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Review

Propeptide-like cysteine protease inhibitors: Structural properties, mechanisms of inhibition and emerging roles in biological tissues

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ABSTRACT

Propeptides of cysteine proteases including papain and cathepsins B, K, L, and S are selective inhibitors of their cognate cysteine proteases. A new class of endogenous inhibitors homologous to the propeptide regions of cysteine proteases has been identified and characterized in the past few decades. These include the mouse cytotoxic T-lymphocyte antigen-2 (CTLA-2), *Bombyx* cysteine proteinase inhibitor (BCPI), *Drosophila* crammer, and salmon salarin. They have been categorized as I29 (CTLA family) in the MEROPS peptidase database. In this review, we summarized experimental findings on their molecular forms, inhibition mechanisms, and biological functions. The overall properties of these inhibitors, molecular structures and inhibition mechanisms were found to be similar to those of propeptides of cysteine proteases. CTLA-2 has been shown to possess a unique inhibition mechanism by blocking its cognate enzyme, cathepsin L, through oxidizing the active thiol residue of the enzyme with its own thiol residue. The divergent biological functions of these inhibitors have been determined based on their inhibitory activities towards cathepsin L-like cysteine proteases. CTLA-2 is strongly expressed in the placenta, and may play roles in implantation and decidualization. It is also an inducer of Treg cells in the eyes, and has been shown to induce apoptosis in murine T-lymphoma cells and cardiac fibroblasts. In the brain, CTLA-2 transcript is strongly expressed in neuronal cell bodies while the protein is localized in dendrites and fibre bundles. BCPI has been demonstrated to exhibit anti-parasitic activity and thus thought to act as a negative regulator of silk gland histolysis. Crammer has been identified in mushroom bodies (brain) of *Drosophila melanogaster* as one of the proteins essential for long-term memory formation through regulation of cathepsin activity in the insect. These findings suggest that the inhibitors are novel proteins that participate in various physiological actions in different organisms. Their emerging roles in normal biological tissues, diseases and as potential targets for drug development are discussed in detail.

KEYWORDS: inhibitor, cysteine protease, BCPI, propeptide, cathepsin L, CTLA-2, crammer

1. Introduction

Cysteine peptidases are widely distributed in a variety of organisms, where they are involved in the process of intra- and extra-cellular protein degradation and turnover [1-3]. Cysteine proteases such as cathepsins B, K, L, and S, belonging to the papain subfamily (C1A) have been studied extensively and documented in the MEROPS peptidase database (http://merops.sanger.ac.uk) [4-6]. Cathepsins of mammalian origin are considered to perform several important functions, including bone protein

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turnover, antigen processing and presentation, prohormone processing and disease-related tissue remodeling [7, 8]. Previous studies show that cysteine proteases in parasites facilitate the parasites to invade tissues [9]. Most C1A cysteine proteases are expressed as inactive precursor proteins with N-terminal propeptides [10-12]. In the processing of the proteases, the propeptides are released by proteolytic cleavage and the precursor proteins become active matured enzymes, eliciting their biological activities at their destination [13-16]. The propeptides are known to exhibit specific inhibition to their cognate cysteine peptidases. Inhibition mechanisms of the propeptides have been demonstrated [17-20], suggesting that the propeptides could probably be useful as therapeutic agents against parasitic pathogens or for the treatments of diseases such as cancer [21-25]. In the past three decades, novel cysteine peptidase inhibitors, with amino acid sequences homologous to the propeptides, have been reported. Cytotoxic T-lymphocyte antigen-2 (CTLA-2) was first discovered in mouse-activated T-lymphocytes [26]. A similar protein, *Bobyx* cysteine proteinase inhibitor (BCPI) was also found in the silkmoth *Bombyx mori* [27, 28]. Other homologous inhibitor proteins, crammer [29] and salarin [30], were also identified in *Drosophila* (*D. melanogaster*) and the Atlantic salmon (*Salmo salar*), respectively.

In our previous report, we presented detailed comparison between these inhibitor proteins and propeptide inhibitors [31]. Discussion was focused on how the inhibitor proteins emerged evolutionally as "functional propeptides", resulting into a new class of inhibitors, "propeptide-like cysteine protease inhibitors". In 2003, the inhibitors were categorized as I29 (CTLA family) in the MEROPS peptidase database. In the present review, we addressed recent advances in research on the inhibitor proteins, with a focus on their structural properties, inhibition mechanisms and emerging functions in biological tissues.

2. Molecular forms and inhibition mechanisms

The sequence alignments of propeptide-like cysteine protease inhibitors are shown in fig. 1. The inhibition constants of these inhibitors towards cysteine proteases are summarized in table 1.

