Published in final edited form as:

J Health Care Poor Underserved. 2010 February ; 21(1 Suppl): 51–70. doi:10.1353/hpu.0.0257.

Cystatin Superfamily

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Abstract

Cystatins, the classical inhibitors of C1 cysteine proteinases, have been extensively studied and reviewed in the literature. Over the last 20 years, however, proteins containing cystatin domains but lacking protease inhibitory activities have been identified, and most likely more will be described in the near future. These proteins together with family 1, 2, and 3 cystatins constitute the cystatin superfamily. Mounting evidence points to the new roles that some members of the superfamily have acquired over the course of their evolution. This review is focused on the roles of cystatins in: 1) tumorigenesis, 2) stabilization of matrix metalloproteinases, 3) glomerular filtration rate, 4) immunomodulation, and 5) neurodegenerative diseases. It is the goal of this review to get as many investigators as possible to take a second look at the cystatin superfamily regarding their potential involvement in serious human ailments.

Keywords

Cystatin; fetuin-A; metalloproteinases; neurodegenerative; tumorigenesis

Structure-Function relationships of Cystatins

The cystatins are the reversible competitive inhibitors of C1 cysteine proteases. The major cysteine proteases which interact with cystatins include the plant derived papain and the mammalian cathepsins, B, H and L.¹ Since their discovery, the cystatin family has grown and is now a superfamily that can be categorized into three major families. These are: 1) Stefins (stefin A and B; also known as cystatin A and B) that belong to family 1. They are unglycosylated inhibitors of ~11 kDa, lack signal sequence and disulfide bonds and are generally expressed intracellularly. 2) Family 2 (cystatins) have molecular masses in the range of 13–14 kDa, contain signal sequence and disulfide bonds at the carboxy terminus of the molecule. Some members of the family are glycosylated,^{2,3} and the family is represented by cystatin C, D, S, SA, and SN. 3) Family 3 (kininogens) have molecular weights in the range of 88–114 kDa, are glycosylated and have three family-2 cystatin domains, two of which (domains 2 and 3) have protease inhibitory activities (Table 1).

Amino acid sequence alignments of the family 1 cystatins (Stef A and B) show extensive sequence identity (>50%) and similarities. Mutagenesis and X-ray crystallographic studies have unraveled three conserved motifs in cystatins which form a wedge-shaped structure that blocks the active site of C1 cysteine proteases.¹ These are an N-terminal glycine, a glutamine-valine-glycine (Q-X-V-X-G) loop and a second c-terminus hairpin loop consisting of proline-typtophan (PW) residues (Figure 1A). It should, however, be noted that in human stefins, the PW motif is replaced by PG in stefin A and PH in stefin B. These conserved residues are also reflected in the ClustalW alignment⁴ of the amino acid sequences of the family-2 cystatins (Figure 1B).

Although family-1 cystatins share conserved amino acid residues with members of family-2 proteins, their 3-D structures^{5,6} are distinct. Each cystatin structure has a core of five-stranded anti-parallel β -sheets wrapped around a core of a central helix as shown by the ribbon and space filling models of the structures (Figure 2).

Apart from the three established members of the superfamily, there are several other proteins that have been identified that contain cystatin like sequences but lack cysteine protease inhibitory properties and thus represent new families. The evolution of cystatins predicts that archetypal cystatin gave rise to the family-1 cystatins. The introduction of disulfide bonds then gave rise to family-2 members, and then gene duplication events gave rise to family-3 cystatins via a two cystatin domain intermediate.² The two cystatin domain intermediate with inhibitory activities is, however, missing in the puzzle.^{2,7} This 'missing link' would have given rise to a fourth family with new functions (Figure 3). For example, Histidine-rich glycoprotein (HRG) contains two cystatin-like domains and the conserved disulfide bond but are not inhibitory in protease assays.⁷ The physiological function of HRG is not known. On the other hand, a related protein known as fetuin-A,^{7,8} has been shown to be involved in a number of physiological functions including some that it shares with the other cystatin-family members and discussed in this review. Like HRP, bovine fetuin-A and its human homologue alpha 2HS glycoprotein (ahsg) contain two cystatin like domains but lack the cysteine protease inhibitory activities. Sequence similarities among family 3 cystatins, HRG and fetuin-A is depicted in Figure 4 and the dendrogram showing how the cystatin family members are related is depicted in Figure 5.

