J. Biochem. 2010;147(3):393–404 doi:10.1093/jb/mvp188

JB THE JOURNAL OF BIOCHEMISTRY

Identification of essential residues of CTLA-2α for inhibitory potency

Received September 12, 2009; accepted October 18, 2009; published online November 11, 2009

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To identify functionally essential sequences and residues of CTLA-2α, *in vitro* mutagenesis was carried out. The coefficient of inhibition (K_i) was determined towards rabbit cathepsin L using Z-Phe-Arg-MCA as the substrate. Recombinant CTLA-2α inhibited the enzyme potently ($K_i = 15$ nM). A truncated mutant, lacking the N- and C-terminal Ala1–Asp9 and Leu80–Glu109 regions, was also a potent inhibitor ($K_i = 10$ nM). Subsequent short deletions in the central region (Asn10–Ser79) showed three functionally essential distinct regions: Asn10–Phe19, His30–Ala44 and Ser55–Ser79. These regions cover sequences corresponding to three helices (α1, α2 and α3) and sequences that interact with the cognate enzyme. Alanine scanning showed that replacement of one of three conserved Trp residues increased the K_i by 15–20-fold; whereas, replacement of two/three Trp residues at once caused complete loss of potency, as did replacing Cys75 with Ala or Ser. The proteins from wild-type (WT) CTLA-2α and mutant C75A were stable overnight when incubated with cathepsin L; whereas, proteins from mutants W12A, W15A and W35A were quickly digested. Incubation of cathepsin L/WT CTLA-2α formed a complex; whereas, C75S did not form a complex. Our overall results point to a critical role of W12, W15, W35 and Cys75 residues in CTLA-2α.

Keywords: cysteine protease inhibitor/cathepsin L/CTLA-2/site-directed mutagenesis.

Abbreviations: CP1, cysteine proteinase 1; BSA, bovine serum albumin; suffix 'E', residues of enzyme.

Cysteine proteinases 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*). The papain family is the largest family, the members of which include a wide range of enzymes from both prokaryotes and eukaryotes, encompassing bacteria, plants, vertebrates and invertebrates (*2*), and are essentially synthesized as inactive proenzymes that require processing to form the active enzyme (*3*). The proteinases of this family are implicated in a number of degradative, invasive and pathological processes (*4*). Therefore, cysteine proteinases of the papain family represent attractive targets for the development of therapeutic inhibitors because of their involvement in abnormal physiological processes. However, since they are involved in important roles in normal protein turnover process and also due to their broad substrate specificity, the development of highly selective inhibitors has been a great challenge (*5*, *6*). Structurally, these proteinases comprise three parts: an N-terminal signal sequence is followed by the proregion and the third part represents the mature enzyme (*5*, *6*). The proregion is required for the *in vivo* expression of the zymogen, *i.e.* for its correct folding, stability and functioning as an intra-molecular chaperone in the refolding process (*7–11*). More importantly, a number of studies have shown that the proregion is a potent and often highly selective inhibitor of the cognate enzyme (*12–18*).

Over the past two decades, several cysteine proteinase inhibitor proteins homologous to the proregion of papain like cysteine proteinases (propeptide-like cysteine proteinase inhibitors) have been reported (*19*): *bombyx* cysteine proteinase inhibitors (BCPIs) (*20–22*), cytotoxic T-lymphocyte antigen-2s (CTLA-2s) (*23–29*) and cer protein (*30*, *31*). Comprehensive details are available in the MEROPS peptidase database under clan JF family 129 (http://merops.sanger.ac.uk). BCPI (BCPI-α and β) are cysteine proteinase inhibitor proteins, which are found in the hemolymph of the silkmoth *Bombyx mori* and are homologous to the proregion of certain cysteine proteinases including *Bombyx* cysteine proteinase and are highly selective inhibitors of cathepsin L-like cysteine proteinases (*21*, *22*). The sharp modulation of the expression of cer mRNA (CG10460 gene product) in the *Drosophila melanogaster* brain is accompanied by specific long-term memory formation suggesting its role in memory formation through the regulation of cathepsin L (*30*). The amino acid sequence of cer protein is significantly similar to the proregion of *Drosophila* cysteine proteinases such as CP1 (*31*). CTLA-2 (α and β) genes are mapped to the C1 band of mouse chromosome number 13, which encodes 109 amino acid residues with additional amino-terminal hydrophobic amino acid sequences, the transcripts of which were originally found in mouse activated T-lymphocytes and mast cells (*23*). The amino acid sequences of CTLA-2 are highly homologous to those of mouse cathepsin L