2.1. CTLA-2

Two similar, but distinct CTLA-2s namely, CTLA-2α and CTLA-2β, have been detected in mouse tissues [26]. CTLA-2α has an N-terminal hydrophobic peptide and localizes both intra- and extracellularly [32]. CTLA-2α has one internal cysteine residue (C75), while recombinant CTLA-2α expressed in the *E. coli* system has two molecular forms, a monomer and disulfide-bonded dimer [32, 33]. CTLA-2α acts as a monomer and dimer when expressed in HEK293 cells and mostly as a dimer in cell media, suggesting that it has the ability to change its molecular forms

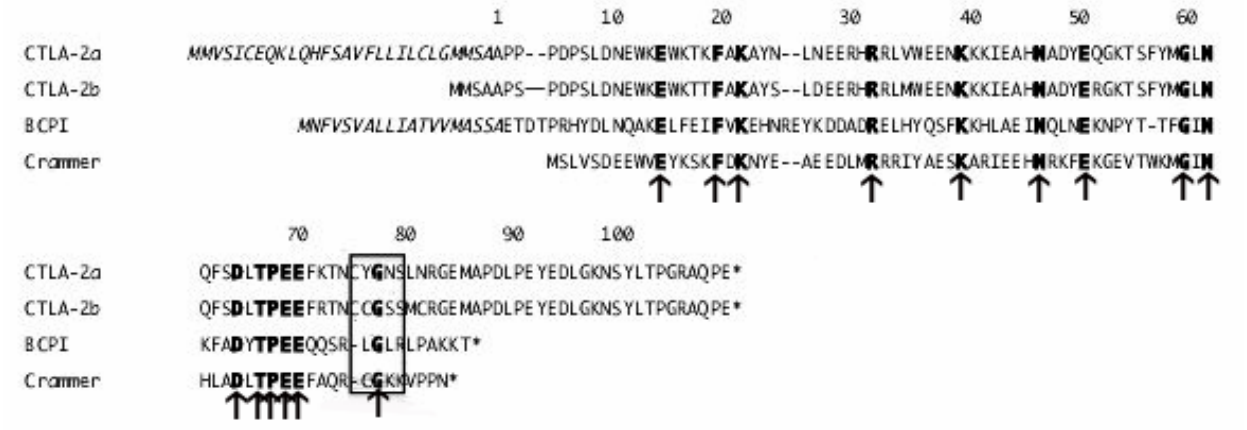

Fig. 1. Alignments of propeptide-like cysteine protease inhibitors. Well conserved amino acid residues among the proteins are shown by arrow. Gaps introduced to optimize the alignment are marked with dashes. N-terminal hydrophobic peptides based on mature CTLA-2α are shown in italics. Sequences interacting with the substrate binding clefts of the enzymes are boxed.

Table 1. Inhibition constants of propeptide-like inhibitors towards cysteine peptidases.

	Ki (nM)					Reference
	Cts L	**BCP**	**Cts H**	**Cts B**	**Papain**	
CTLA-2α	0.38	0.23	86	>1000	560	[33]
CTLA-2β	24	nd	67b	1000a	25	[34]
BCPIβ	0.036	0.0059	82	>4000	>1000	[41]
Crammer	25	nd	nd	30	1000a	[29]
Crammer	3.9	4.7	7.8	15	>1000	[42]
Crammer	1.4	nd	nd	790	nd	[43]

a: No inhibition was observed up to the concentration indicated. b: IC_{50}. nd: Not determined.

in a manner that depends on the physiological conditions present [32]. CTLA-2β does not have an N-terminal hydrophobic peptide and contains two internal cysteine residues (C75 and C76). Although recombinant CTLA-2β expressed in the baculovirus system acted as a dimer under physiological conditions, this form was not covalently bonded [34].

CTLA-2α is a potent and selective inhibitor of cathepsin L-like cysteine proteases. Ki values were previously reported to be 0.38 nM for cathepsin L and 0.23 nM for *Bombyx* cathepsin L-like cysteine proteinase (BCP) [33]. BCP has been purified from the eggs of the silkmoth *Bombyx mori* [35]. Its specificity is similar to that of cathepsin L, and has been classified as cathepsin L1 (arthropod-type) in the MEROPS peptidase database (C01.092). A previous study reported that CTLA-2α does not inhibit cathepsin B. However, it showed weak inhibition activity to papain [33]. Furthermore, CTLA-2β was found to be less selective to cathepsin L than CTLA-2α, with Ki values of 24 nM for cathepsin L and 25 nM for papain [34]. CTLA-2α and β both showed significant inhibition to cathepsin H with Ki values of approximately 86 nM [33, 34].