Other proteins that contain cystatin-like domains but lack the inhibitory activities are the cystatin-related protein (CRP), an androgen-regulated protein expressed in prostate^{9,10} and Spp24, a bone phosphoprotein.¹¹ These duos contain disulfide bonds, but only a single cystatin domain which lack inhibitory activity and may have evolved from a family-2 precursor. Other cystatins that may eventually be classified as a new sub-group are cystatin E/M and F. Although they are capable of the protease inhibitory function, they have low sequence homology with the other family-2 members.^{3,12,13} Even more intriguing is cathelin, which inhibits cathepsin L and papain, but lacks all the three cystatin inhibitory motifs.^{14,15} Yet another sub-group of family-2 proteins is represented by cystatin-related epididymal spermatogenic (CRES) proteins.¹⁶ The CRES proteins contain only the C-terminal PW sequence and lack the Q-X-V-X-G. These differences are reflected on Table 1.

Role of Cystatins in tumorigenesis

It is established that cysteine proteinases play key roles in the progression of a variety of tumors. Tumor progression leading to metastasis involves the destruction and remodeling of the extracellular matrices during local invasion, angiogenesis, intravasation, and extravasation.^{17,18} These processes are facilitated by matrix metalloproteases,¹⁹ serine,²⁰ aspartic,²¹ and cysteine proteases.²⁰ The prototype cysteine protease which plays a key role in tumor cell invasion is cathepsin B.^{22,23} Even though the cathepsins are generally regarded as intracellular proteases in normal cells, they are expressed extracellularly on the surface of tumor cells. The enzymes have been shown to be concentrated in the leading edge of motile metastatic cells where they dissolve the ECM proteins to pave the way for the movement of the cells.^{24,25} It is in this micro-environment that the activity of cysteine proteases are inhibited by cystatins, particularly Cyst C, which is also present extracellularly.²⁶

The expression of these proteinases and their natural inhibitors *in vivo* are under tight regulatory controls for obvious reasons.^{27–29} For example, a number of studies have suggested an inverse relationship between the stage of tumor progression and the levels of cystatins in the tumor microenvironment. As tumors progress towards the metastatic end stage, the levels of the cystatins in both the cytosol and extracellular spaces are drastically reduced.^{30,31} In addition,

studies have demonstrated a direct correlation between high cystatin C levels and improved tumor prognosis.²⁶

The mechanisms by which cystatins modulate tumorigenicity are not only attributed to their inhibitory roles against cysteine proteinases. Studies by Sokol et al.³² identified Cyst C as a novel antagonist of TGF- β signaling. They determined that Cyst C physically interacts with TGF- β receptor II, thereby abrogating the binding of TGF- β . It is known that TGF- β has growth suppressing properties, particularly in normal epithelial cells,^{17–19} but that during tumor progression the tumor suppressing properties of TGF- β is frequently subverted, and it becomes a powerful progression factor for the transformed cells.^{33,34} The ability of Cyst C to down-regulate the growth promoting properties of TGF- β is very provocative in view of the fact that ahsg (a family-3 cystatin) has also been shown to down regulate tumorigenesis, presumably by a similar mechanism. Swallow et al.,³⁵ in elegant experiments using ahsg mutant mice, showed that this family-3 cystatin down-regulates colon carcinogenesis by antagonizing TGF- β activity. Moreover, two other notable studies, by Zhang et al.³⁶ and Hsu et al.³⁷ have demonstrated that cystatin M and fetuin-B (family-3 cystatin) also act as tumor suppressor proteins in lung and skin tumors, respectively.

Assuming that cystatins generally suppress tumorigenicity, it is difficult to explain why sera of patients with melanoma and colorectal cancers contain more Cyst C than normal volunteers.^{28,38} Experimentally, when syngeneic tumor cells were inoculated subcutaneously into Cyst C wild-type and null mice, no difference in the latency and growth of the primary tumors were observed in the two groups of mice. However, lung colonization by the same syngeneic cells following experimental metastasis protocol was suppressed in Cyst C-deficient mice in comparison with their wild-type counterparts.³⁹ We have recently demonstrated that fetuin-A is a potent promoter of tumor cell growth both *in vitro* and *in vivo*.⁴⁰ The role of fetuin-A in cell growth, particularly *in vitro*, has been controversial, to say the least.⁴¹ Its ability to associate with a number of adhesive proteins and growth factors fueled this controversy because it was difficult to delineate whether the growth promotion was due to fetuin-A *per se* or the co-purifying proteins. We have demonstrated that the highly malignant Lewis lung carcinoma cells have reduced growth potential in fetuin-A null C57BL/6 mice while they rapidly and substantially colonize the lungs, livers, and subcutaneous tissues of the wild type animals.⁴⁰ The data suggest that in highly tumorigenic cells, the growth promoting potential of fetuin-A far exceeds its ability to downregulate the effects of TGF- β . Therefore, depending on the particular circumstances, cystatin family members may provide both favorable and unfavorable microenvironments for tumor growth.

