Chapter 18
Post-translational Proteolytic Processing on Intracellular Proteins by Cathepsins and Cystatins

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18.1 Antigen Processing and Proteolytic Modification of Biologically Active Proteins by Cathepsins in Health and Diseases

18.1.1 The Importance of Sequential Limited Proteolysis in the Post-translational Processing of Proteins

Almost all proteins are synthesized from pre-pro-proteins and then processed to biologically active mature proteins. This processing is performed in specific intracellular locations by specific proteinases and, therefore, specific cathepsins play important roles in this respect. Here we show an interesting abnormal processing of serum albumin, called abnormal proalbuminemia Tokushima (Matsuda 1986). This inborn error of metabolism was found in a specific family in Tokushima. The metabolic disorder patients showed a weak resistance for heavy metal intoxication. This abnormal proalbumina shows Arg·Gly·Val·Phe·*His*·Arg/Asp·Ala – , while normal proalbumin shows Arg·Gly·Val·Phe·*Arg*·Arg/Asp·Ala – as shown in Fig. 18.1. Therefore, the processing protease can not cleave off the proalbumin part and, therefore, this abnormal proalbumin variant is secreted into the serum. Interestingly, the same abnormal proalbuminemia was found in Lille in France, suggesting a genealogical connection.

Also, we found that the intracellular degradation of cathepsins themselves are initiated from the nicking of a special ordered bond in the lysosome, such as the peptide bond after the 47th amino acid in the case of mature cathepsin B, the peptide bond after the 177th amino acid in mature cathepsin H, and the peptide bond after

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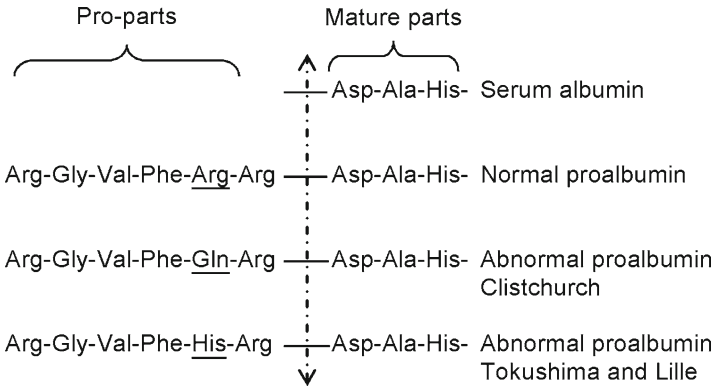


Fig. 18.1 Abnormal amino acid sequence of the pro-parts of albumin in abnormal proalbuminemia

the 178th in mature cathepsin L. Therefore, the intracellular degradation of biologically active proteins and enzymes occurs via ordered limited proteolysis processes in relevant working organelles.

18.1.2 Functional Shear of Individual Cathepsins from Their Morphological Aspects

From the enzyme chemical aspects, each cathepsin has a different cleavage bond to make different product peptides. Furthermore, cathepsins B, H, and L are located in different parts of hepatocytes; these differences are apparent when hepatocytes are stained using antibodies to each cathepsin, as shown in Fig. 18.2. Each cathepsin is located in different lysosome particles, as shown by double immunocytochemical staining with different sizes of gold particles, using an electron microscopic technique (Fig. 18.3). Cathepsin B and cathepsin H in the islet cells of the pancreas are located in separate lysosomes, so a selective incorporation mechanism of substrate proteins is very important. The mechanisms of post-translocational proteolytic processing are not simple and the selective incorporation mechanism of substrate proteins is still unknown.

18.1.3 Antigen Processing by Lysosomal Cathepsins

Allergies and immunity are expressed through the antigen-antibody reaction. Antibody production is initiated by antigen processing by cathepsins in antigen-presenting cells, such as macrophages and dendritic cells. Proteins as an

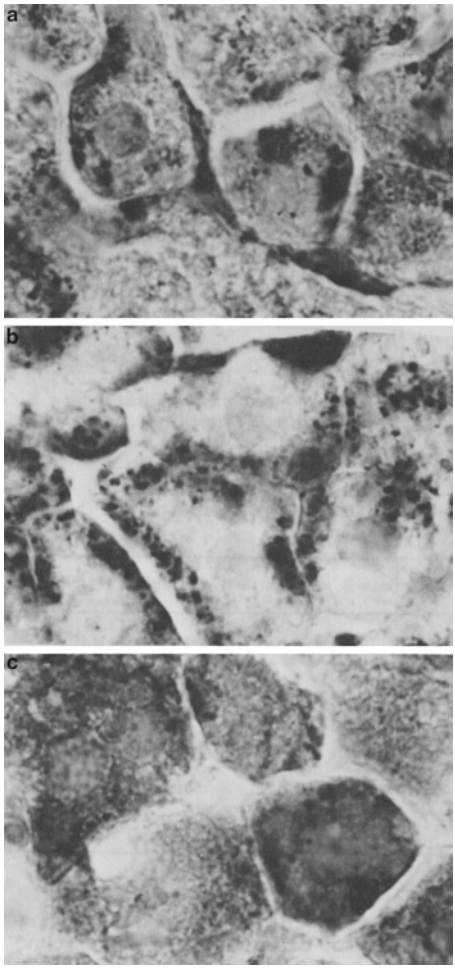


Fig. 18.2 Different intracellular localizations of cathepsin B (*upper*), cathepsin H (*center*), and cathepsin L (*lower*) in hepatocytes, determined with antibodies

antigen are processed to a 15-mer antigenic peptide (epitope) by various cathepsins in the lysosomes and then the processed signal peptides (epitope) are presented to MHC class II in the cells. These signals are further transduced to helper T lymphocytes and then their signals are transduced to B-lymphocytes to produce the antibodies. The classes of antibodies to be produced are determined by the signals of the presented epitopes. Therefore, lysosomal cathepsins play an important role in the class-switching of antibody production. The type of cathepsin determines the kind of antibodies to be formed (Katunuma 1997, 2003).

Cysteine cathepsins are a protease family located in lysosomes; eleven kinds of cysteine cathepsins are presently registered in the human genome database (Turk

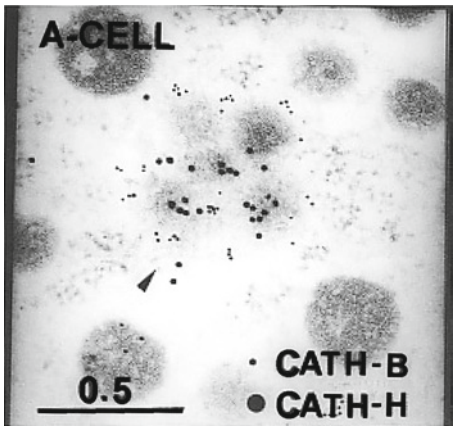


Fig. 18.3 Electron-microscopy showing double immunostaining of cathepsins B and H labeled with gold particles in islet cells of the pancreas

et al. 2001). Lysosomal cathepsins play a role not only in general protein catabolism, but also in the production of bioactive proteins and peptides by their limited proteolysis. One of their most important roles is antigen processing by limited proteolysis.

Each cathepsin has an individual susceptible bond on the substrate proteins; different cathepsins produce different individual biologically active peptides. As a result, the different expressions of cathepsins induce individual physiological or pathological conditions. Therefore, the development of specific inhibitors for individual cathepsins or the development of knockout mice lacking genes for individual cathepsins are essential to analyze the individual roles of cathepsins and to clarify the pathogenesis of the specific diseases mediated by the abnormal expression of cathepsins.

18.1.4 Development of Specific Inhibitors for Individual Cathepsins to Analyze and Regulate the Post-translational Processing

The development of specific inhibitors for the cathepsin family began with the discovery of two kinds of natural cathepsin inhibitors from soil bacteria (Pedolobacteria). As Fig. 18.4 shows, the first group contains amino-acid derivatives of aliphatic aldehydes, such as leupeptin and antipain, that make a thioether bond with the SH-Cys active site of cysteine proteases (cathepsins); those were developed by Umezawa's group (Umezawa 1972). The second group contains amino-acid derivatives of epoxysuccinate, such as E-64, that make a thiohemiacetal bond with the SH-Cys active site of cathepsins; this was discovered by Hanada et al. (1978) and developed as a cathepsin inhibitor by Katunuma's group (Hashida et al. 1980; Hara et al. 1988). These inhibitors were specific for the cysteine protease