The generation of deletion mutants (Fig. 2) has led to three separate regions of CTLA-2α being identified as essential for its inhibitory effects [36].

Since CTLA-2α is homologous to the proregion of cathepsin L, a molecular modeling analysis was performed using procathepsin L as a template [17-19, 36]. The first (N10–F19) and second (H30–A44) regions included large portions of the α1 and α2 helix sequences, respectively. The third region (S55–S79) included an α3 helix sequence and sequences interactive with the prosegment binding loop (PBL) and substrate-binding cleft of the enzyme. The extended C-terminal portion (L80–C-terminal) was not required for its inhibitory activity, because the two insect inhibitors (BCPI and crammer) do not have the C-terminal extensions as CTLA-2α. Alanine scanning experiments performed in our previous studies [32, 36] to identify the critical amino acid residues in these regions revealed three tryptophan residues: W12 and W15 in the α1 helix and W35 in the α2 helix (Table 2). Critical amino acid residues for crammer and the sequences interactive with the prosegment binding loop (PBL) of the enzymes are indicated in fig. 2. The interaction of crammer critical amino acid residues with the PBL produce the hydrophobic core of the α1/α2 helix cross and maintain its globular domain (Fig. 2, 3) [36, 37].

The tryptophan mutants of CTLA-2α not only lost their inhibitory activities, but also their resistance to the enzymes, suggesting conformational changes. Previous studies reported that these residues functioned to produce the hydrophobic core of the α1/α2 helix cross by hydrophobic interactions and also maintain its globular domain (Fig. 2, 3) [36, 37]. Although the α1/α2 helix core did not interact directly with the enzyme, it has been shown to significantly contribute to its inhibitory potency. These findings were consistent with those reported for propeptide inhibitors [37-39].

The most important finding of these studies was the function of the cysteine residue (C75) [32, 36]. A single mutation in the cysteine residue (C75A and C75S) led to the complete loss of inhibitory activity (Table 2). The CTLA-2α/cathepsin L complex, in which CTLA-2α was covalently bound to cathepsin L

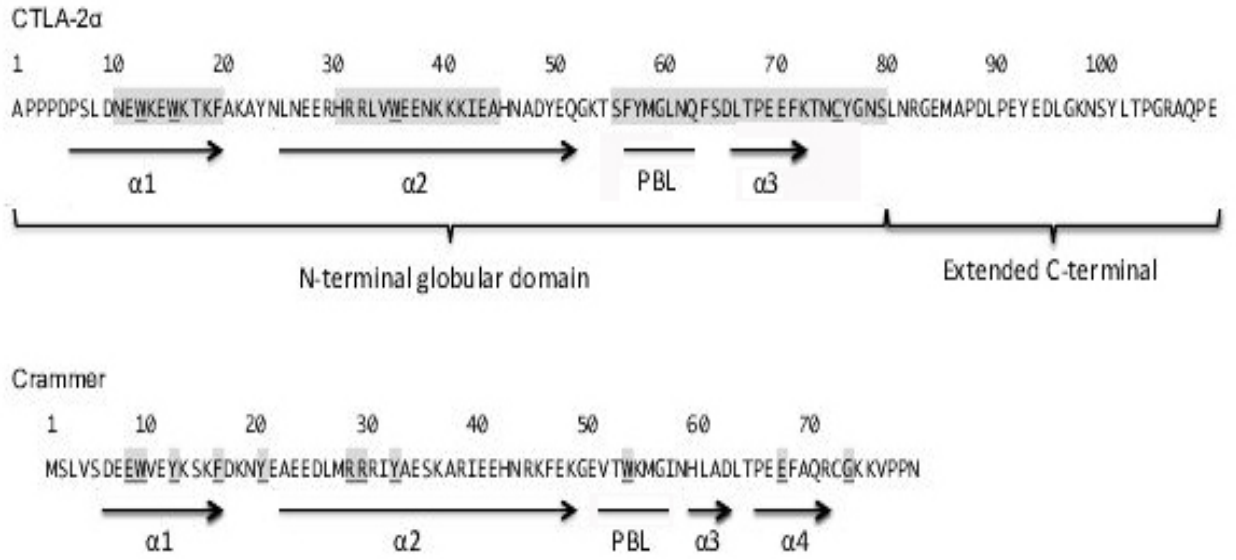

Fig. 2. Essential amino acid residues for inhibitory potency. CTLA-2α: Three essential regions are highlighted by gray boxes. Critical tryptophan residues (W12, W15, and W35) and an essential cysteine residue (C75) are underlined. Crammer: Critical amino acid residues are highlighted by gray boxes. α1, 2, 3, 4 indicate α helixes. PBL: sequences interactive with the prosegment binding loop of the enzymes.