Stabilization of Matrix Metalloproteinases by Cystatins

Matrix metalloproteinases (MMPs) are homologous Zn²⁺-dependent proteinases that participate in the physiological and pathological processes, which require tissue remodeling.⁴² Their expression profiles are highly regulated and defects in their regulatory pathways are likely to give rise to diseases ranging from cancer, arthritis, and cardiovascular disorders to periodontal diseases.^{43–46} Matrix metalloproteinases, like other proteinases, are secreted as zymogens and assume their activities in the extracellular milieu after activation processes.⁴⁷ For some MMPs such as MMP-9, the activation process is mediated by other MMPs.⁴⁸ Once activated, several proteinases including matrix metalloproteinases have the capacity for autolytic inactivation when they are in their active state.^{49,50} There are key mechanisms that have evolved *in vivo* to regulate the activities of these enzymes and prevent their autolytic inactivation. Tissue inhibitors of matrix metalloproteinases (TIMPs), the natural inhibitors of MMPs, interact with the enzymes *in vivo* and inhibit their activities. In the absence of these inhibitors, the activated enzymes if left unchecked, may turn against themselves and undergo autolysis. Therefore, under physiological conditions, which favor the active state of the

enzymes and limited expression of the TIMPs, matrix metalloproteinases are unstable and can easily undergo autolysis unless some other protective mechanisms are in place.

Studies in our laboratory and others, have determined that apart from TIMPs, members of the cystatin superfamily can stabilize and protect matrix metalloproteinases without affecting their activities towards their natural substrates, such as gelatin.^{50,51} Purified MMP-2 and MMP-9 in their active state are rapidly inactivated within hours unless they are in the presence of any of the cystatin family members or TIMPs. The MMPs, particularly MMP-9, interact with cystatins with dissociation constants in the range of 25 nM to 1.9 μ M. However, the ability of fetuin-A to protect MMP-9 from autolysis requires a molar ratio of at least 8:1 (fetuin-A:MMP-9). Interestingly, the molar ratio of fetuin-A: MMP-9 *in vivo*, far exceeds the minimal ratio of [8:1].⁵⁰ This is also true for other members of the family.⁵⁰

Cystatins do occur in all body compartments and fluids where matrix metalloproteinases are present. These include blood, urine, saliva, and cerebrospinal, seminal, and synovial fluids. The enzymes in these compartments are protected from autolytic degradation but maintain the capacity to digest their preferred substrates including a myriad of proteins that they modify *in vivo* such as galectin-3.⁵² We therefore envision an *in vivo* scenario where MMPs, TIMPs, cystatins, and other proteases interact with each other to regulate extracellular remodeling and to proteolytically modify a host of housekeeping proteins in both normal and pathophysiological conditions. For example, Mai et al.⁵³ recently demonstrated that cathepsin B is present on the surface of tumor cells where it co-localizes with annexin-2 tetramer. Annexin-2 tetramer also serves as a receptor for tPA, plasminogen, tenascin C, and plasmin.^{54,55} Additionally, other studies have also demonstrated that annexin-2 on the cell surface can serve as receptor for fetuin-A.^{56,57} Therefore matrix metalloproteinases, which normally co-localize with fetuin-A,^{58–60} are likely to be part of this annexin-2 tetramer complex on the surface of tumor cells. The cystatins in these complexes may inhibit the activity of cathepsin-B while allowing the activity of matrix metalloproteinases.

Role of Cystatin C as a New Marker of Glomerular Filtration rate

For many years, serum creatinine has been the benchmark marker for the renal function assay also known as glomerular filtration rate (GFR).^{61,62} The ideal marker for GFR should be produced endogenously and at a constant rate, regardless of age, sex, weight, and disease state. It should be filtered and excreted by the kidney only without renal tubular secretion and reabsorption, and once in urine it should be stable to allow for later analysis. Although creatinine meets some of these conditions, it has a number of limitations. Creatinine is produced in muscle, and the amount produced depends on muscle mass. It does not bind plasma proteins, and is freely filtered by the kidney although it is also secreted by the renal tubules. One major drawback for creatinine as a marker for GFR is that it is not a sensitive marker of early GFR, necessitating the collection of timed urinary samples and using a formula that requires measurement of serum and urinary forms of creatinine. Rhabdomyolysis and eating uncooked meats can dramatically raise serum creatinine, which can skew the readings of urinary creatinine. Because of these drawbacks related to creatinine, other endogenous markers for GFR have been evaluated. So far, the most promising marker which can potentially replace creatinine is cystatin C.