propeptides (*23*). CTLA-2α is an independently expressed separate protein whose exact biological function has not yet been discovered. Recombinant CTLA-2α is a potent, highly selective inhibitor of cathepsin L-like cysteine proteinases (*26*), while recombinant CTLA-2β is a non-selective inhibitor of papain-like cysteine proteinases (*24*). It is reported that CTLA-2α expression is high in the late pregnant uterus (*25*), and the CTLA-2β expressed in early pregnant uteruses appears to be involved in controlling implantation (*27*). Recently, we identified the expression pattern of CTLA-2α mRNA in a neuronal population of the central nervous system (*28*), and we also found that the protein is mainly localized in the dendritic and axonal components of neurons in the mouse brain (*29*). Our very recent studies have shown that retinal pigment epithelium derived CTLA-2α induces TGFβ-producing T regulatory cells, suggesting a novel function of the inhibitor protein (*32*).

Although, many attempts have been made to explore more functionally specific sequences or residues for inhibition in the proregion of papain family (*14*, *15*, *33–35*). So far no detailed systematic study aimed at defining the functionally important sequences or residues responsible for the inhibition by these inhibitor proteins has been performed. The propeptide-like inhibitor proteins may be more advantageous for such studies than the propeptide inhibitors since they are separate proteins that are expressed independently (*26*). In this sense, our interest was developed to further elucidate the inhibitory mechanism and functionally essential regions of CTLA-2α. In the present study, we report the identification of the functionally important regions and residues of CTLA-2α by *in-vitro* mutagenesis. Truncation studies revealed that neither the short N- nor long C-terminals are functionally essential; whereas, deletion of five amino acids motifs led to the identification of three distinct essential regions within the central region. In addition, amino acid replacement showed that Trp and Cys residues play critical roles in inhibitory potency.

Experimental Procedure

Substrate, inhibitor and enzymes

The enzyme substrate, carboxybezoxy-L-phenylalanyl-L-arginine-4-methyl-coumaryl-7-amide (Z-Phe-Arg-MCA) and a specific inhibitor of cysteine proteinases, L-3-trans-carboxyoxirane-2-carbonyl)-L-leucylagmatine (E-64), were purchased from the Peptide Institute (Osaka, Japan). Cathepsin L was purified from a rabbit's liver (*36*, *37*). The concentrations of active enzymes were determined by active-site titration using E-64 (*38*).

Construction of cDNA for CTLA-2α and in vitro mutagenesis

Mutant CTLA-2α cDNA were constructed using routine molecular cloning techniques (*39*). We have established a bacterial expression system for CTLA2-α (*26*). The bacterial expression vector pET16b (Novagen, WI, USA) was modified to express a His-tagged CTLA-2α fusion protein (*26*). The construct corresponding to the WT CTLA-2α was used for all mutant constructs. The subsequent deletion of five amino acids was accomplished using the Transformer site-directed mutagenesis kit (Clontech, CA, USA) as described in the instruction manual. A selection primer was employed for mutagenesis at the Ssp1 restriction site, and mutagenic primers were employed for the desired mutagenesis. After amplification of the desired mutant strand, the DNA was subjected to Ssp1 digestion. The digested DNA was transformed into *E. coli* mutS cells for mismatch repair. Afterwards, plasmid DNA was extracted and subjected to Ssp1 digestion again, followed by transformation into *E. coli* Nova blue cells (Stratagene, CA, USA). In the case of point mutation, the site directed mutagenesis was carried out using the QuikChange II site-directed mutagenesis kit (Stratagene) as described by the manufacturer. By using mutagenic primers the desired mutations were obtained. After the mutagenic reaction, the mixture was subjected to Dpn1 digestion for elimination of methylated parental templates followed by transformation into *E. coli* XL1- blue cells. All mutant cDNA sequences were verified by DNA sequencing. After verifying the success of the mutagenesis, transfection into *E. coli* BL21 (DE3) pLysS (Novagen) cells was performed for expression of the mutant proteins.