Table 2. Inhibition of cathepsin L by CTLA-2α mutants. Digestion: CTLA-2α mutant proteins were incubated continuously with cathepsin L overnight, and protein bands were evaluated by SDS/PAGE.

Mutant	Ki (nM)	Digestion
Wild	15 ± 4.2	N
W12A	228 ± 3.5	Y
W15A	188 ± 17.6	Y
W35A	250 ± 9.1	Y
W12A/W15A	500 ± 0.1	Y
W12A/W35A	>1000	Y
W15A/W35A	>1000	Y
W12A/W15A/W35A	>1000	Y
C75A	>1000	N
C75S	>1000	N

N, not digested. Y, completely digested.

via the cysteine residue (C75), was isolated using purified proteins. These complexes were also isolated from mouse tissues [40]. As shown in fig. 3, the C75 residue is located close to the catalytic cysteine residue ($C25^E$) of the enzyme in the complex. Based on these finding, CTLA-2α has been suggested to inhibit cathepsin L-like cysteine proteases by oxidizing the active thiol residue of the enzyme with its own thiol residue [32, 36]. This may be a unique property of CTLA-2 because no cysteine residues are present in these regions in most cathepsin L proregions. Similar to propeptide inhibitors, CTLA-2α was not inhibitory towards its cognate enzyme cathepsin L, under neutral pH conditions, but actually stabilized it [20, 32]. The tryptophan mutant (W12A/W15A/W35A) completely lost its stabilizing activities while the C75 mutants (C75A and C75S) exhibited full activities, suggesting that the α1/α2 helix core domain is essential for this stabilizing function, whereas the cysteine residue is not involved in this function.

Novel interactive proteins with CTLA-2α identified recently are cathepsin C (dipeptidyl peptidase 1, C01.070) and tubulointerstitial nephritis antigen-like 1 (TINAGL1, C01.975), both belong to the papain subfamily (C1A) [40]. Complexes of CTLA-2α/cathepsin C and CTLA-2α/TINAGL1 have also been isolated from tissues and cells. The inhibitory and interaction mechanisms of these proteins with CTLA-2α have not yet been elucidated in detail.

2.2. BCPI

Two similar, but distinct BCPIs (BCPIα and BCPIβ) inhibiting BCP were purified from the hemolymph of the silkmoth *Bombyx mori*, and extensive kinetic

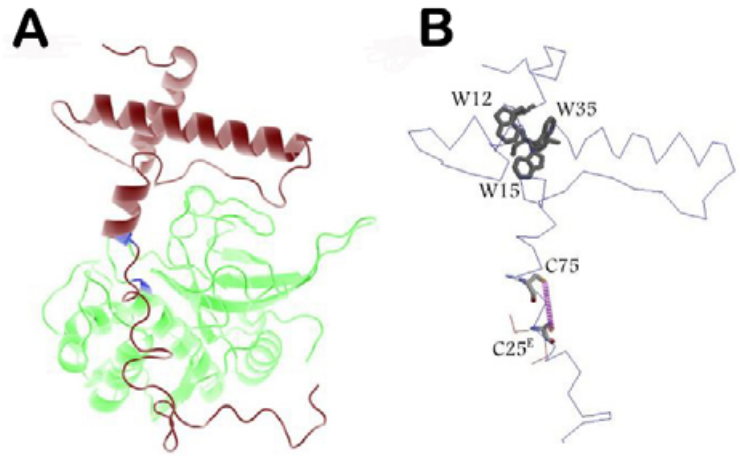

Fig. 3. Tertiary structure of the complex of CTLA-2α and mouse cathepsin L. (A) Structures of CTLA-2α and cathepsin L are shown in red and green, respectively. Cysteine residues (C75 of CTLA-2α and $C25^{E}$, a catalytic Cys of cathepsin L) are shown in blue. (B) Backbones of CTLA-2α and cathepsin L are shown in blue and red, respectively. Tryptophan residues are shown with thick wireframes. A possible disulfide bond is shown in dashed lines. (Reproduced from Deshapriya, R. M. C., Yuhashi, S., Usui, M., Kageyama, T. and Yamamoto, Y. 2010, J. Biochem., 147, 393, with permission from Oxford University Press.)

studies were conducted on BCPIβ [27, 41]. BCPIs are secretary proteins with N-terminal hydrophobic peptides. Unlike CTLA-2, BCPIs do not have internal cysteine residues for the formation of disulfide-bonded dimers. BCPIβ is a strong, highly selective inhibitor of cathepsin L-like cysteine proteases, with previously reported Ki values of 0.036 nM for cathepsin L and 0.0059 nM for BCP [41]. It does not inhibit cathepsin B or papain. By generating mutants of recombinant BCPIβ, a sequence with four amino acid residues, $L^{77} - G - L - R^{80}$, was identified as an essential region for its inhibitory potency [32, 41]. This region is considered to correspond to the sequence interactive with the substrate-binding cleft of the enzyme (Fig. 1) [31].