Cyst C has proven to be a suitable marker because it satisfies most of the above established criteria. Simonsen et al.⁶³ first noted the excellent correlation between Cyst C secretion and GFR when compared with the gold standard exogenous markers of GFR, such as⁵¹ Cr-EDTA. Cyst C is present in high concentrations in serum, saliva, and seminal, synovial, and cerebrospinal fluids.⁶⁴ It is produced and secreted at a constant rate by most nucleated cells and is freely filtered by the glomerular because of its small size. Unlike creatinine, serum Cyst

C is not secreted by renal tubular epithelial cells, although they reabsorb and catabolyze it so that Cyst C does not return to the bloodstream.⁶⁵

Immunomodulatory Properties of Cystatins

Fetuin-A and its human homologue (ahsg) are defined as negative acute-phase proteins. Normal circulating levels of the proteins ~0.5 mg/ml fall significantly (by approximately 40%) during injury and infection.^{66,67} These cystatins have been shown to be immunomodulators that can mediate bacterial phagocytosis by neutrophils⁶⁸ and promotion of endocytosis by mouse macrophages.^{69,70} Studies by Wang et al.⁷¹ demonstrated that spermine, a ubiquitous biorganic polyamine that accumulates at sites of injury or inflammation, inhibits macrophage cytokine synthesis only in the presence of fetuin-A. Thus, during inflammation, fetuin-A down-regulates the production of pro-inflammatory cytokines and prevents excessive inflammation.^{71,72} Another study by this group determined that fetuin-A modulates macrophage deactivation by opsonizing both endogenous (spermine) and therapeutically administered cationic cytokine synthesis inhibitors (CNI-1493) that restrain the innate immune response.⁷³

Apart from the fetuin-A, family-2 cystatin members have also been shown to be important immunomodulatory proteins. For example, during inflammatory processes, cystatin C release is down-regulated, contributing to increased cysteine protease activities in the macrophage microenvironment.⁷⁴ Cyst C is a powerful inhibitor of cathepsins S and L. High levels of Cyst C are detectable in class II-positive lysosomes of immature dendritic cells (DCs) and Langerhans cells. The maturation process of DCs leads to reduced levels of cyst C, and allows the up-regulation of cysteine proteases cathepsins L and S. For this reason, it has been suggested that Cyst C plays a role in the intracellular control of invariant chain (Ii) degradation and antigen presentation.⁷⁵ One of the initial steps of an antigen-specific T cell response to external antigens, is the formation of peptide-MHC class II complexes in antigen presenting cells (APCs) such as macrophages, DCs, and T cells. In this process, cysteine proteases play a key role in two important processes. Firstly, they degrade the proteins within the endosomal-lysosomal compartments of APCs. The resulting peptides then bind to MHC class II molecules that are later targeted to the cell surface. Secondly, cysteine proteases are involved in the cleavage of MHC class II-associated Ii, leading to the formation of clip associated MHC-molecules. The generation of clip that binds to the antigen-binding groove of MHC class II molecules subsequently allows the binding of peptides to the MHC class II molecule. It has been documented that cathepsin S promotes the generation of clip fragment in B cells and DCs,⁷⁶ while cathepsin L is active in thymus epithelial cells^{77,78} and cathepsin F in macrophages.

The inhibition of immunologically relevant cysteine proteases by cystatins secreted by parasites such as filarial nematodes, could lead to drastic changes in antigen processing and presentation by APCs of the host. Apart from their ability to inhibit proteases, cystatins emanating from parasitic nematodes can also affect the production of cytokines by host macrophages, resulting in anti-inflammatory responses. For example, it was demonstrated by Schonemeyer et al.⁷⁹ that *Onchocerca volvulus* cystatin induces early TNF-alpha response in human peripheral blood mononuclear cells (PBMC), followed by a down-regulation of the IL-12 production and a massive increase in IL-10 production by monocytes. High levels of IL-10 are characteristic for filarial worm infection, and increased IL-10 production of unstimulated or antigen-stimulated PBMC of lymphatic filariasis patients coincides with T Cell hypoactivity.

Members of the three cystatin families have been shown to up-regulate the release of nitric oxide (NO) from IFN-gamma activated macrophages.^{80,81} This feature applies only to natural cysteine protease inhibitors, as synthetic inhibitors are unable to up-regulate NO productions.⁸² The inducible NOS (iNOS) is induced by IFN-gamma and accounts for rapid production of

large quantities of NO.⁸³ The increased NO production by chicken cystatin was demonstrated to be potent enough to cure mice from potentially lethal visceral Leishmaniasis.⁸⁴ Interestingly, NO has been demonstrated to induce a strong inhibition of lymphocyte proliferation *in vitro* and to modulate cytokine gene expression in various cell types.^{85,86} It is important to note that cystatin induced up-regulation of NO release by activated macrophages is stimulated via the synthesis of TNF-alpha and IL-10 and not due to the conserved inhibitory domains (QXVXG). We conclude that while the cystatins produced by the parasites ensure their intracellular survival in the host cells, the host cysteine proteases speed up the antigen presentation events culminating in the elimination of the parasite by the host immune system.