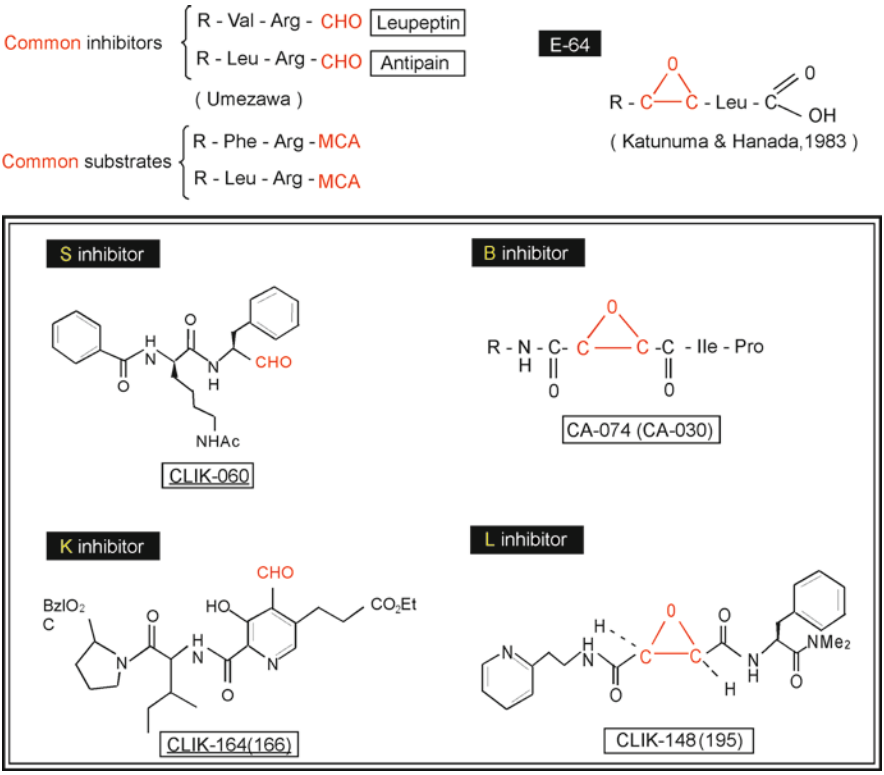


Fig. 18.4 Design of specific inhibitors for individual cysteine cathepsins. Epoxysuccinate or aldehyde serves as the active site to be bound with the active thiol group of cathepsins

group, but unfortunately, not specific for individual cathepsins. Using these two frame compounds as the active site of inhibitors, we designed compounds inhibitors that exhibit specificity for individual cathepsins, based on their different tertiary structures of substrate binding pockets of cathepsins, determined using X-ray crystallographic analysis by Turk et al. (1995). These designed inhibitors were then chemically synthesized and their specificities were tested by our group.

As Fig. 18.4 shows, individual cathepsin-specific inhibitors were developed. The epoxysuccinate derivatives CA-074 and CA-030 were designed as specific inhibitors for cathepsin B (Katunuma 1995) and CLIK-148 and CLIK-195 (CLIK: Cathepsin L Inhibitor Katunuma) were designed as specific inhibitors for cathepsin L (Katunuma 1999). The aldehyde derivative CLIK-60 was designed as a cathepsin S-specific inhibitor. As pyridoxal propionate (aromatic aldehyde) derivatives, CLIK-163 and CLIK-166 have been developed. These compounds were designed based on the structures of their substrate binding pocket (Katunuma 1995, 1999, 2000).

These inhibitors easily pass through the cell membrane and are effective not only in vitro, but also in vivo. These inhibitors showed 100% inhibition at concentrations of 10^{-6}–10^{-7} M, both in vitro and in vivo, as shown in Table 18.1.

Table 18.1 Inhibition specificities for various cathepsins

	10^{-x} M	B	L	S	K
CA-30	–7	100	0	0	0
CA-74	–7	100	0	0	0
CLIK-148	–6	0	100	30	0
	–7	0	63	0	0
CLIK-195	–6	0	100	25	0
	–7	0	85	0	0
CLIK-60	–6	25	30	100	10
	–7	0	0	86	0
CLIK-164	–5	0	20	60	100
	–6	0	0	20	60
CLIK-166	–4	0	0	17	100
	–5	0	0	0	100

The mechanisms to show the cathepsin B specificity by CA·030 were clarified by X-ray crystallography (Musil et al. 1991).

18.1.5 The Role of Special Cathepsins for Antigen Processing and Presentation

Immunological events are started from the phagocytosis of antigens. The antigenic proteins are then processed to antigenic 15-mer epitopes by various cathepsins to present to MHC class II genes. There are many unknowns regarding the selective incorporation of antigen proteins to be incorporated into relevant lysosomes. The resulting antibodies and cytokines are determined by signals of the epitopes that are processed and the signals are transduced to the helper T cells (Th-1 or Th-2). For instant, ovalbumin as an antigen is able to produce not only IgE and INFα, but also IgG1 and IL-4 by the different epitopes produced by distinct cathepsins.

The immune response to rabies vaccine was used as a model for antigen processing by cathepsin B. CA-074 as a cathepsin B-specific inhibitor, the rabies vaccine, and its epitope $ER_{281–299,}$ (EECLDALESTMTTKSVSFR) derived from rabies glycoprotein, were used to challenge 2C5 strain or B6 strain cells, which respond specifically to rabies vaccine. As shown in Fig. 18.5, when rabies vaccine or antigenic epitope $ER_{281–299}$ was incubated with antigen-presenting cells (2C5 strain or B6 strain), [H^3]-thymidine incorporation by rabies vaccine was suppressed by the addition of CA-074 in a dose-dependent manner, but the proliferation response by the processed antigenic peptide $ER_{281–299}$ was not inhibited by CA-074.

Importantly, the C-terminus sequence of the antigenic peptide has an FR (phenylalanine-arginine) structure in the C-terminus that is susceptible to cleavage by cathepsin B. This response of the 2C5 clone to rabies vaccine was also suppressed by the F(ab)′ of cathepsin B-specific antibody or by Z-RR-MCA (Z-Arg·Arg-MCA), a specific substrate for cathepsin B, but methylcoumarinamide (MCA) itself showed no effect, as shown in Fig. 18.6. This further confirmed that

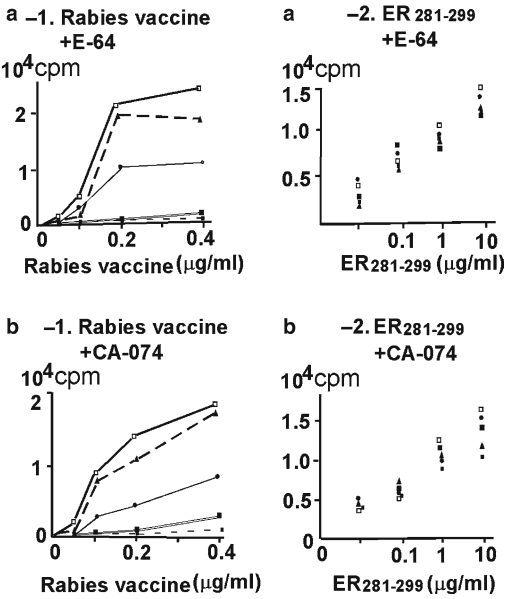


Fig. 18.5 Inhibitory effects of E-64d and CA-074 on the proliferative response of human T cell clones by re-challenge with a rabies vaccine or antigenic peptide. The 3×10^4 cells of 2C5 clone were incubated for 72 h with rabies vaccine [(**a**)-1, (**b**)-1] or with the synthetic peptide ER·281–299 [(**a**)-2, (**b**)-2] in the presence of 1×10^5 cells of Rx-PBMC plus various concentrations of cathepsin B inhibitors E-64d or CA-074. E-64d was used in (**a**)-1 and (**a**)-2 and CA-074 was used in (**b**)-1 and (**b**)-2. (▲···▲) Pre-pulsed Rx-PBMC + 5 μg/ml of inhibitor; (●——●) Rx-PBMC + antigen + 1.25 μg/ml of inhibitor;(■===■) Rx-PBMC + antigen + 2.5 μg/ml of inhibitor;(▪▪) Rx-PBMC + antigen + 5 μg/ml of inhibitor;(□——□) Rx-PBMC + antigen

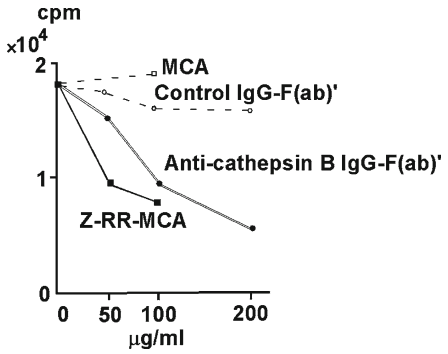


Fig. 18.6 Inhibition of T cell proliferation by the antibody fragment of anti-cathepsin B antibody and the specific substrate of cathepsin B, Z-RR-MCA. Cells (3×10^4) of 2C5 clone were incubated for 72 h with 1×10^5 cells of Rx-PBMC and 0.2 μg/ml of rabies vaccine in the presence of various concentrations of the F(ab)' of anti-cathepsin B antibody IgG. The cells were pulsed with 1 mCi/well of [^{3}H] thymidine for the last 12 h and harvested

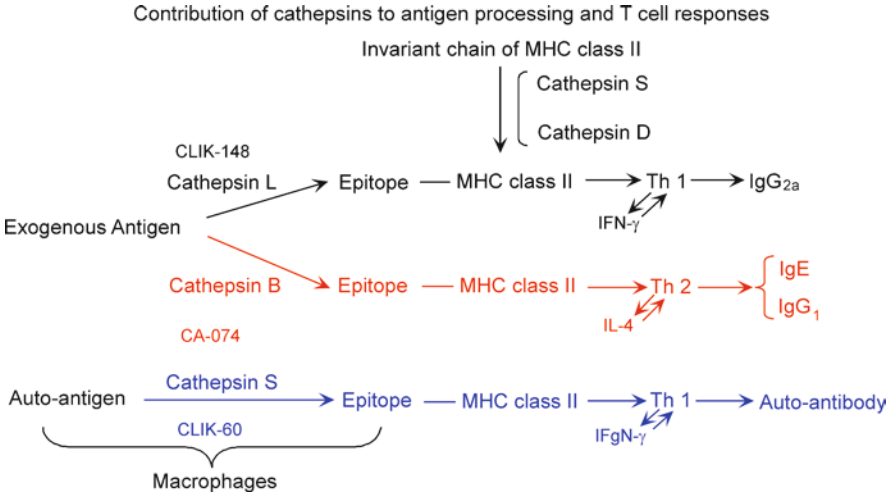
			57						63			
MHC-II, β·chain	R	P	V	A	E	S	W	N	S	Q	K	D
Cathepsin B	W	L	V 217	A	N	S	W	N	T 223	D	W	G
Cathepsin L	W	L	V	K	N	S	W	G	K	E	W	G
Cathepsin II	W	I	V	K	N	S	W	G	S	N	W	G
Cathepsin S	W	L	V	K	N	S	W	G	L	H	F	G
Cathepsin J	W	I	V	K	N	S	W	G	S	Q	W	G
Papain	I	L	I	K	N	S	W	G	T	G	W	G