2.3. Crammer

Crammer was first reported as a *Drosophila* CTLA-2-like protein (D/CTLA-2) by searching the SwissProt database [31], and was identified as a CG10460 gene product of *Drosophila melanogaster* [29]. Crammer does not have an N-terminal hydrophobic peptide, suggesting that it is located in the cytoplasm. Crammer has one internal cysteine residue (C72), and recombinant crammer expressed in the *E. coli* system showed two molecular forms, a monomer and disulfide-bonded dimer [42, 43]. Crammer was previously reported to be predominantly monomeric under acidic conditions and dimeric at neutral and basic pH values [43]. Furthermore, the monomer and dimer were found to be actively inhibitory towards BCP [32, 42]. On the other hand, only the monomeric form was active towards *Drosophila* cathepsin L-like cysteine protease [43]. No reasonable explanation currently exists for this discrepancy. Only the monomeric form was detected in a western blot analysis of *Drosophila* tissue extracts under non-reducing conditions [42, 43]. Crammer may be monomeric, not dimeric, under physiological conditions. Tseng *et al.* elucidated the three-dimensional structure of crammer using NMR spectroscopy, and found that it was very similar to that of the prosegment of procathepsin L [43]. They also demonstrated that crammer was a monomeric molten globule under acidic conditions, and underwent a molten globule-to-ordered structural transition while binding to cathepsin L.

Unlike CTLA-2 and BCPI, recombinant crammer inhibited not only cathepsin L (Ki = 25 nM for cathepsin L), but also cathepsin B (Ki = 30 nM for cathepsin B) (Table 1) [29]. Similar Ki values of 3.9 nM for cathepsin L, 4.7 nM for BCP, and 15 nM for cathepsin B were obtained by another group [42]. Crammer only weakly inhibited papain. However, in contrast, a previous study reported that crammer strongly inhibited *Drosophila* cathepsin L-like cysteine protease with a Ki value of 1.4 nM, but

moderately inhibited *Drosophila* cathepsin B-like cysteine protease with a Ki value of 790 nM [43]. Deshapriya *et al*. attempted to purify cysteine proteases inhibited by crammer from *Drosophila* tissue [42]. *Drosophila* cysteine proteinase 1 (CP1) was identified as a target enzyme of crammer with a Ki value of 12 nM. CP1 is homologous to BCP, and has been classified as cathepsin L1 (arthropod-type) in the MEROPS peptidase database (C01.092). These findings suggest that crammer is also a selective inhibitor of cathepsin L-like cysteine proteases in *Drosophila* cells, similar to CTLA-2α and BCPI.

Tseng *et al.* recently performed alanine scanning experiments in order to identify the amino acid residues of crammer that are critical for its inhibitory activity [44]. Similar to CTLA-2α, W9, Y12, F16, Y20, and Y32, aromatic amino acids in the hydrophobic core of α1/α2 helix, were detected (Fig. 2). Mutations of charged residues (E8, R28, R29, and E67) in salt bridges also significantly decreased inhibitory activities. They further showed that W53 was essential for the interaction between crammer and PBL of the enzyme. Separate alanine scanning experiments were performed to identify the critical amino acid residues for inhibition, with a focus on the sequence of $C^{72} – G – K – K^{75}$, which is known to interact with the substrate-binding cleft of the enzyme (Fig. 1) [32]. In contrast to CTLA-2α, the cysteine residue (C72) was not essential for inhibitory potency. A mutant of C72A was fully active. Instead, a single mutation to G73 (G73A) markedly reduced inhibitory activity, suggesting that the glycine residue (G73) is one of the essential residues for the interaction with the substrate-binding cleft of the enzyme.

2.4. Salarin

Salarin was first purified from the skin of the Atlantic salmon (*Salmo salar*) [30]. It has a higher molecular mass of 43 kDa on sodium dodecyl sulfate/polyacrylamide agarose gel electrophoresis (SDS/PAGE) containing repeated amino acid sequences than those of other inhibitory proteins. cDNA cloning and gene analyses of salarin revealed that it has a 19-amino acid signal sequence and 323-amino acid-matured protein consisting of four almost identical domains (designated as salarins 1, 2, 3, and 4) [45]. A previous study showed that the amino acid sequences of the four salarins were homologous to those of the propeptides of salmon and certain cathepsins. Although salarin was also found to exhibit inhibitory activities towards papain [30], kinetic studies have yet to be conducted.