Expression and purification of the recombinant proteins

The expression and purification were performed according to a previously described method (*26*) with slight modifications. In summary, *E. coli* strain BL21 (DE3) pLysS cells transformed with the constructed plasmid were grown in 200 ml of Luria–Bertani broth (LB broth) containing ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml) with vigorous shaking at 37°C. Protein expression was induced by the addition of isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 1 mM when A_{600} reached 0.6. After 2.5 h, the bacterial cells were harvested by centrifugation. The cell pellets were washed twice with cold TBS (20 mM Tris–Cl at pH 7.4 and 0.15 M NaCl), suspended, and sonicated in 20 ml of 20 mM Tris–Cl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, 0.1% Triton X-100 containing 0.1 mM phenyl methylsulphonyl fluoride (PMSF) and 0.1 mg/ml lysozyme. The suspension was centrifuged, the supernatant was filtered, and the clear filtrate was applied to a His-Bind resin column (HisTrap HP, GE Healthcare, UK) that had been pre-equilibrated with binding buffer (20 mM Tris–Cl at pH 7.9, 0.5 M NaCl and 5 mM imidazole). The unbound proteins were removed by washing with 30 ml of wash buffer (20 mM Tris–Cl at pH 7.9, 0.5 M NaCl and 60 mM imidazole), and the target proteins were eluted with an elution buffer (20 mM Tris–Cl at pH 7.9, 0.5 M NaCl and 1 M Imidazole). The elution was monitored by measuring absorbance at 280 nm, and the highest concentrated fractions (2.5 ml) were applied to a desalting column (PD-10, GE Healthcare, UK) equilibrated with 20 mM Tris–HCl (pH 7.4), 1 mM EDTA and 0.15 M NaCl, and were eluted with the same buffer (3.5 ml). Pooled protein fractions were stored at −20°C for further analysis.

Enzymatic assays and kinetic analysis

The activity of cathepsins L was determined as described previously by Barrett and Kirschke (*40*). The inhibition properties were studied for each mutant as described earlier (*26*). Briefly, the assay buffer consisted of 0.1 M sodium phosphate (pH 5.8), 1 mM EDTA, 8 mM cysteine and 0.1% Brij®-35. In the assay, 10 μl (0.011 pmoles) of rabbit cathepsin L were pre-incubated for 5 min at 37°C in the assay buffer (390 μl), and the enzymatic reaction was started by adding 100 μl from the mixture containing the substrate (Z-Phe-Arg-MCA) and inhibitor proteins at varying concentrations. Then, the mixture was incubated for a further 5 min before the reaction was stopped by adding 0.1 M sodium monochloroacetate/ 0.1 M sodium acetate buffer (pH 4.3). The amount of MCA produced by the substrate hydrolysis was monitored by measuring the absorbency at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using a spectrophotometer (model F2000, Hitachi Tokyo, Japan). For each mutant, two or three separate measurements were taken from the same batch of protein. The coefficient of inhibition (K_i) values were obtained from Dixon's plots (*41*) of the form $1/V$ versus [inhibitor] at three different substrate concentrations (1–10 μM). The student's *t*-test was used to determine the statistical significance of the increased K_i values.