Role of Cystatins in Neurodegenerative Diseases

Cerebrovascular amyloid deposition (CAA) is a disease characterized by amyloid protein deposition in the blood vessels of the brain. Severe forms of the disease may cause cerebrovascular disorders such as lobar cerebral hemorrhage and leukoencephalopathy and may present with dementia such as that observed in Alzheimer's disease (AD).⁸⁷ Several cerebrovascular amyloid proteins have been characterized and include amyloid- β -protein type (A β), cystatin C, prion protein, variant transthyretins (ATTR) in meningoarteriosclerosis, mutated gelsolin (AGE1) in familial amyloidosis of Finnish type, disease associated prion protein (PrP(Sc)) in a variant of the Gerstmann-Strausler-Scheinker syndrome.^{87,88} Among the several types of CAA, that of the A β type is the most commonly found in elderly individuals and in patients with AD and mutations found in the genes encoding amyloid precursor proteins are associated with hereditary CAA.⁸⁷

Studies have shown cystatin C to be immunohistochemically co-localized with A β in sporadic CAA^{89,90} and to co-immunoprecipitate with amyloid-beta precursor protein.⁹¹ However, in such cases examined to date, cystatin C has not been shown to be an intrinsic component of the amyloid fibrils and more definitive studies must be conducted to elucidate the significance of the immunohistochemical co-localization of cystatin C. The association of cystatin C with A β in AD patients may have a hitherto unexplored role. As we have discussed above, members of the cystatin superfamily can stabilize and protect matrix metalloproteinase from autolytic degradation. These enzymes have also been shown to associate with A β proteins and can specifically cleave them.^{92–94} This cleavage or degradation may modulate or increase the clearance of the amyloid bodies, thereby reducing their detrimental effects in AD patients. In support of this notion, Mendes Sousa et al.⁹⁵ have demonstrated an up-regulation of gelatinase-associated lipocalin and matrix metalloproteinase-9 in familial amyloid polyneuropathy. Interestingly, lipocalin—which is distantly related to the cystatin family—has been shown to stabilize and protect MMP-9 from autolytic degradation.⁹⁶

Apart from simply being co-localized with A β in the brain, there are studies showing that cystatin C can also polymerize and give rise to amyloid bodies. In hereditary cerebral hemorrhage with amyloidosis icelandic-type (HCHWA-1), an autosomal dominant disorder in icelanders, cystatin C is directly involved in the pathogenesis of CAA. This disorder is associated with a (68Leu—Gln) mutation and a loss of 10 amino acids in the N-terminus of the cystatin C.^{97,98}

Concerning the formation of A β fibrils in the brain, it is generally believed that globular amyloidogenic proteins partially unfold and then switch into alternatively folded conformations to self assemble into fibrils (amyloid deposits).^{99,100} More specifically, cystatins and other amyloidogenic proteins have been shown to participate in domain-swapping, which leads to formation of stable dimers and eventually stable fibrils in amyloidogenic deposits.^{101,102} Domain-swapping, as the name implies, is a process in which a domain in a protein breaks its non-covalent bonds with the rest of the molecule and has its

place taken by the same domain of a second molecule. The theoretical consideration of domain-swapping in cystatins began with the studies of Ekiel and Abrahamson,¹⁰³ where they demonstrated that human cystatin C had the propensity to form inactive dimers under pre-denaturing conditions. Subsequent studies involving human stefins confirmed the initial observations.^{104,105} For each of the cystatins, NMR chemical shift changes indicated that dimerization involved no structural rearrangement of the main fold. There were, however, rearrangements in the active site regions of the molecules. The rather slow kinetics and high activation energy of dimerization were suggestive of domain-swapping instead of simple association.^{104,106}

It is possible that the behavior of domain-swapping is shared by other members of the cystatin superfamily including fetuin-A, which can also form aggregates under favorable conditions as demonstrated in our laboratory (unpublished information). Moreover, conditions that favor domain-swapping are also ideal for amyloidogenesis.¹⁰¹ For normal cystatin molecules, the phenomenon does not occur readily under physiological conditions but human cystatin C with the L68Q mutation, dimerizes readily under physiological conditions. Thus, certain mutations destabilize the monomeric structures, making it possible for the mutant proteins to form dimers readily by the domain-swapping mechanism.^{101,102}

Other than amyloidogenesis, cystatin C has been shown to be associated with multiple sclerosis (MS), another neurodegenerative disease. Studies have demonstrated that the level of cystatin C is highly reduced in the cerebrospinal fluid of individuals suffering from MS compared with healthy controls.¹⁰⁷ The reduced levels of cystatin C in CSF of these patients was accompanied by increased activity of cysteine proteases in the fluid compared with normal subjects, pointing to the role of this cysteine proteinase inhibitor in the pathogenesis of MS. A possible role of cystatin C in MS therefore is to modulate the activities of cathepsin B and possibly other cysteine proteases and is not an active participant in the pathogenesis of the disease.