Fig. 18.7 One of the active sites of cathepsin B shares homology with a part of the antigenic peptide binding domain (desetope) of MHC class II, but no homology with the other cathepsins

cathepsin B is the determinant protease responsible for the antigen processing of vaccines of rabies to be presented to the MHC class II region. Furthermore, a part of the active site of cathepsin B, V_{217}-N_{223} (VANSWNT), shows high homology with a part of the binding domain (desetope) of the MHC class II β·chain, V_{57}-N_{63} (VAESWNS), because the alanine and the asparagine are the same amino acids on cathepsin B and the MHC class II region, although the alanine and the asparagine in the same active domain of the other cathepsins are commonly substituted by lysine and glycine, respectively, as shown in Fig. 18.7. This is also one important reason why only cathepsin B specifically makes antigenic peptides bind with the MHC class II region. We conclude that the antigenic fragments of hepatitis B surface antigen and rabies vaccines are processed by cathepsin B specifically and show common affinity to bind to the desetope of the MHC class II β-chain (Matsunaga et al. 1993).

18.1.6 Class-Switching of Antibody Formation by Different Cathepsins

Different cathepsins produce different antigenic epitopes (peptides) to present to the MHC class II region, and then the different signals are transduced to the different helper T lymphocytes (Th-1 or Th-2). Because the cathepsins participate in antigen processing, the inhibitors of individual cathepsins regulate the class-switching of antibody formation through the formation of different signal peptides. For instance, cathepsin B participates in antigen processing for the signal transduction to helper T2 type (Th-2) cells; as a result, IgG2a and IFNα formation are stimulated and IgE, IgG1, and IL-4 productions are suppressed. Cathepsin L participates in expression to helper Th1 type (Th-1) signal transductions to produce IgE, IgG1, and IL-4. Therefore, the classes of immune responses were switched between Th-1 and Th-2 types by various cathepsin inhibitors as follows:



Scheme

Class-switching of antibody formation to ovalbumin was observed by administration of a cathepsin B-specific inhibitor, such as CA-074, in vivo. The Th-2 type of immunoglobulins and cytokines, such as IgG1 and IgE, the passive cutaneous anaphylaxis reaction, and IL-4 levels were decreased and IgG2a and IFN-α levels were elevated in the CA-074-treated ovalbumin-immunized rabbits, as shown in Figs. 18.8 and 18.9. The immune responses were opposite in rabbits treated with CLIK-148, which is a cathepsin L-specific inhibitor (Katunuma et al. 2003).

18.1.7 Auto-Antigen α-Fodrin was Processed by Cathepsin S in Sjögren's Disease

Autoimmune diseases are expressed by specific auto-antigens processed by specific cathepsins. An inhibitor of one specific cathepsin (in this case, the cathepsin S inhibitor CLIK-60) prevented the expression of Sjögren's syndrome. The auto-antigen α-fodrin of Sjögren's syndrome is processed by cathepsin S to make autoantibody in the mouse model of Sjögren's syndrome, as shown in Fig. 18.10 and Table 18.2.

The acceleration of [H^3] thymidine incorporation into splenocyte T cells of the mouse model of Sjögren's syndrome was assayed. The [H^3] thymidine incorporation was stimulated by α-fodrin, but not by ovalbumin. Thymectomized mice with Sjögren's syndrome were used, as shown in Fig. 18.10. The [H^3] thymidine incorporation was suppressed completely by CLIK-60, which is a cathepsin S-specific inhibitor and the other cathepsin inhibitors had no effect. The pathological signs

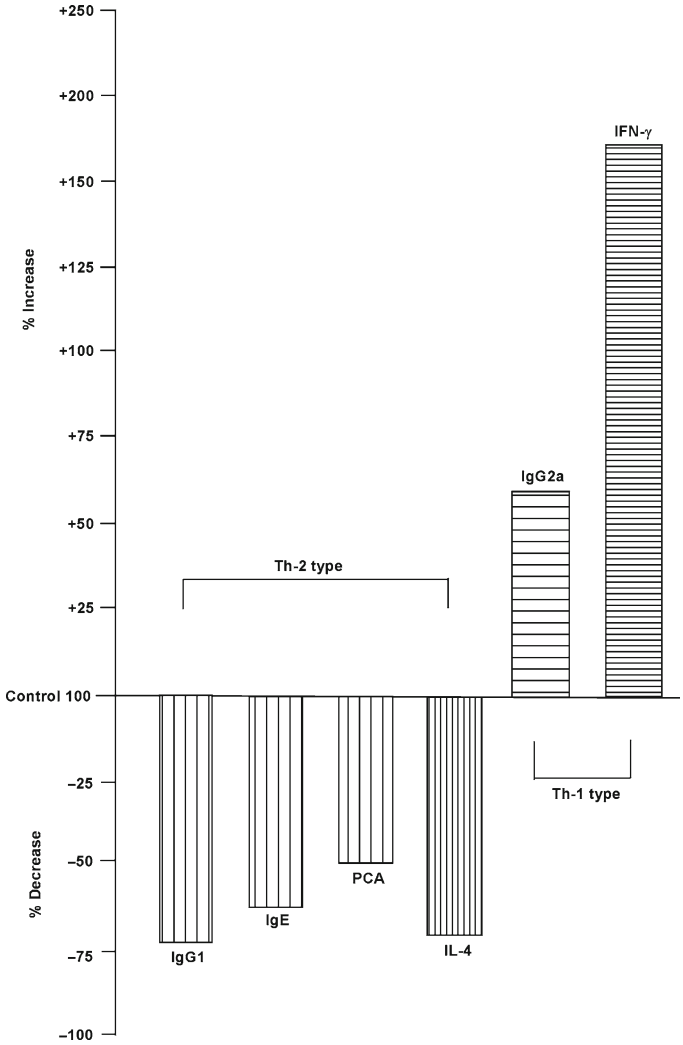


Fig. 18.8 Changes in the ovalbumin-dependent production of cytokines and immunoglobulins by CA-074 administration

in mice with Sjögren's syndrome and their prevention by CLIK-60 are shown in Table 18.2. Pathological lesions and increased secretion volumes of saliva and tears were almost completely prevented by treatment with CLIK-60 (Maekawa et al. 1998; Saegusa et al. 2002; Ishimaru et al. 2004).

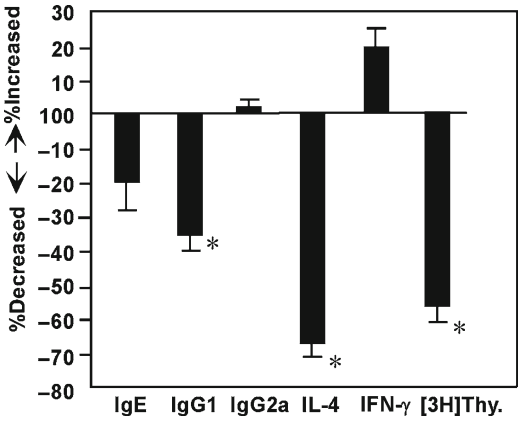


Fig. 18.9 Changes in ovalbumin-dependent immune responses in mice by peroral administration of pyridoxine in a high-protein diet. Percent changes in productions of immunoglobulins and cytokines by 6.0-mg pyrodoxine diet in 70% casein were compared with 0.58-mg pyridoxine diet in 70% casein. Each group consisted of eight mice. A statistically significant difference was found between the standard and excess pyridoxine diets. $*P<0.05$ (for details, See Katunuma et al. 2000)

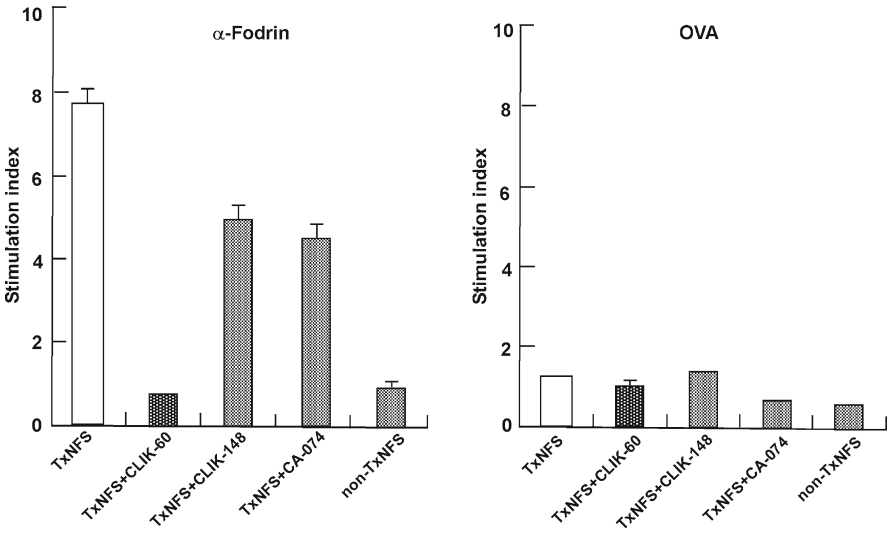


Fig. 18.10 Suppressed expression of Sjögren's syndrome in the mouse model of Sjögren's syndrome by the cathepsin S inhibitor CLIK·060. Response to autoantigen α·fodrin (*left panel*) and ovalbumin (*right panel*). TxNFS: thymectomized mice with Sjögren's syndrome 3 days after birth. Stimulation indices: accelerated [^{3}H] thymidine incorporation in T cells of the spleen from mice with Sjogren's syndrome

Table 18.2 Pathological symptoms of Sjogren's syndrome model mice and their suppression by CLIK-60

	Grade of legions	
Pathological legion grade	Lacrimal G.	Submandibular
Non-Tx SS	0.3	0.3
Tx	3.8	3.0
SS + CA-074	2.8	2.5
SS + CLIK-148	3.5	2.8
SS + CLIK-060	0.9	1.0
Secretion volume of saliva and tears		
	Saliva	Tears
Non-Tx SS	9.0	3.0
Tx	2.0	1.0
SS + CLIK-060	7.0	2.9

Txx-SS: Thymectomized Sjogren's model mice. Secretion volume given in μl/20 min.