Treatment of recombinant inhibitor proteins with cathepsin L

Fifteen microlitre (1.65 pmoles) of rabbit cathepsin L were incubated with an equal volume of two times digestion buffer (0.2 M sodium phosphate at pH 5.8, 2 mM EDTA, 16 mM cysteine and 0.02% Brij®-35) for 5 min at 37°C, and digestion was started by mixing it with 120 μl of digestion buffer [0.1 M sodium phosphate (pH 5.8), 1 mM EDTA, 8 mM cysteine and 0.01% Brij®-35] containing 5 μg (300–444 pmoles) of mutant protein. After the incubation, an

aliquot (20 µl) of the reaction mixture was taken, and the reaction was stopped by adding an equal volume of two times SDS sample loading buffer containing E-64 (1.4 mM). As a control, 1 mM E-64 was added to a fraction of the reaction mixture (20 µl) and incubated overnight. Then, the samples were stored at −20°C until the analysis by SDS–PAGE.

Determination of cathepsin L/ CTLA-2α complex by immunoblotting

For determination of complex of cathepsin L and CTLA-2α, rabbit cathepsin L (25 pmoles) and CTLA-2α (2.5 nmoles) were pre-incubated at 37°C in 500 µl of the pre-incubation buffer (0.1 M Na-phosphate, pH 5.8, 1 mM EDTA, 8 mM cysteine). After 5 min, the buffer was quickly exchanged with 0.1 M Na-phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.15 M NaCl by gel filtration with a PD MiniTrap G-25 column (GE Healthcare, UK). The sample was further incubated for 20 min at 37°C. Then, 10 µl of the sample was mixed with 10 µl of 2× SDS-sample buffer (with or without 10% β-mercaptoethanol), and heat treated (100°C, 5 min). Then, aliquot of 20 µl was subjected to SDS–PAGE (10% gel). It was followed by electro-blotting onto a polyvinylidene fluoride membrane and detection using BM Chemiluminescence Western Blotting Kit (Roche Applied Science, USA), according to the manufacture's instructions. Affinity purified rabbit anti-CTLA-2α specific antibody that was prepared as described previously (*29*) and anti-cathepsin L antibody (anti-cathepsin L, CPLH-3G10, Santa Cruz, USA) were used to detect CTLA-2α and cathepsin L, respectively. The analysis was done using LAS-3000mini (FUJIFILM, Japan).

Molecular modeling

The tertiary structural model of the complex of mouse CTLA-2α (NM_007796) and cathepsin L (BC068163) was constructed with a program Modeller (*42*) using crystal structures of human procathepsins L (pdb ID, 1CS8), S (2C0Y) and K (1BY8). Images were created using Pymol ver. 1.1 and Rasmol ver. 2.7.

Results

Production of mutant CTLA-2α

Studies on the propeptides of cysteine proteinases have elucidated the contribution of the N- and C-terminals to the overall inhibition (*14*, *15*). Also, the CTLA-2α sequence is highly homologous to mouse cathepsin L propeptide (Fig. 1A). By preliminary experiments, we have found that both N- and C-terminals of CTLA-2α are not functionally essential and only a polypeptide of 70 residues (Asn10–Ser79), the central region of the CTLA2-α protein, exerts inhibitory activity almost identical to that of WT CTLA-2α ($K_i = 10$ nM), which consists of 109 residues. Therefore, we first tried to construct mutants by sequential deletion of five amino acids in the central region (Asn10–Asn79). Next, we carried out alanine scanning and other replacements in the regions, which were identified as essential by the deletion experiments.

The recombinant mutant proteins were extracted from the bacteria as soluble fractions and purified using His-affinity chromatography. Each purified mutant protein (0.5–4 mg) was obtained from 200 ml of bacterial culture. The homogeneity and molecular weights of the proteins were ascertained by SDS–PAGE. The protein concentrations of the recombinant proteins were determined by the method of Bradford using BSA as a standard (*43*) and the predicted molar extinction coefficient at 280 nm (*44*). Based on the three Trp, six Tyr and one Cys residue contained in the sequence, the molar extinction coefficient of the recombinant wild CTLA-2α was estimated to be 21,870 $M^{-1}\,cm^{-1}$. In the case of the mutants in which those residues had been replaced, the calculation was made by subtracting the molar extinction coefficients of the respective residues.