In other studies of patients with inflammatory neurological disorders, such as Guillain-Barre syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy, levels of cystatin C were shown to be consistently lower in the CSF compared with control subjects.¹⁰⁸ In the neurodegenerative disorder known as progressive myoclonus epilepsy of Unverricht-Lundborg type (EPMI), a significant reduction in cystatin B activity was recorded in lymphoblastoid cells of EPMI.¹⁰⁹ Concomitant to the decreased level of cystatin B, levels of cathepsin B, L, and S shot up significantly, as expected. Furthermore, mice with disruptions in the cystatin-B gene (loss of function mutation) display myoclonic seizures, progressive ataxia and cerebellar pathology, which closely parallels EPMI in humans.¹¹⁰

Conclusion

In summary, cystatins are at the heart of a number of normal and pathological conditions, and should not be regarded merely as inhibitors of C1 cysteine proteases. Particularly their roles in tumorigenesis and neurodegenerative diseases should be given increased attention, since we now have animal models for mechanistic studies. It is also our hope that in future more proteins with cystatin-like domains will be identified and their physiological roles described.

Acknowledgments

This work was supported by NIH grants IU54 CA091408 (J.O. and G.C.).

Notes

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A. Family I Cystatins (Intracellular)

CystatinA **IP**GLSEAKPATPEI**Q**EV**K**K**Q**LEEK**T**NETY**G**KLE**AV**Q**Y**K**T**Q**V**VAGT**N**Y**I**K
 CystatinB **MC**GAP**S**AT**Q**PAT**A**ET**Q**H**I**AD**Q**RS**Q**LEEK**E**N**K**K**F**P**V**F**K**AV**S**F**S**Q**V**VAGT**N**Y**F**I**K**
 *: * . * : : * * * . * . * . * : : * * * * * : : * : * : * * * * * : *

CystatinA **N**K**Y**M**H**L**K**V**F**K**S**L**P**G**Q**N**E**D**L**V**I**T**G**Y**Q**V**D**K**N**D**E**L**T**G**F**
 CystatinB **E**D**F**V**H**I**R**V**F**Q**S**L**P**H**E**N**K**P**L**T**I**S**N**Y**Q**T**N**K**A**K**H**D**E**L**T**Y**F**
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B. Family II Cystatins (Extracellular)