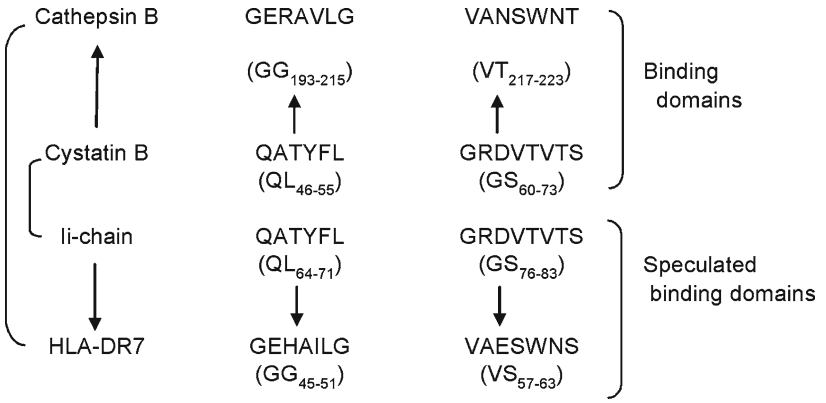


Fig. 18.11 Possible binding domains between Ii-chain and HLA·DR7. The domains $GG_{193–215}$ and $VT_{217–223}$ of cathepsin B that correspond to the second and the third active sites of cathepsin B are exposed on the surface of the substrate binding section, as shown by X-ray crystallography. It is well known that the two domains of cystatin B, $QL_{46–55}$ and $GS_{60–73}$, which show the highest homology with the corresponding two domains of the Ii-chain, are binding domains with two active sites of cathepsin B. The domain $VS_{57–63}$ of HLA-DR7 is generally considered to be the desetope that binds with the antigenic peptide

18.1.8 Immunological Significance of the Relationship Between the Invariant Chain of the MHC Class II Region and Cathepsins

The primary structure of p31 of the invariant chain (Ii-chain) shows about 50% homology with those of the cystatin family, as shown in Fig. 18.11. At a concentration of 3.8×10^{-7} M, the Ii-chain inhibited 75% of cathepsin L and 55% of cathepsin H activities, while cathepsin B activity was not inhibited at all, as shown in Fig. 18.12.

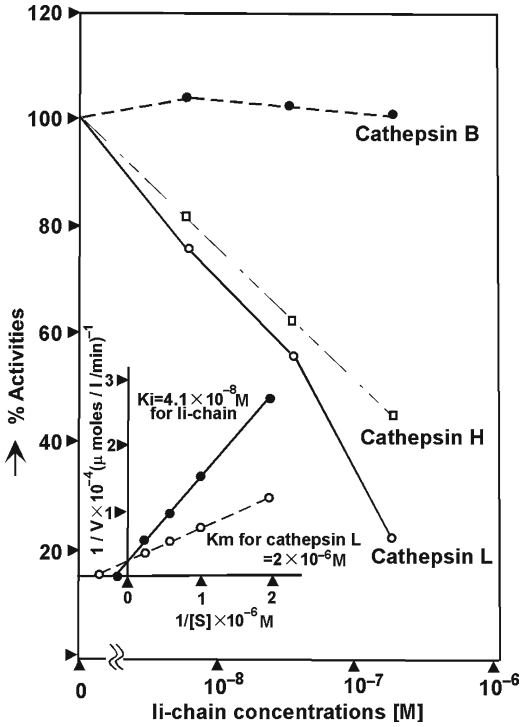


Fig. 18.12 Inhibition profiles of cathepsins L, H, and B by purified human Ii-chain. The reactions were carried out in 50 mM acetate buffer, pH 5.5, with 10^{-3} M of L-cysteine for 30 min and the released methylcoumarinamide was fluorometrically assayed. The molar concentrations of the Ii-chain added were calculated as 60-kDa dimer form. The K_m for cathepsin L was 1.97×10^{-6} and the K_i value for Ii-chain was 4.1×10^{-8} M

The inhibition mode of cathepsin L by the Ii-chain shows typical competitive kinetics and the Ki value of the Ii-chain for cathepsin L was 4.1×10^{-8} M. The antigen processing by cathepsin B is apparently not deterred by the released Ii-chain; in addition, further degradation of processed antigenic peptides by cathepsin L or H is protected by the Ii-chain (Zhang et al. 2000).

18.1.9 Cathepsin L Activity Controls Adipogenesis and Glucose Tolerance

The important role that cathepsin L activity plays in the control of adipogenesis and glucose tolerance has been recently elucidated by Guo-Ping Shi and his group (Yang et al. 2007). They have been demonstrated in vivo a role for cathepsin L in the degradation of essential proteins for adipogenesis and glucose metabolism, such as the matrix protein fibronectin, as well as the insulin receptor (IR) and the

insulin-like growth factor 1 receptor (IGF-IR). Cathepsin L inhibition by CLIK-195 leads to the reduction of preadipocyte adipogenesis or lipid accumulation, protection of fibronectin from degradation, accumulation of IR and IGF-IR-β subunits, and an increase in glucose uptake, as shown in Figs. 18.13–18.16. Cathepsin

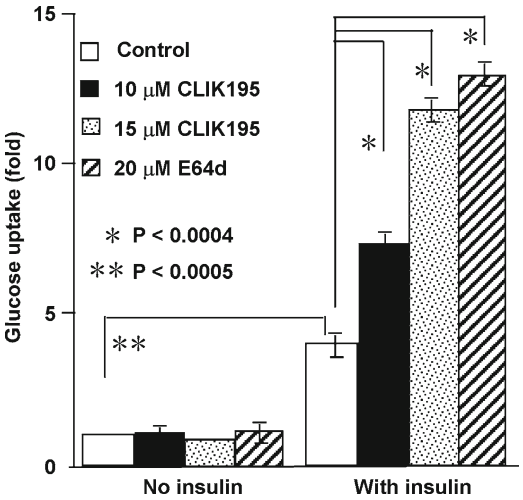


Fig. 18.13 Cathepsin L inhibition affects 3T3-L1 cell adipogenesis and insulin receptor proteolysis. Cathepsin L inhibition with CLIK-195- or E-64d enhanced insulin-induced glucose uptake in 3T3-L1 adipocytes. Data are presented as the increase in glucose uptake (cpm) relative to the counts per minute from untreated cells (mean ± s.e.m. of quadruplicate experiments)

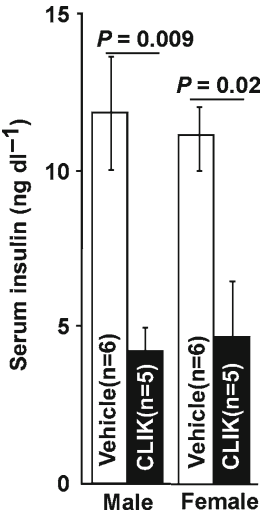


Fig. 18.14 Pharmacological syndrome inhibition of cathepsin L reduces body weight gain and glucose intolerance in CLIK-195-treated male and female *ob/ob* mice

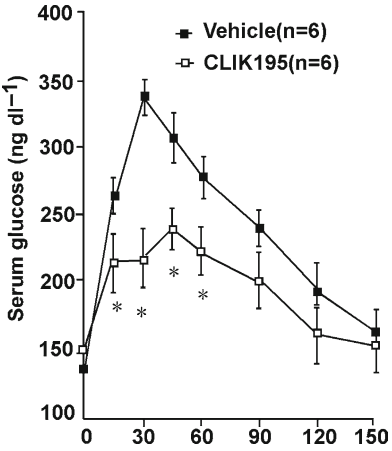


Fig. 18.15 CLIK-195 reduced glucose intolerance in male *ob/ob* mice

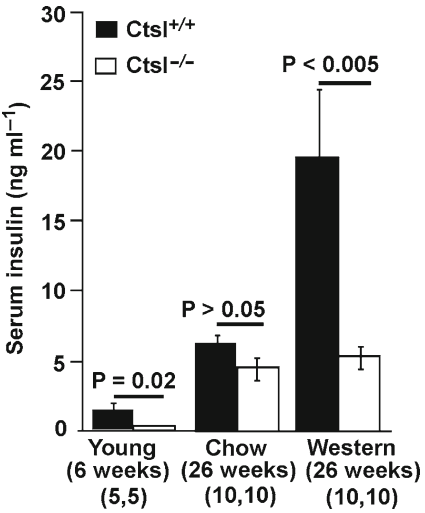


Fig. 18.16 Cathepsin L deficiency increased glucose tolerance. Serum insulin level was significantly lower in *Ctsl*$^{-/-}$ mice compared with *Ctsl*$^{+/+}$ mice at a young age and after 26 weeks of consuming a Western diet

L-deficient mice have reduced levels of serum glucose and insulin, but increased levels of muscle IR β-subunits, fibronectin, and glucose transporter (Glut 4) in muscle (Figs. 18.17 and 18.18). The inhibition of cathepsin L demonstrated reduced bodyweight gain and serum insulin levels and increased glucose tolerance, and increased levels of muscle IR β-subunits, fibronectin, and Glut 4. Cathepsin L is a novel target for diabetes therapy (Yang et al. 2007).