Inhibitory profiles

Initially, we determined the residual activity of cathepsin L against different concentrations (1–1000 nM) of inhibitor proteins under a constant substrate concentration (3 µM) to assess its inhibitory potency. Its residual activity against some important mutants is illustrated in Fig. 2A. In a previous report, we showed that recombinant CTLA-2α (WT) is a potent, selective inhibitor of human cathepsin L and that the inhibition obeys slow-binding kinetics (*26*). CTLA-2α fully inhibited rabbit cathepsin L, but its inhibition kinetics were different. Under the

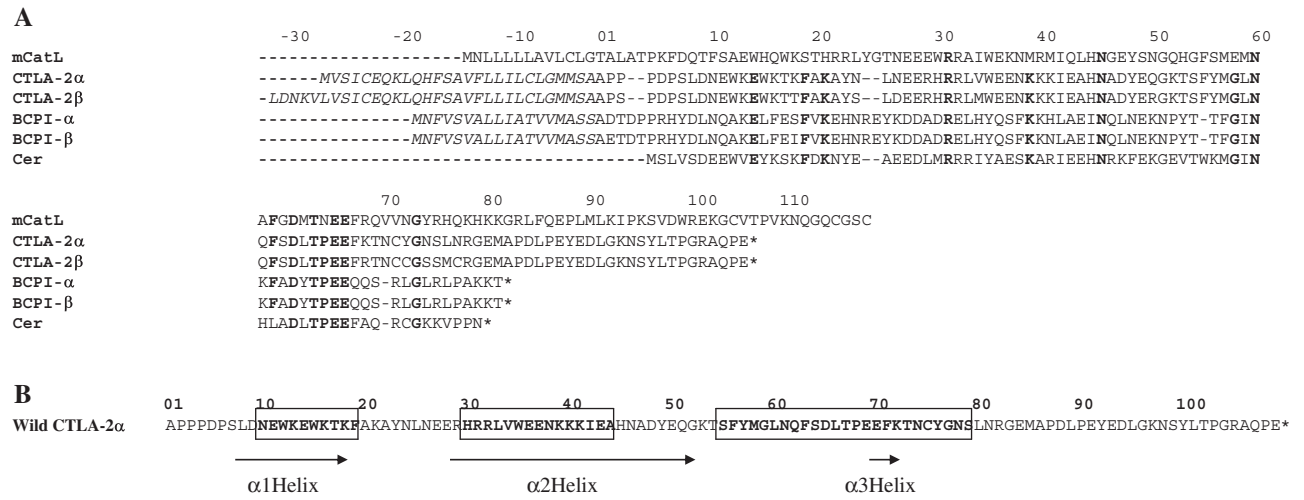

Fig. 1 Alignment of propeptide-like cysteine proteinase inhibitor proteins and identified essential sequences of CTLA-2α. (A) the highly conserved amino acids residues are shown in bold. Gaps introduced to optimize the alignment are marked with dashes. N-terminal numberings based on the mature CTLA-2α. Genbank accession numbers: mcatL (mouse cathepsin L) NP034114, CTLA-2α S04924, CTLA-2β p12400, BCPI-α AU401721, BCPI-β CAB41937, Cer AAF57567. (B) In CTLA-2α, three helices α1, α2 and α3 (arrows), and three essential regions (bold-letters inside boxes) were defined.