CystatinC ---MAGPLRAPLLLLLAILAVALAVSPAAGSSPGKPPRLV**G**PM**D**AS**V**EE**E**G**G**RR**L**
 CystatinD ---MMWPMHTPLLLLTALMVAVAGSASAQSR----TL**A**G**I**H**A**D**L**DN**D**K**S**VR**L**
 CystatinE MARS**N**L**P**L**A**L**G**L**A**V**F**C**L**L**A**L**P**R**D**A**R**A**R**P**Q**E---RMV**G**EL**R**DL**S**PD**D**P**Q**V**K**A
 CystatinF ---MRAAGTL**A**F**C**CL**V**L**S**T**T**G**G**P**S**P**D**T**C**S**Q**DL**S**R**V**K**P**C**F**P**K**T**I**K**T**ND**P**G**V**L**Q**A
 CystatinM MARS**N**L**P**L**A**L**G**L**A**V**F**C**L**L**A**L**P**R**D**A**R**A**R**P**Q**E---RMV**G**EL**R**DL**S**PD**D**P**Q**V**K**A
 CystatinS ---MAR**P**CT**L**LL**L**MA**T**LAG**A**L**A**SS**S**KE**E**N**R**----I**P**G**G**I**Y**D**A**DL**N**DE**W**VR**L**
 CystatinSA ---MAW**P**CT**L**LL**L**LA**T**Q**A**VAL**A**W**S**P**Q**E**D**R----I**E**G**G**I**Y**D**A**DL**N**DE**W**VR**L**
 CystatinSN ---MA**Q**HL**S**T**L**LL**L**LA**T**VAL**A**W**S**P**K**E**D**R----I**P**G**G**I**Y**N**A**DL**N**DE**W**VR**L**
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CystatinC GEY**N**K**A**S-ND**M**Y**H**S**R**AL**Q**V**V**R**A**R**K**C**I**V**A**G**V**N**I**FL**D**VE**L**GR**I**T**C**T**T**Q**P**N-L**N**CP**F**
 CystatinD SEY**N**K**V**IN**K**DE**Y**S**R**PL**Q**V**M**A**Y**Q**C**I**V**G**G**V**N**I**F**N**V**K**F**GR**I**T**C**T**T**Q**P**N-L**N**CP**F**
 CystatinE ASY**N**M**G**S-NS**I**Y**F**RD**T**H**I**I**K**Q**S**L**V**A**G**I**K**Y**F**LT**M**EM**G**S**I**DR**T**RV**T**-G**H**VD**L**
 CystatinF EK**F**N**N**CT-ND**M**FL**F**K**E**S**R**IT**R**AL**V**C**I**V**K**GL**K**Y**M**LE**V**E**I**GR**I**T**C**K**N**Q**H**LR**L**DC**D**F
 CystatinM ASY**N**M**G**S-NS**I**Y**F**RD**T**H**I**I**K**Q**S**L**V**A**G**I**K**Y**F**LT**M**EM**G**S**I**DR**T**RV**T**-G**H**VD**L**
 CystatinS SEY**N**K**A**T-EDE**Y**Y**R**RL**Q**VL**R**ARE**C**TF**G**V**N**I**F**D**V**EV**G**RI**T**Q**P**N-L**T**CA**F**
 CystatinSA SEY**N**K**A**T-EDE**Y**Y**R**RL**Q**VL**R**ARE**C**I**V**G**G**V**N**I**F**D**I**EV**G**RI**T**Q**P**N-L**T**CA**F**
 CystatinSN SEY**N**K**A**T-KDD**Y**Y**R**RL**Q**VL**R**AR**Q**TV**G**V**N**I**F**D**V**EV**G**RI**T**Q**P**N-L**T**CA**F**
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CystatinC HLK---RK**A**F**S**F**Q**I**Y**A**V**P**W**Q**G**T**M**TL**S**K**S**T**C**Q**D**A
 CystatinD KLK---EE**E**F**S**F**Q**I**N**E**V**P**W**ED**K**IS**I**L**N**Y**K**CR**K**V
 CystatinE LAAG**A**Q**Q**E**K**LR**D**FE**V**LV**V**P**W**Q**N**SS**Q**LL**K**H**N**C**V**Q**M**
 CystatinF TLK---Q**T**LS**Y**SE**V**W**V****V**P**W**L**Q**H**F**EV**P**VL**R**CH--
 CystatinM LAAG**A**Q**Q**E**K**LR**D**FE**V**LV**V**P**W**Q**N**SS**Q**LL**K**H**N**C**V**Q**M**
 CystatinS ELQ---KK**Q**L**S**F**E**I**Y**E**V**P**W**ED**R**MS**L**V**N**S**R**C**Q**E**A**
 CystatinSA ELQ---KK**Q**L**S**F**Q**I**Y**E**V**P**W**ED**R**MS**L**V**N**S**R**C**Q**E--
 CystatinSN ELQ---KK**Q**L**S**F**E**I**Y**E**V**P**W**EN**R**RS**L**V**K**S**R**C**Q**ES
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Figure 1.

Figure 2.

Figure 3.

FetuinA -MKSIVLLLLCLAQWGCHSAPHGPGLIYRQPNCDDEPETEEAALVVIDYINQNLPW
FetuinB MGLLLPLALCILVLCCGKSPQQLALNPSALLSR--GCNDSVLA VAGFALRDIKDRKD
HRGP -----MKALIAALLLITQYSCAVSP-----TDCSAVEPEAEKALDLIKRRRD
Kininogen1 -----MKLITILFLCSRLLSLTQESQSEE----IDCNDKDLFKA VDAALKKYSQNQS

: : * : . *: *.

FetuinA GYKHTINQIDEVKVWPQQPSGELFEIEIDTLETTCHVLDPTPVARCS---VRQLKEHAVE
FetuinB GYVLRINRVNDAQEYRRGGLGSLFYLTLDVLETDCHVLRKKA WQDCG---MRIFVESVYG
HRGP GYLFQLLRIADAHLD RVEN-TTVYYLVLDVQESDCSVLSRKYWNDCPPDSRRPSEIVIG
Kininogen1 NNQFVLYRITEATKT--VGSDTFYSFKYEIKEGDCPVQSGKTWQDCG---YKDAAKAAT

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FetuinA GDCDFQLLKLKGKFSVVYAK--CDSSPDSAEDVRKVCQDCPLLAPLN--DTRVVHAAKAA
FetuinB -QCKAIFYMNNPSRVLYLAAYNCTLRPVSKKKIYMTCPDCPSSIPTDSSNHQVLEAATES
HRGP QCKVIATRSHESQDLRVIDFNCTTSSVSS--ALANTKDSPVLIDFFEDTERYRKQANKA
Kininogen1 GECTATVGK-RSSTKFSVATQTCQITPAEGPVVTAQYDCLGCVHPISTQSPDLEPILRHG

. . * . .