3. Tissue distribution and biological functions

3.1. CTLA-2

CTLA-2α is strongly expressed in the placenta, brain, and eyes. These organs are immune-privileged sites in which immunotolerant mechanisms that operate to protect tissues from immune-mediated damage have been established [46, 47]. This finding suggests that CTLA-2α functions as an immunoregulator. Furthermore, cathepsin L which is strongly inhibited by CTLA-2α is shown to be expressed during onset of autoimmune diseases such as diabetes type 1 [48, 49].

3.1.1. Placenta

Campo *et al*. first reported that, among mouse tissues, the uterus was a major site for the gene expression of CTLA-2α, and its expression was up-regulated during pregnancy [50]. Cheon *et al.* demonstrated that CTLA-2β mRNA was specifically expressed in the uterus during early pregnancy, and was dependent on progesterone [51]. Its expression was initially detected on day 4 of pregnancy, increased further on days 5-8, and then declined abruptly to an undetectable level by day 10. Its mRNA was localized in the stromal cells surrounding the implanted embryo. Cathepsin L has also been identified as a target protein of CTLA-2β in the uterus. Campo *et al*. showed that CTLA-2β regulated implantation of the embryo by inhibiting cathepsin L-like proteases. Unlike CTLA-2β, the expression of CTLA-2α mRNA increased 10-12 days after completion of the implantation period (day 8), and was then maintained during mid- and late-pregnancy [52]. During these periods, the CTLA-2α protein was consistently localized on the maternal side of the placenta, including the decidua basalis, metrial gland, and myometrium layers, but was not detected on the embryonic side (Fig. 4) [40]. Cathepsin L co-localized with CTLA-2α in these regions. Cathepsin L has been identified as a protease that is important for the proteolytic processing of extracellular matrices, cellular invasion, and migration [7, 8, 53]. Large amounts of cathepsin L have been detected in the placenta, and are secreted in order to allow the

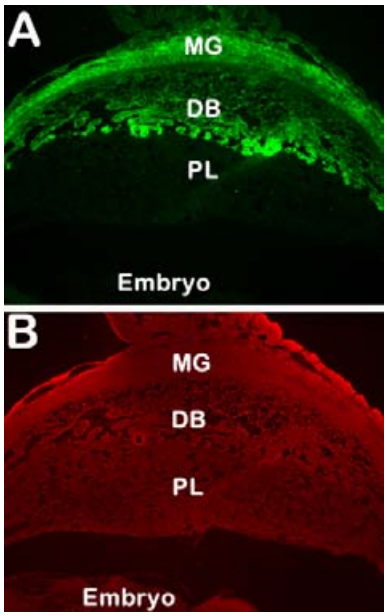

Fig. 4. Distribution of CTLA-2α and cathepsin L in the mouse placenta (10 dpc). Immunofluorescence signals for CTLA-2α (green) and cathepsin L (red) are shown. MG, metrial gland; DB, decidua basalis; PL, placental labyrinth.

blastocyst to penetrate into the maternal endometrium, which later becomes the decidua [54]. CTLA-2α, with its ability to inhibit cathepsin L, has been suggested to play a role in the decidualization reaction and normal placental formation.

Both cathepsin C and TINAGL1 were also co-localized with CTLA-2α in these regions [40]. Cathepsin C, also known as dipeptidyl peptidase 1, is a cysteine protease, the function of which has been characterized in detail in immune systems [55]. In the placenta, cathepsin C has been shown to localize to the decidua [56]. CTLA-2α, which interacts with cathepsin C, may contribute to protecting the embryo from the maternal immune system. The inhibitory mechanisms of CTLA-2α towards cathepsin C *in vitro* and *in vivo* have been attracting increasing attention, but have not yet been elucidated in detail. Tubulointerstitial nephritis antigen-like 1 (TINAGL1, also known as adrenocortical zonation factor, AZ-1, or lipocalin-7) is a non-catalytic cathepsin B-like protein [57, 58]. It is a matricellular protein that regulates cell adhesion and spreading. It has also been shown to interact with laminin 1 [59] and promote integrin-mediated cell adhesion and angiogenesis [60, 61]. A previous study demonstrated