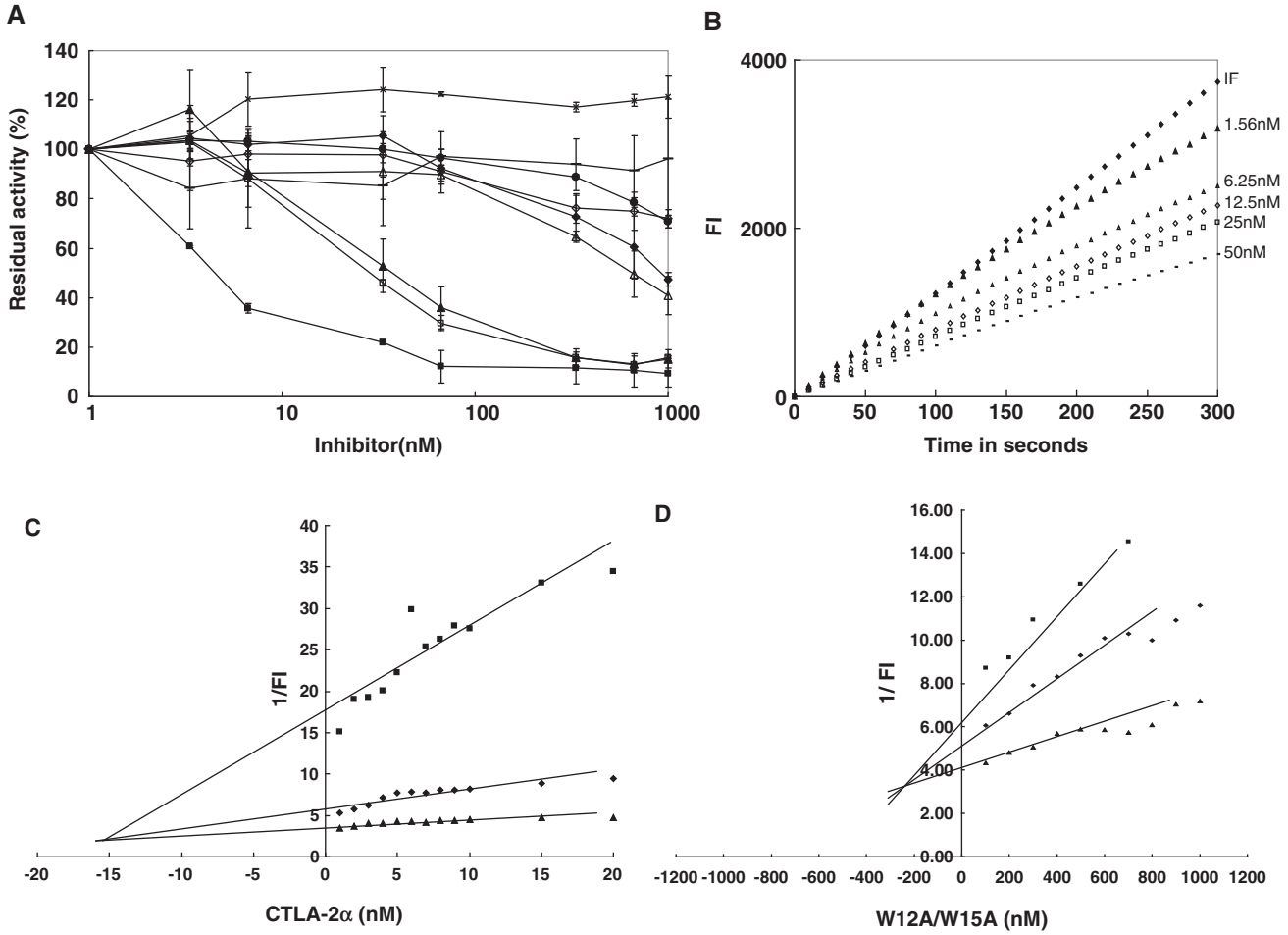

Fig. 2 The effects of the CTLA-2α recombinant proteins on cathepsin L. (A) residual activity as a percentage of the total activity against different concentrations of inhibitors. (filled square) WT CTLA-2α, (open square) mutant W15A, (filled triangle) mutant W12A, (open triangle) mutant W12A/W35A, (filled diamond) mutant W12A/W15A/W35A, (open diamond) mutant W12A/W15A, (filled circle) mutant W15A/W35A, (−) mutant C75A, (×) mutant C75S, ($n = 3$). (B) progress curves obtained with increasing concentrations of CTLA-2α (WT) were shown to be linear. (C,D) Dixon's plot representations of the inhibition of cathepsin L by WT CTLA-2α, and mutant W12A/W15A, respectively. The slope of the each curve was used to derive the K_i values under three different substrate concentrations [(filled square) 3 μM, (filled diamond) 5 μM, (filled triangle) 10 μM]. FI denotes fluorescence intensity.