FetuinA LAAFNAQNN-GSNFQLEEISRAQ-LVPLP-PSTYVEFTVSGTDCVAK---EATEAAKCNL
FetuinB LAKYNNENT-SKQYSLFKVTRAS-SQWVVGPSYFVEYLIKESPTKS---QASSCSLQ-S
HRGP LEKYKEENDDFASFRVDRIEVARVRGGEGTGYFVDFSVRNCPRHHF---PRHPNVFGFC
Kininogen1 IQYFNNNTQHSSLFMLEVKRAQ-RQVVAGLNFRITYSIVQTNCSKENFLFLTPDCKSLW

: :: :. : : : *. . : : :

FetuinA LAEKQYGFCKATLSEKLGGAEVAVTCTVFQTQPVTSQ-----
FetuinB SDSVPVGLCKGSLRTHWEKFVSVTCDFFESQAPATG-----
HRGP RADLFYDVEALDLES---PKNLVINCEVFDPQEHENINGVPPHLGHPFHGGHERSSTTK
Kininogen1 NGDTGECTDNAYIDIQLRIASFSONCDIYPGKDFVQP-----

. : . *. : :

FetuinA PQPEGAN-EAVPTVVDPDAPP-SPPLGAPG-LPPAG---SPPD SHVLLA-----
FetuinB SENS AVNQKPTNLEKVEESQKNTPTDPSKAGPRGSVQYLPD LDDKNS-----
HRGP PPFKPHGSRDHHHPHKPHEHGPPPPDERDHSHPPLPQGPPLLPMS C SSCQHATFGTN
Kininogen1 -PTKICVGCPRDITNSPELEETLHTITIKLNAENNATFYFKIDNVKKARVQVVAG----

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FetuinA -----APPGHQLHR-AHYDLRHTFMGVV
FetuinB -----QEKGPQEAFPVHLDLTTNPQGET
HRGP GAQRHSHNNSSDLHPKHHSHEQHPHGHHPHAHHPHEHDTHRQHPHGHHPHGHHPHGHH
Kininogen1 -----KKYFIDFVARETTCSKESNEELTESCETKK

:

FetuinA SLGSP-----
FetuinB LDIS-----
HRGP PHGHHPHGHHPHCHDFQDYGPCDPPPHNQGHCCGHGPPPGHLRRRGPGKGPRPFHCRQI
Kininogen1 LGQSLDCN-----AEV

FetuinA SGEVSHPRKTRTVVQPSVGAAAGPVVP**P**CPGRIRHFKV-----
FetuinB -FLFLEPMEEKLVVLPFPKEKARTAEC**P**GPAQNASPLVLPP-----
HPRG GSVYRLPPLRKGEVLPLPEANFPSFPL**P**HHKHPLKPDNQPFQSVSESCPGKFKSGFPQV
Kininogen1 YVVPWEKKIYPTVNCQPLGMISLMKR**P**GFSPFRSSRIGEIKEETTSHLRSCEYKGRPPK

*

FetuinA -----
FetuinB -----
HPRG SMFFTHTFPK--
Kininogen1 AGAEPASE

Figure 4.

Figure 5.

Table 1

LE 1, THE CYSTATIN INHIBITORY MOTIFS IN THE DOMAINS OF THE VARIOUS CYSTATIN FAMILIES AND SUB-GROUPS

Cystatin-family group	Inhibitory sequence motif
Family 1 (stefins)	D1 (G—Q-V-G—PW)
Family 2 (Cystatins)	D1 (G—Q-V-G—PW; S-S bonds present)
Family 3 (Kininogens)	D1 (empty); D2 (G—Q-V-G); D3 (G—Q-V-G—PW); S-S
HRG (histidine-rich glycoprotein)	D1 (empty); D2 (empty); S-S bonds
Fetuiins (ahsg)	D1 (empty); D2 (empty); S-S bonds
CRP (cystatin-related protein)	D1 (empty); S-S bonds
Spp24	D1 (empty); S-S bonds
CRES (cystatin-related epididymal spermatogenic)	D1 (Q-X-X—PW); S-S bonds