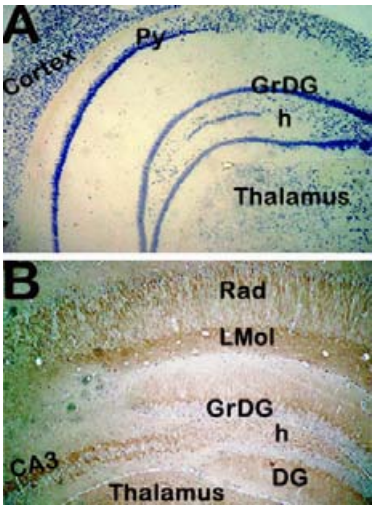

Fig. 5. Distribution of CTLA-2α mRNA and its protein in the hippocampus of the mouse brain. *In situ* hybridization histochemistry (A) and immunoprecipitates (B). The strong expression of CTLA-2α mRNA was observed in the cell bodies of pyramidal neurons (Py) throughout the Connu Ammonis (CA1, CA2, and CA3) fields and in the granular layer of the dentate gyrus (GrDG) and the dentate gyrus (DG). CTLA-2α proteins are located in dendrites radiating from pyramidal neurons and also in Lacunosum Moleculare (LMol) and the CA3 pyramidal neurons.

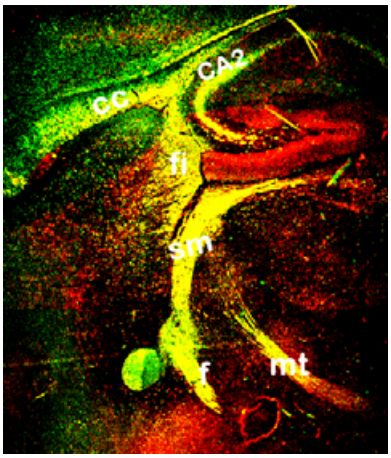

Fig. 6. Double labeling immunofluorescence merged image of Cathepsin L (green) and CTLA-2 (red) showing immunoreactivity in sagittal sections from various regions of the mouse brain. Very high immunoreactivity to indicate co-localization (yellow) is seen in external capsule (ec), corpus callosum (cc), fimbria of hippocampus (fi), Cornu Ammonis 2, 3 fields of hippocampus (CA2 and CA3), stria medullaris of thalamus (sm) and mammillothalamic tract (mt).

that TINAGL1 was expressed in the decidualized endometrium during the post-implantation period [62]. CTLA-2α interacts with TINAGL1 *in vitro* and possible *in vivo*, and is co-localized in the decidual regions. However, its function remains unknown.

3.1.2. Brain

Luziga *et al.* examined the distribution of CTLA-2α mRNA and its protein in the mouse brain [63, 64]. The distribution pattern of CTLA-2α correlated well with that of cathepsin L. Intense expression of cathepsin was observed within cerebral cortices, pyramidal and granular layers in the hippocampus, and the choroid plexus and Purkinje cells in the cerebellum [65, 66]. These regions were also found to express high levels of CTLA-2α, suggesting that the fine equilibrium between the synthesis and secretion of cathepsin L and CTLA-2α is part of the various brain processes maintaining normal growth and development (Fig. 5).

In the hippocampus, the simultaneous inhibition of multiple caspases, a family of cysteine proteases, was found to block long-term, but not short-term spatial memory [67]. Similar findings were reported by Comas *et al.*, who showed that a sharp modulation in the expression of crammer was accompanied by specific long-term memory formation in *Drosophila* [29]. The expression of CTLA-2α, a potent and selective inhibitor of cathepsin L, in the hippocampus has led to a deeper understanding of the relationship between cysteine protease inhibitors and memory formation. The relationship between CTLA-2α and cathepsins in memory formation and establishment was confirmed by co-localization of cathepsin L (a family of cysteine proteases) with CTLA-2α protein in the mouse brain (Fig. 6) [64]. Understanding the cellular relationship of cathepsin inhibitory activity of CTLA-2α in light of the emerging roles of cathepsins in memory formation and establishment is essential in the development of treatments for degenerative diseases associated with learning and memory loss.

3.1.3. Eyes

The ocular pigment epithelium is known to contribute to immune tolerance in the eyes, and retinal pigment epithelial (RPE) cells are the principal mediators of this reaction [68, 69]. RPE cells have been shown to suppress the activation of T cells by releasing soluble factors such as TGFβ, an immunosuppressive cytokine. Sugita *et al.* identified CTLA-2α as a novel immunosuppressive factor in the eyes [70], and found that RPE cells produced and secreted CTLA-2α, which, in turn, induced $CD4^+CD25^+Foxp3^+$Treg cells producing TGFβ. TGFβ also up-regulated the expression of CTLA-2α in RPE cells. CTLA-2α may function as an inhibitor of cathepsin L, with reductions in cathepsin L activity being shown to induce Treg cells in some populations of $CD4^+$T cells. An experimental autoimmune uveitis (EAU) animal model and cathepsin L knockout mice were used in order to confirm this in more detail [71]. The suppression of ocular inflammation was significantly greater in EAU mice with the knockout of cathepsin L than in EAU normal mice. Furthermore, the administration of the recombinant CTLA-2α protein to EAU mice significantly suppressed ocular inflammation. The findings indicated that CTLA-2α, acting as a cathepsin L inhibitor, contributed to the establishment of immune tolerance in the posterior segment of the eye. CTLA-2α may also be functional in the anterior segment of the eye. The same group reported that corneal endothelial cells produced CTLA-2α on their surfaces, and functioned to promote the generation of Treg cells [72].