experimental conditions used here for the inhibition of rabbit cathepsin L, the progress curve for substrate hydrolysis appeared to be linear, suggesting that the inhibition obeyed classical kinetics (*45*) (Fig. 2B). Therefore, K_i values were determined from Dixon's plot of the form $1/V$ versus $[I]$ (Fig. 2C). All mutants were shown to obey classical binding kinetics and as examples, Dixon's plot for the mutant W12A/W15A is illustrated in Fig. 2D. By referring to the K_i values (Table 1) of the short deleted mutants that showed loss of inhibitory activity, it was revealed that there are three distinct regions that are essential for the inhibitory activity. These sequences are identified as Asn10–Phe19, His30–Ala44 and Ser55–Ser79 as shown in Fig. 1B. Further, sequence alignment (Fig. 1A) showed that there are highly conserved and other functionally critical residues within these three regions. Therefore, in an attempt to elucidate their functional contribution, some of them were substituted. Initially, each amino acid was replaced with an Ala residue. Ala was chosen because it is the least structurally disruptive amino acid (*46*). The results

Table 1. The K_i values of five amino acid deletion mutants.

Mutant	Number of residues	K_i (nM)
Wild type	109	15 ± 4.2
Δ10–14	104	800
Δ15–19	104	800
Δ20–24	104	90
Δ25–29	104	33
Δ30–34	104	>1000
Δ35–39	104	>1000
Δ40–44	104	600
Δ45–49	104	40
Δ50–54	104	25
Δ55–59	104	>1000
Δ60–64	104	>1000
Δ65–69	104	>1000
Δ70–74	104	>1000

The mutants are named base on deleted sequences. Remaining number of residues are also indicated.

revealed that replacing three Trp residues (W12A, W15A, W35A) in this manner significantly enhanced the K_i values, which became 228, 188 and 250 nM, respectively (Table 2). This was 15–20-fold higher

Table 2. The K_i values and amino acids sequences of single amino acid replacement mutants aligned with wild CTLA-2α.

Mutant	Amino acid sequence							K_i (nM)
	20	30	40	50	60	70	79	
Wild type	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	15 ± 4.2
W12A	NE**A**KEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	228 ± 3.5
E14A	NEWK**A**WKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	9 ± .01
W15A	NEWKE**A**KTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	188 ± 17.6
F19A	NEWKEWKTK**A**A	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	20 ± 14
R31A	NEWKEWKTKFA	KAYNLNEERH	**A**RLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	9 ± 0.7
W35A	NEWKEWKTKFA	KAYNLNEERH	RRLV**A**EENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	250 ± 0.1
N38A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEE**A**KK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	18 ± 2
K39A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEEN**A**K	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGNS	25 ± 4.2
F56A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTS**A**YMGL	NQFSDLTPEE	FKTNCYGNS	10 ± 6.7
M58A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFY**A**GL	NQFSDLTPEE	FKTNCYGNS	9 ± 1.2
G59A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYM**A**L	NQFSDLTPEE	FKTNCYGNS	10 ± 0.7
N61A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	**A**QFSDLTPEE	FKTNCYGNS	12 ± 0.7
F63A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQ**A**SDLTPEE	FKTNCYGNS	11 ± 0.7
D65A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFS**A**LTPEE	FKTNCYGNS	4 ± 1.06
T67A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDL**A**PEE	FKTNCYGNS	6 ± 1.06
E69A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTP**A**E	FKTNCYGNS	7 ± 1
E70A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPE**A**	FKTNCYGNS	13 ± 1.5
C75A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTN**A**YGNS	>1000
C75S	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTN**S**YGNS	>1000
Y76A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNC**A**GNS	18 ± 1.6
G77A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCY**A**NS	9 ± 0.35
N78A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYG**A**S	4 ± 1.7
S79A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	FKTNCYGN**A**	15 ± 3.2

The mutants are named base on positions that were replaced by alanine. The replaced amino acids are bold. K_i values are indicated as mean ± SD.