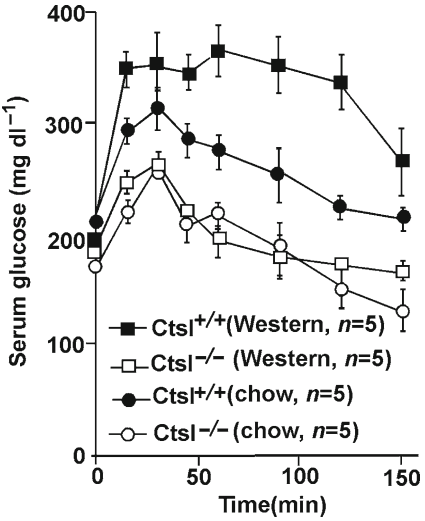


Fig. 18.17 Cathepsin L deficiency increases glucose tolerance. (**a**) Glucose tolerance testing showed increased glucose tolerance in *Ctsl*$^{-/-}$ mice that had consumed either chow or Western diets

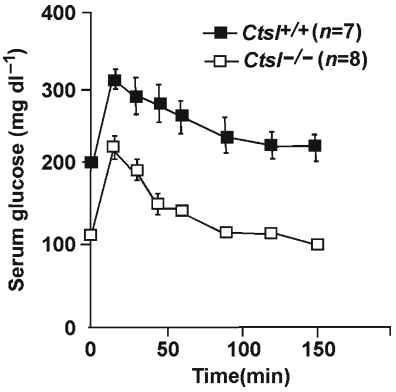


Fig. 18.18 Reduced glucose intolerance

18.1.10 Cleavage of Retinoid X Receptors α by Lysosomal Cathepsin L

Retinoid X receptors (RXRs) belong to the steroid/thyroid hormone receptor superfamily and their endogenous ligand has been shown to be 9-cis-retinoic acid (9-cisRA). We characterized a protease responsible for the cleavage of the 9-cisRA

receptor α (RXRα) in two human delivered cell lines, HepG2 and JEC-3. The presence of the protease in the cytoplasm was confirmed by incubating full-length S^{35}RXRα with each fraction. The cytoplasmic fraction cleaved RXRα into small pieces with molecular sizes of 45, 43, and 31 kDa. To characterize the RXRα cleaving protease, a series of protease inhibitors was added in the reaction of S^{35}RXRα with the cytoplasm of JEG-3 cells. The proteolytic cleavage was inhibited by cysteine protease inhibitors, but not by serine protease inhibitors, aspartic protease inhibitors, metalloprotease inhibitors, or members of the calpain family. As shown in Fig. 18.19, RXRα was specifically inhibited by cathepsin L inhibitors, such as CLIK-088, -112, and -121. In addition, Nagaya et al. (1998) reported the intracellular proteolytic cleavage of RXRα by cathepsin L using CLIK-148 and suggested the potentiality of this process for modulating thyroid hormone action. Inhibition of cathepsin L activity in the monolayer of hepatocytes resulted in increased nuclear RXRα protein and augmentation of T3-dependent induction of spot-14 mRNA.

18.1.11 Short Summary and Discussions

All proteins and biologically active peptides are synthesized as their pre-pro forms, therefore to make mature active forms proteolytic processing is indispensable processes. Intracellular cathepsins play a major role in the intracellular processing of proteins and peptides, not only intracellular proteins catabolism. We developed the specific inhibitors for individual cathepsins using the structure based new inhibitor design. Using these specific inhibitors, we clarified the mechanisms of processing

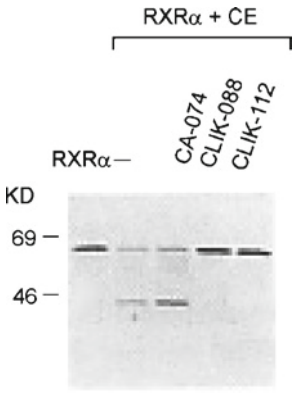


Fig. 18.19 Characterization of a cytoplasmic protease in hepatocytes cultured as a monolayer. When cathepsin B (CA-074) or L-type-specific inhibitors (CLIK-088 and CLIK-112) were added to the incubation reaction, cathepsin L-type-specific inhibitors CLIK-088 and CLIK-112, but not cathepsin B-specific CA-074, inhibit proteolytic cleavage of RXRα by cytoplasmic extract, indicating that cathepsin L-type protease is the enzyme that cleaves RXRα at its amino terminus

of various proteins (enzymes) and biologically active peptides. Also these inhibitors participate to the elucidation of pathogenesis of various diseases and therapy for the special diseases. Especially, we put the focus on the antigen processing mechanisms and autoantigen processing in autoimmune diseases. The most important question to be clarified at the present is the following problem; The individual cathepsin (B, L, H) are located in different particles (lysosomes) from their electron-microscopic pictures (labeled by gold). The selection incorporation mechanisms of the objective substrate proteins into target lysosomes are unknown. These recognition mechanisms of target substrate proteins have been entirely unknown.

18.2 Post-translational Covalent Modifications of Cystatin Family

18.2.1 Intracellular Cathepsin Inhibitors Such as Cystatins

The cysteine protease family (cathepsins) plays an important role in intracellular protein metabolism and cathepsin activities are regulated by cystatins, which are endogenous cysteine protease inhibitors. Among them, more than ten kinds of endogenous cysteine cathepsin inhibitors have been reported at the present. Cystatin α (A) and cystatin β (B) are well known endogenous cysteine protease inhibitors with molecular weights of 10–15 kDa; cystatin α is located only in the epidermis and cystatin β is located in all parenchymal cells ubiquitously.

18.2.2 History of Cystatin Studies

In 1970, endogenous cysteine protease inhibitors of the cystatin family began to be studied as cathepsin inhibitors in various mammalian organs by Järvinen et al. (1976) and Lenny et al. (1979). Then Katunuma and co-workers in 1982, and Turk and colleagues in 1983 reported the presence of two kinds of endogenous cysteine protease inhibitors in rats and humans, respectively (Kominami et al. 1982 Machleidt et al. 1983). They were named "cystatin α" and "cystatin β" in the case of rats by the Katunuma's group, and cystatin A and cystatin B in the human case by the Turk's group. Cystatin α and cystatin A or cystatin β and cystatin B are the same kinds of cysteine protease inhibitors and their amino acid sequences show strong homology.

More than ten kinds of cystatins have been reported in many organs and secretory fluids, and the individual cystatins show different inhibitory specificities against various cathepsins. We focused on the regulation of the inhibitory activities by their covalent modification of these cystatins at the molecular level. The inhibition mechanism and the regulation mechanism of cathepsin activity by cystatins in situ are very important for the regulation of protein catabolism; however, little is known.

We found that cystatin α is phosphorylated by protein kinase C and then targeted in the cornified envelope in skin; then the phosphorylated cystatin α is conjugated with a filaggrin linker segment peptide mediated by transglutaminase. The phosphorylated cystatin α in skin inhibits bacterial growth and plays an important role in the protection against bacterial infection. On the other hand, cystatin β is distributed in all parenchymal cells of various animal organs and plays a role in the regulation of intralysosomal cathepsin activities. The cysteine residue located in the third position of the N-terminus of the cystatin β molecule is reversibly modified to make mixed disulfate derivatives with glutathione and the glutathionated cystatin β. The changes between the glutathionated form and the deglutathionated form of cystatin β and also their dimers are regulated by the redox potential in the cells (Wakamatsu et al. 1984; Tsukahara et al. 1987). Therefore, the intracellular cathepsin activities should be regulated by the redox potential in the cells.

18.2.3 Classification of Cystatin Family and Inhibition Mechanisms of Cystatins

As shown in Fig. 18.20, the cystatin family is divided into two groups; one is located inside cells and the other is secreted into various body fluids. The intracellular group includes cystatin α and β and the secreted group includes cystatin S in saliva (Isimura et al. 1984), cystatin C and γ-trace in cerebrospinal fluid (Barrett et al. 1984), and egg white cystatin (Turk et al. 1983). Another secreted group is a high molecular weight cystatin group, including kininogen (Ohkubo et al. 1984). Cystatins are classified expediently into three groups: family 1, including cystatin α and β (A and B); family 2, including cystatin S and egg white cystatin; and family 3, which are high molecular weight inhibitors like kininogen.

