. Progressive myoclonus epilepsy (EPM1)

This type of epilepsy is exhibited by a group of inherited diseases characterized by myoclonic seizures, generalized epilepsy and progressive neurological deterioration. One of the five major types of this epilepsy is Unverricht-Lundborg disease (EPM1), a rare autosomal recessive disease with onset at the age of 6 to 15 years. Patients later generally develop ataxia and mild dementia.

Genetic analysis revealed that mutations in the gene for stefin B (cystatin B) are responsible for the disease (149, 150). Stefin B inhibitory activity decreased in EPM1 pathogenesis, in contrast to increased activity of cathepsins S and L (151). Very recently, two novel associations of EPM1 with stefins have been reported, one of them involving the conserved QVVAG region in the stefin family that is essential for binding to cathepsins (152).

7. 3. Other diseases and cystatin C clinical diagnostics

There are many other diseases with decreased cystatin levels, such as inflammatory diseases, osteoporosis, arthritis, and diabetes, as well as a number of other neurodegenerative diseases.

It is noteworthy that cystatin C was the first protein to be used in clinical diagnostics. There are over 300 clinical papers concerned with diagnosis of different diseases on the basis of cystatin C. In clinical practice cystatin C is primarily known as a reliable marker for glomerular filtration in kidney dysfunction (153).

8. PERSPECTIVES

The last 25 years have seen a crucial contribution to our understanding of the cystatin superfamily, their related protein inhibitors, and their target cysteine proteinases, including several cathepsins. During this time the basic mechanism of the interaction between protein inhibitors and their cognate enzymes has been established. An important contribution has been made to understanding the role of cystatins in normal biological processes and in diseases such as cardiovascular, inflammatory and infectious diseases, neurodegenerative disorders and cancer. At present, we are witnessing the discovery of new cystatin-related proteins in humans and other organisms, including a variety of pathogenic organisms.

However, although cystatins can inhibit their target enzymes very effectively, they have seldom been used as leading compounds in drug discovery. Instead, proteases and their protein inhibitors can be used as biomarkers for early diagnosis and prognosis, for example in some forms of cancer and in parasitic infections (2, 4, 11, 141). Nowadays, most inhibitor-oriented research in pharmaceutical companies and other laboratories is directed towards small molecule design. Nevertheless, some selective cathepsin B inhibitors have been synthesized, based on the sequence of the propeptide added to an E-64 type inhibitor (154). Similarly, based on the N-terminal sequence RLVG of cystatin C, small molecules have been synthesized that effectively inhibit cathepsin K and bone resorption (155). Further development of small compounds, based on the structures of inhibitors from microorganisms such as E-64 and its analogues, has proved to be a promising approach (156, 157), as exemplified by the crystal structure of cathepsin B in complex with epoxysuccinyl inhibitor CA030, derived from E-64 (158). However, it was recently shown that small fluoromethyl- or chloromethyl-ketone caspase inhibitors inhibit irreversibly most of the cathepsins, and to some extent, legumain (159).

Many new compounds have been synthesized as possible protease blockers and are currently being tested in a number of laboratories. Therefore, new therapies based on protease inhibition can be expected (2, 11, 160). Emerging technologies for high-throughput assays will greatly assist providing the data necessary for profiling proteases in proteomes and for identifying substrates and inhibitors, thereby defining new therapeutic targets (161-163). Only combined interdisciplinary strategies, including translational medicine, will provide the ultimate tools to prevent, control and cure diseases.

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Abbreviations: HCC, human cystatin C; Ki, inhibition constant; Mr, relative molecular mass; pI, isoelectric point

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