3.1.4. Others

Insel's group demonstrated that cAMP increased the expression of CTLA-2α in murine T-lymphoma cells and cardiac fibroblasts, and this increase contributed to cAMP/PKA-promoted apoptosis in these cells [73, 74]. This CTLA-2α-promoted apoptosis was independent of the inhibition of cathepsin L activity, thereby suggesting that other unknown enzymes participate in this process. Although cathepsin C or tubulointerstitial nephritis antigen-related protein 1 (TINAGL1) may be a candidate, no evidence currently exists to support this.

3.2. BCPI

BCPI is a strong inhibitor of BCP [41]. It is mainly expressed in the fat body, a homologous tissue to adipose tissue/the liver, secreted in the hemolymph, and then incorporated into developing oocytes [75]. BCPI was purified from the hemolymph of spinning pupae. It is expressed in the fat body and hemocyte,

and is secreted in the hemolymph [27, 28, 76]. A previous study demonstrated that BCP was present as an inactive proenzyme in the hemolymph [75]. Furthermore, BCPI has been suggested to prevent proBCP from uncontrolled activation in the hemolymph [27].

BCPI also exhibits anti-parasitic activity. Yoshiyama and Yamakawa injected the protozoan parasite *Trypanosoma brucei* into the larvae of the silkmoth [76]. They found that the expression level of BCPI significantly increased following the parasite challenge, suggesting that BCPI may contribute to defense mechanisms against invading parasites. The cysteine proteases of protozoan parasites are known to play important roles in host invasion [9]. Since the amino acid sequence of BCPI is homologous to that of the prosegment of the trypanosomal cysteine protease, BCPI may prevent trypanosomal invasion by inhibiting its cysteine protease [21, 76]. Furthermore, these findings suggest a novel role for BCPI in the development of peptidyl inhibitors against invading parasites.

The involvement of cathepsin L-like cysteine proteases in silk gland histolysis has been reported in *Bombyx mori* [77]. The cathepsin L-like cysteine protease fibroinase, which catalyzes the hydrolysis of fibroin, was detected in the silk gland [78], and found to be identical to the mature, active form of BCP. By using transgenic silkworm overexpressing the Ras1 oncogene, Ma *et al.* recently demonstrated that up-regulating the expression of BCPI prevented destruction of the posterior silk gland and delayed pupation [79]. We have also determined the inhibitory activities of BCPI towards cathepsins in the gland. The findings suggest that BCPI, which inhibits cathepsin L-like cysteine proteases including fibroinase, negatively regulates the self-destructive process of the silk gland. Moreover, the control of BCPI expression may be useful for improving silk yield because the amount of fibroin produced was proportional to the gland size.

3.3. Crammer

Comas *et al.* reported that crammer played an essential role in long-term memory (LTM) formation in *Drosophila* [29]. By analyzing enhancer-trap strains, they isolated an aversive LTM mutant. Gene analyses of the mutant revealed that expression of the CG10460 gene was markedly reduced in the mutant, and crammer was the gene product. Other groups found that appetitive LTM formation was also disrupted in the crammer mutant [80, 81]. The expression of crammer has been detected in mushroom bodies (MBs), the *Drosophila* olfactory memory center, and in glial cells around MBs [29]. Although the co-localization and interaction between crammer and cysteine proteases such as *Drosophila* cysteine proteinase 1 (CP1) in these regions has been attracting interest, no study has been conducted to investigate these phenomena. The expression of crammer was previously observed in the whole life cycle of *Drosophila* [42]. Its expression was found to be strong in garland cells and the prothoracic gland in the late stage of embryonic development. However, the elucidation of interactive cysteine proteases for crammer in these tissues remains challenging.

3.4. Salarin

Salarin localizes in the intercellular space of the epidermis and loose connective tissue of the dermis in the skin of the Atlantic salmon [82]. Salarin is known to be expressed in other tissues, such as in the interstitial capillaries of the kidney and liver hepatocytes. A previous study reported that salarin did not exert inhibitory effects on the growth of fish pathogenic bacteria or viruses [82]. The biological functions of salarin currently remain unclear.

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CONFLICT OF INTEREST STATEMENT

The authors confirm that this article content has no conflict of interest.

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