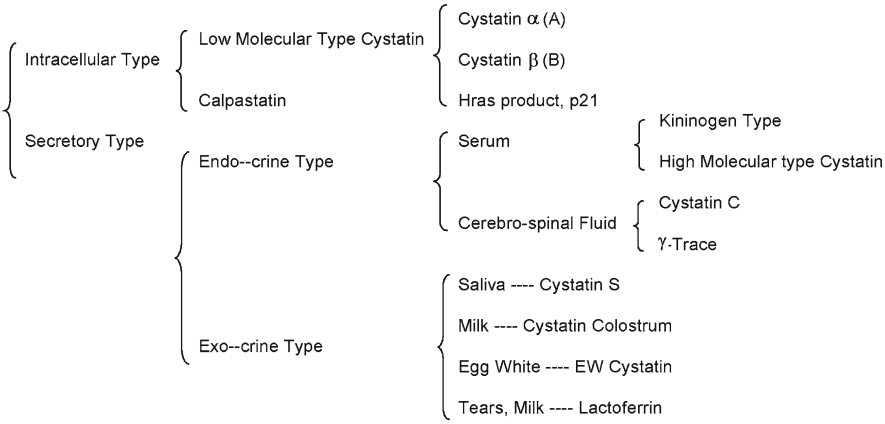


Fig. 18.20 Biological classification of endogenous cysteine protease inhibitors

Each cystatin shows a different affinity for individual cathepsins. For example, egg white cystatin and cystatin α do not inhibit cathepsin B activity, but strongly inhibit cathepsin L activity. In other words, various cystatins share inhibitory functions against individual cathepsins. Turk and co-workers (1983, 1985, 1991) explained the inhibition mechanism of cystatin at the molecular level as follows. The tertiary structure of cystatin C, determined using X-ray crystallography, is shown in Fig. 18.21. The pink loop and green loop in the Fig. 18.21 structure have homologous amino acid sequences in all cystatins and are the common binding sites with cathepsins. The low molecular weight cystatin family has three binding domains showing homologous amino acid sequences, illustrated in Fig. 18.22 with

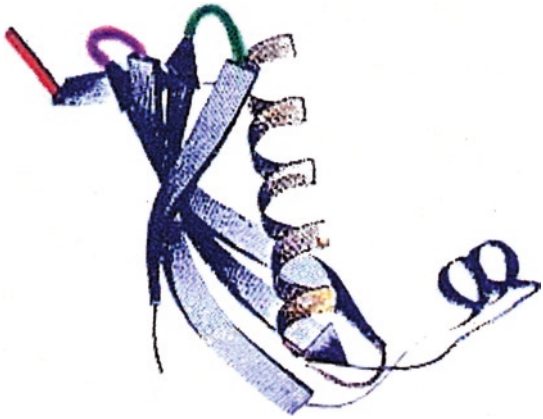


Fig. 18.21 Tertiary structure of egg white cystatin (ribbon-like presentation). The upper left (*red part*) end is the N-terminus. The upper right two loops (*pink* and *green parts*) are binding domains with cathepsins

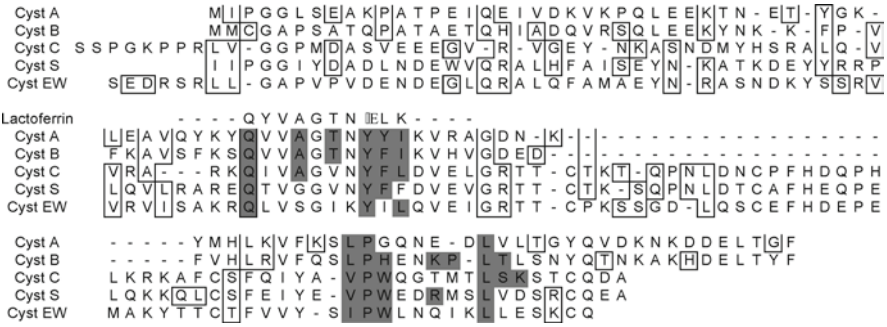


Fig. 18.22 Sequence homology of the cystatin family. The highlighted domains are the common binding domains with cathepsins

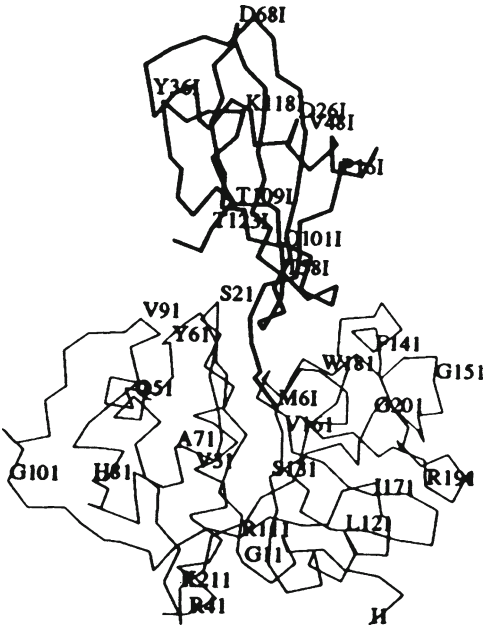


Fig. 18.23 X-ray co-crystallography of cystatin C with cathepsin B; the N-terminus of cystatin is inserted into the substrate binding pocket of cathepsins

red and green as the common binding sites with cathepsin. The N-terminus of cystatins, indicated by a red symbol in Fig. 18.21 is inserted into the substrate binding pocket of the cathepsins. X-ray co-crystallography of the complex between cystatin C and cathepsin B was demonstrated by Turk (1995), as shown in Fig. 18.23.

18.2.4 Inhibition Mechanism of Cathepsin by Cystatins In Vivo

The contribution of the cystatin family to the inhibition of cathepsin activities in situ is very important from the aspect of regulation of protein catabolism. The intracellular localizations of cystatins are shown in Figs. 18.24–18.26. The immunohistochemical examinations of cystatin α and cystatin β in skin are shown in Fig. 18.24 using anti-cystatin α and anti-cystatin β antibodies. Cystatin α is located only in the epidermis. In contrast, cystatin β is ubiquitously detected not only in epidermal cells, but also in hair follicular cells and dermal cells. As shown in Fig. 18.26, anti-cystatin β antibodies were labeled by large gold particles and the anti-insulin antibody wasv labeled by small gold particles. Both sizes of gold particles were observed in the β-cell granules of the islets of Langerhans of the pancreas by immuno-electron microscopy. When cystatin α was injected intravenously, it was incorporated into lysosomes of kidney cells after 30 min. and the intralysosomal cathepsin H activity was reciprocally inhibited by incorporated cystatin α, as shown in Fig. 18.25.

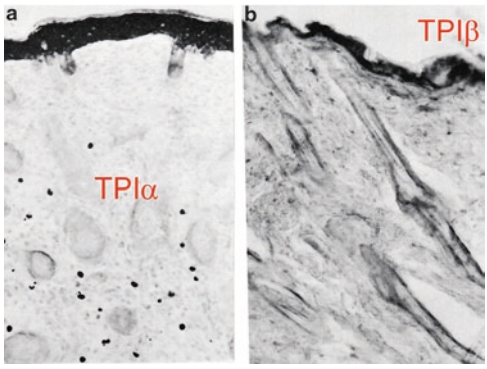


Fig. 18.24 Immunohistochemical staining of cystatin α (TPI-α) and cystatin β (TPI-β) in skin

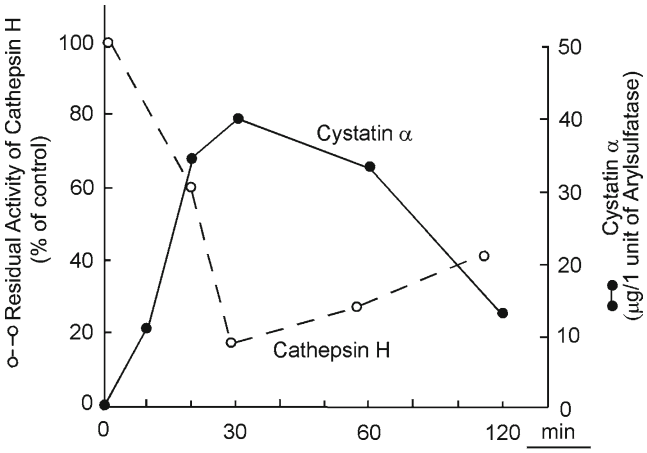


Fig. 18.25 Incorporation of cystatin α into kidney lysosomes and inhibition of intra-lysosomal cathepsin H after intravenous injection of cystatin α

Therefore, as Fig. 18.27 shows, the cystatin β located originally in secretory granules is secreted outside of the cells and then incorporated into lysosomes by exophagy, and the cathepsin activities in the lysosomes are inhibited. We determined the cystatin α gene expression, as shown in Fig. 18.28. When the cystatin α gene plasmid was transfected to cancer cells, surprisingly, not only cystatin α was expressed, but the expression of cathepsin B was also increased. This result indicates the coordinated relationship between the expression of both the cystatin α and cathepsin B genes.

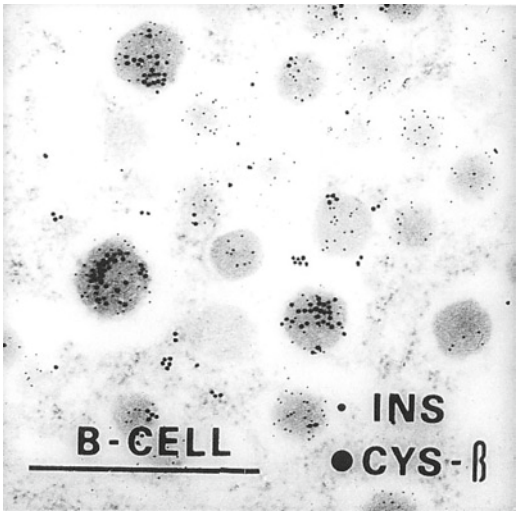


Fig. 18.26 Localization of cystatin β and insulin in β-cells of the island of Langerhans in the pancreas. Cystatin β is labeled by big gold particles and insulin is labeled by small gold particles. Cystatin β is located in secretory granules

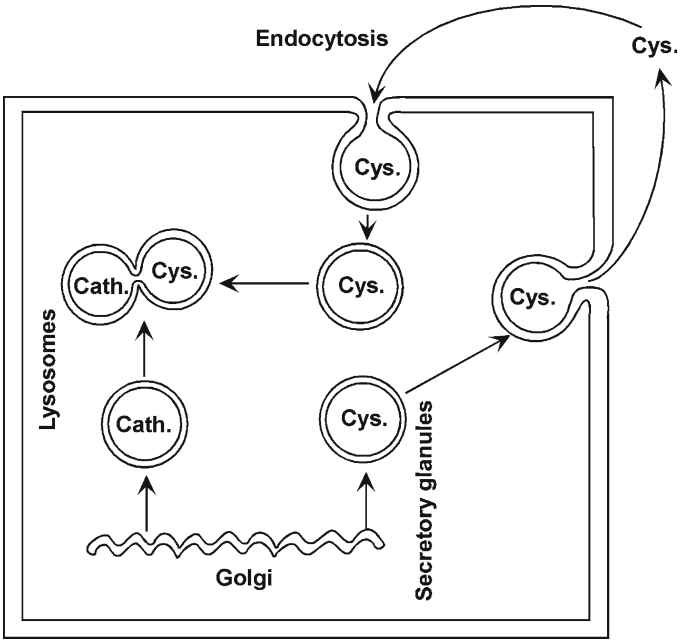


Fig. 18.27 Hypothetical mechanism of lysosomal cathepsin inhibition by cystatin β in situ. The inhibition mechanism of lysosomal cathepsins by cystatins was originally located in secretory granules

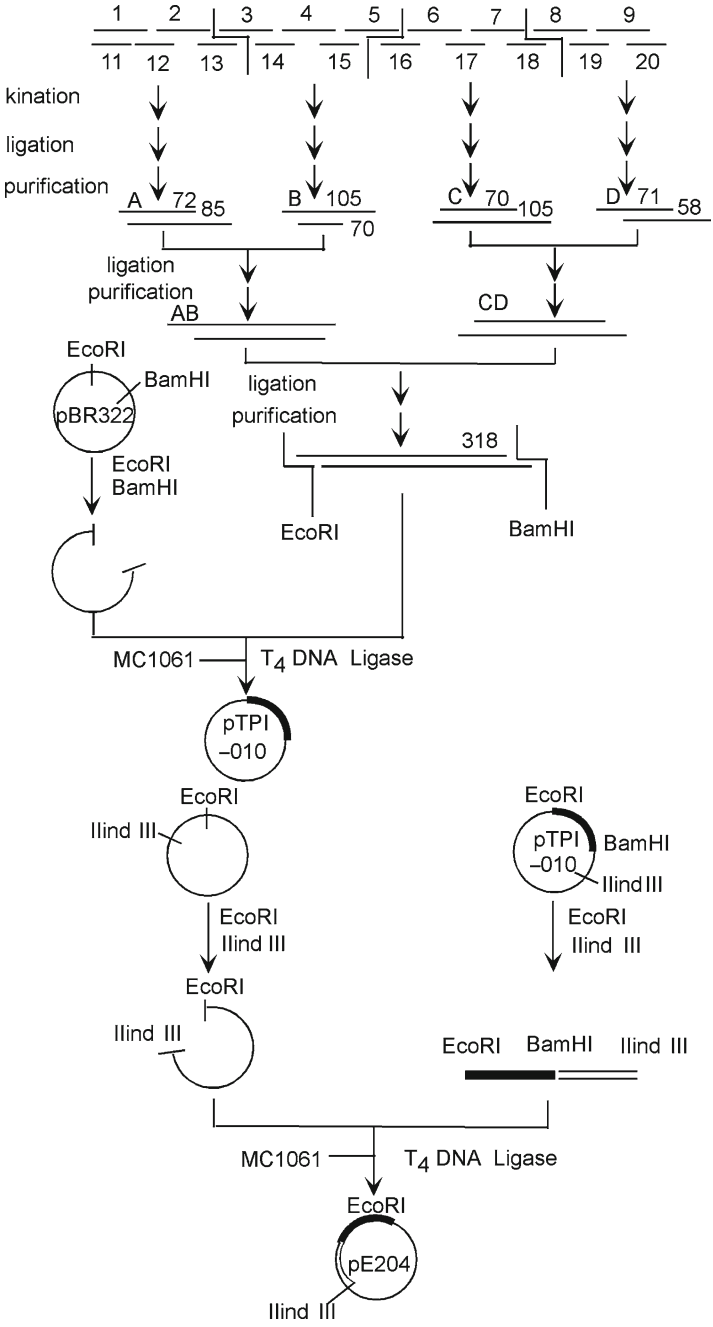


Fig. 18.28 Structures of the cystatin α gene and the expression plasmid of the cystatin α gene

18.2.5 Post-translational Covalent Modification of Cystatin α

When skin was stained by immunohistochemistry using anticystatin α antibody, only the cornified envelope of the skin was stained. The sphingosine treatment of newborn rat skin resulted in the suppression of the targeting of cystatin α into the cornified envelope, as shown in Fig. 18.29. Therefore, cystatin·α was stained in the cornified envelope in the case of untreated skin using anti-cystatin α antibody, because sphingosine is a powerful inhibitor of protein kinase C (PKC) and cystatin α was not phosphorylated.

We found that the hematoxylin stainable protein in newborn rat epidermis was a phosphorylated cystatin α. Alkaline phosphatase treatment of the hematoxylin stainable protein in keratohyalin granules resulted in the release of cystatin α. A threonine residue located in near C-terminus of cystatin α is phosphorylated by PKC (Protein Kinase C) and the phosphorylated cystatin α is then incorporated into the cornified envelope.

The participation of PKC in the phosphorylation of cystatin α was confirmed. The specific inhibitor of PKC, H-7, inhibited the incorporation of ^{32}P into cystatin α, as shown in Table 18.3. The cystatin α was then conjugated with the filaggrin linker segment peptide, which is rich in glutamine residues, mediated by epidermal transglutaminase in the presence of calcium, to yield a high molecular weight protein, as shown in Fig. 18.30 (Takahashi et al. 1994; Takahashi et al. 1999).

The phosphorylated cystatin α possesses the capacity to inhibit the cysteine protease activity of bacteria and viruses. Furthermore, the cornified envelope, which contains cystatin α, shows inhibitory activity to cathepsins, but the cornified envelope from the skin treated with sphingosine lost its inhibitory activity, because

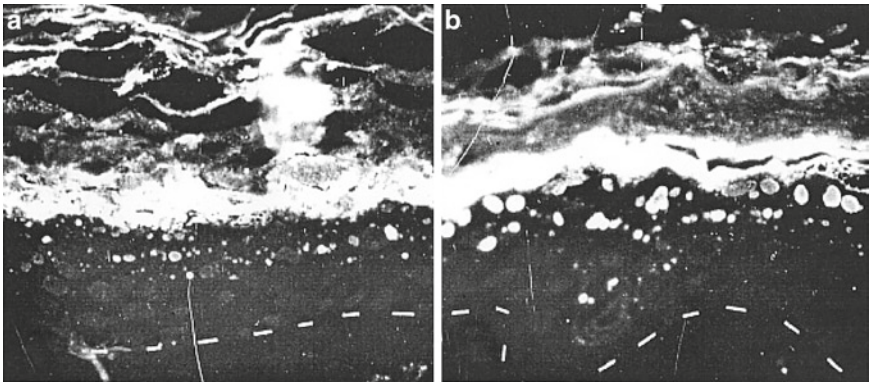


Fig. 18.29 Inhibition of cystatin α incorporation into keratohyaline particles with cathepsin B. Keratohyaline granules of skin detected by sphingosine. Indirect immuno-fluorescence staining of newborn rat skin by using anti-cystatin α antibodies. (**a**) Sphingosine-treated skin. (**b**) Normal control skin

Table 18.3 Incorporation of 32P into cystatin α

Substrate	Inhibitor	32P incorporated into protein fraction (cpm)	32P incorporated into cystatin α or histone (cpm)
–	–	523.9	
–	+H-7	448.0	
Cystatin α (86 μg)	–	1,146.4	622.3
Cystatin α (86 μg)	+H-7	711.4	263.3
Cystatin α (172 μg)	–	1,363.1	839.9
Cystatin α (172 μg)	+H-7	789.1	344.1
Histone (86 μg)	–	6,820.4	6,296.9
Histone (86 μg)	+H-7	792.3	344.3

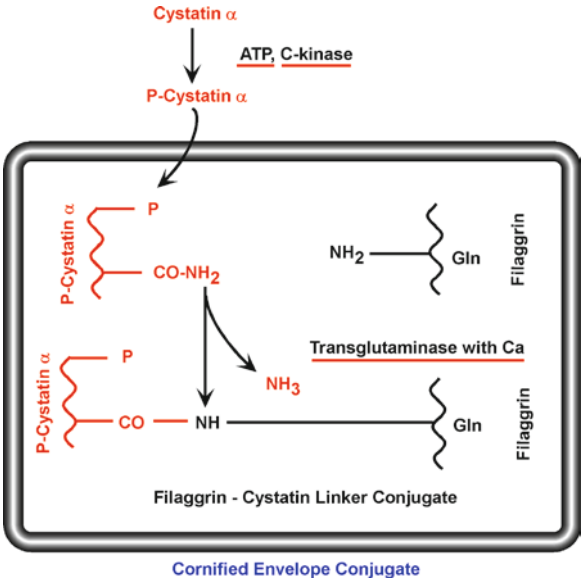


Fig. 18.30 Fate of cystatin α to make filaggrin conjugates in skin

by the treatment, the cystatin α was not incorporated into the envelope. As shown in Fig. 18.31, phosphorylated cystatin α strongly inhibited the activity of cathepsin L, but did not inhibit the activities of cathepsin B or cathepsin H; therefore, cystatin α in the cornified envelope showed a specific inhibition for cathepsin L.

Cathepsin L is an important protease for the survival of bacteria and viruses. In 1994 Katunuma's group reported that cystatin α strongly suppressed the bacterial growth of cultured *Staphylococcus aureus* V8. Thus, when *Staphylococcus aureus* V8 was inoculated onto the sphingosine-treated skin, the incorporation of cystatin α was suppressed and many more colonies were obtained compared with that of normal skin (Takahashi 1994). This anti-bacterial action of cystatin α and its blocking effect on poliovirus proliferation (Korant et al. 1985) led authors to propose that

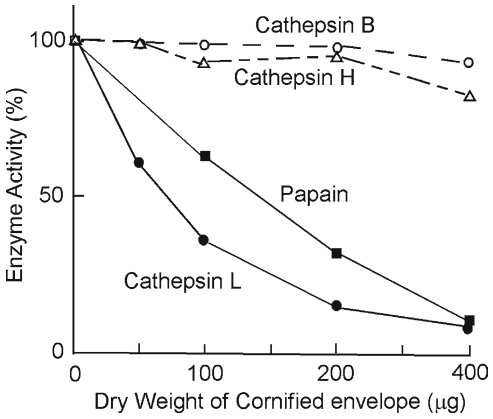


Fig. 18.31 Inhibition of various cathepsins by purified cornified envelope extract

skin cystatin α plays an important role in the protection against bacterial and viral infections. In this context fell our observations regarding the anti-bacteral action of lactoferrin, a member of the cystatin family. We found that lactoferrin strongly inhibits cathepsin L activity ($Ki = 10^{-8}$ M) and, therefore, that the intake of lactoferrin in milk suppresses the growth of *Staphylococcus epidermis* in the small intestine.

18.2.6 Post-translational Covalent Modification of Cystatin β

Cystatin β is located ubiquitously in all cells and regulates proteolysis in their lysosomes (Fig. 18.26). Cystatin β possesses at the third position of the N-terminus a cysteine residue (Fig. 18.32) with which glutathione reacts to form a mixed disulfate complex, as shown in Fig. 18.33 (Katunuma 1985). Since the glutathionated cystatin β did not bind with cathepsins, it loses its inhibitory activity. As mentioned earlier, Turk's group (1995) made it clear using X-ray co-crystallography of cystatin with cathepsin B that while the N-terminus of cystatin was inserted into the binding pocket of cathepsin, the glutathionated N-terminus of cystatin β was unable to bind with the binding pocket. The changes in coefficient of the oxidized and reduced forms of glutathione affected the binding of cystatin β with cathepsins. The coefficient of the oxidized and reduced forms of glutathione regulates the inhibitory activity of cystatin β, as shown in Fig. 18.33. When the oxidized form of glutathione was increased by adding menadione (vitamin K_3) to a cultured macrophages system, the binding of cystatin β with glutathione to make an inactive form of cystatin β was increased, as Figs. 18.33 and 18.34 show (Tsukahara et al. 1987, 1984). Therefore, the activities of cathepsins were regulated by the intracellular redox potentials through the changes of the inhibitory activity of cystatin β, as shown in Fig. 18.34.

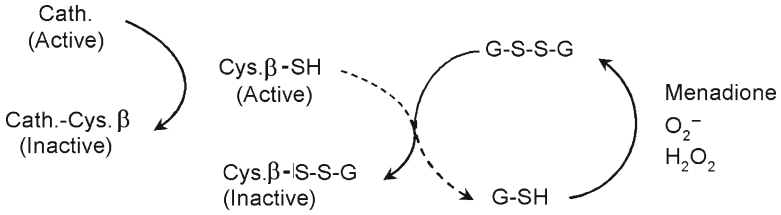


Fig. 18.32 Regulation of cathepsin activity through covalent modification of cystatin β with glutathione. The arrow shows the reaction when menadione is added

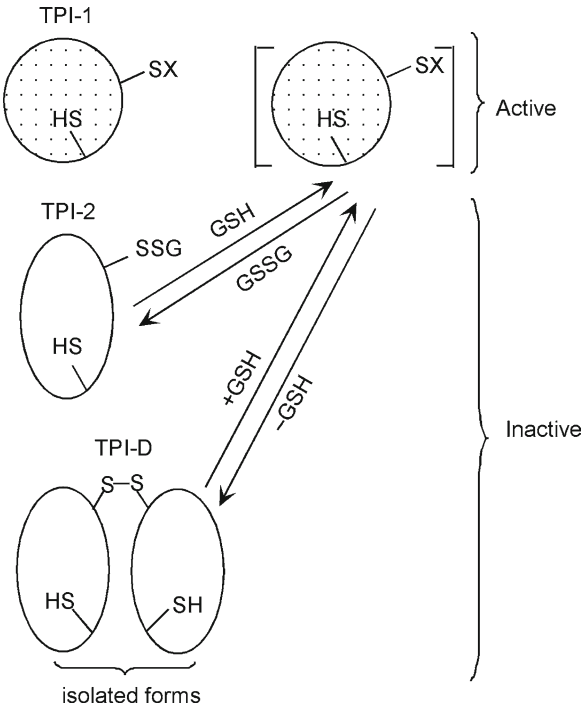


Fig. 18.33 Interconversion of three forms of cystatin β by redox potential

The inhibitory activity of cystatin α did not change under reducing conditions and cystatin α is able to make conjugates with cathepsin H in either condition. In contrast, cystatin β was available to make conjugates with cathepsin H only under reducing conditions; glutathionated cystatin β under oxidizing conditions could not bind with cathepsin H. Therefore, the inhibitory activity of cystatin β depends on the intracellular ratio of oxidized form and reduced form of glutathione. As a result, cathepsin activities are regulated by the level of intracellular redox potential through the changes in cystatin β activities.

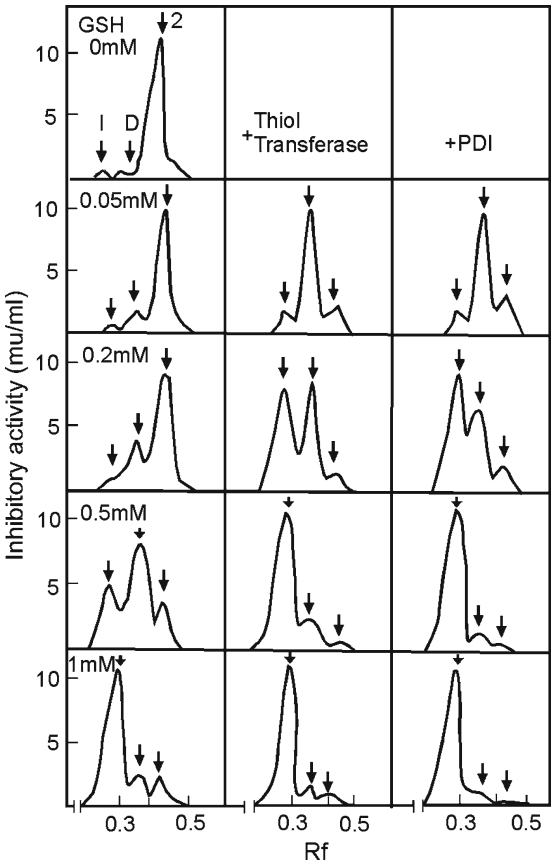


Fig. 18.34 Enzymatic dethiolation of cystatin β. Purified cystatin β (mixed disulfide with glutathione, 10 μg) was incubated with the indicated concentrations of glutathione and thiol transferase (0.2 unit) or protein disulfide isomerase (0.1 unit) in 50 μl of 0.1 M sodium phosphate buffer, pH 7.5, containing 5 mM EDTA for 1 h at 37°C. Then, aliquots of the reaction mixtures were promptly subjected to polyacrylamide gel electrophoresis without sodium dodecyl sulfate at pH 8.0. After electrophoresis, gels were cut into slices and the papain inhibitory activity of their extracts was examined in the presence of 8 mM cysteine

18.2.7 Short Summary and Discussion

At the present, about ten kinds of cystatins are reported. Cystatin β is distributed in all organs, on the contrary, other cystatins are located in special organs or body fluids. Cathepsins are originally located in lysosomes, but the other cystatins are originally located in cytoplasm or special body fluids or serum. Therefore, how to contact between cathepsins and cystatin family in situ is a big question about in general. Cystatin α and cystatin family in milk or serum play a role in the protection from the bacterial infection. Invariant chain is a kind of cystatin family and might play a role in protection from cathepsin action in antigen presentation.

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