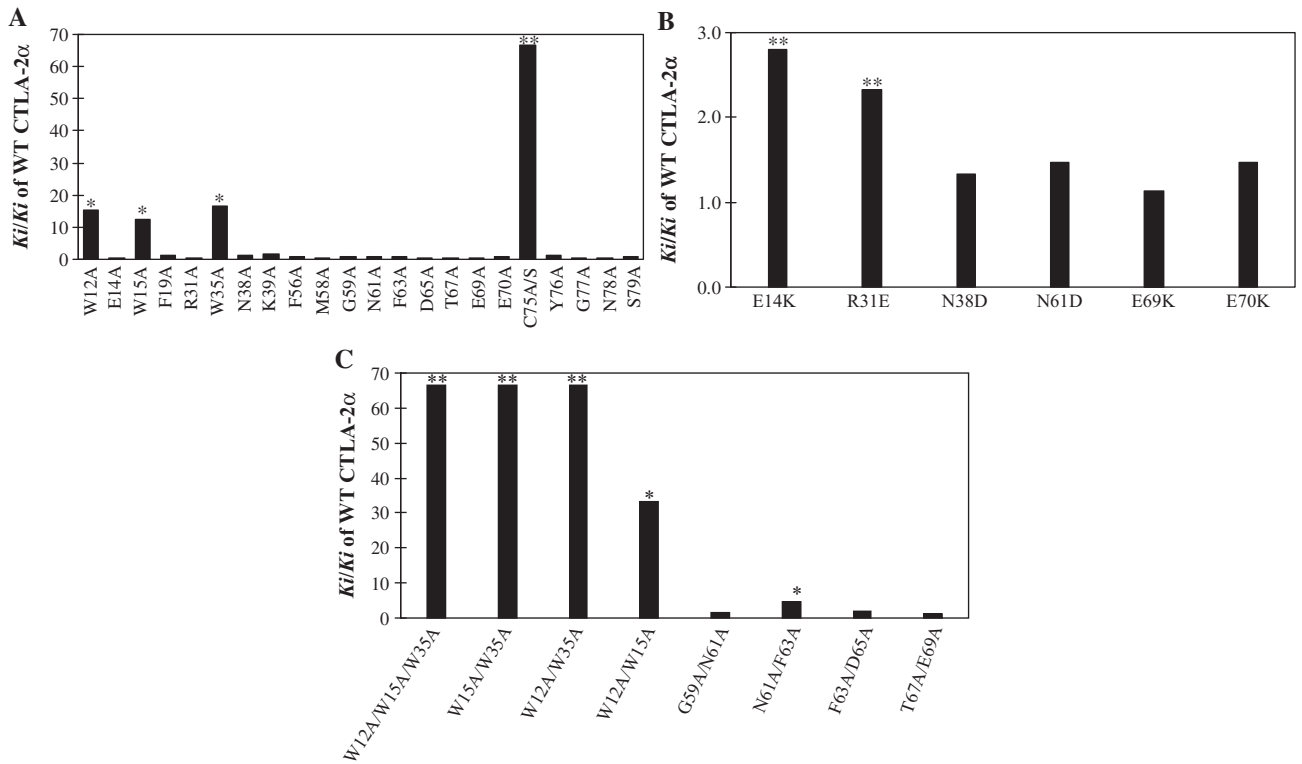

Fig. 3 K_i values of the various CTLA-2α mutant proteins towards cathepsin L. Relative K_i values were obtained against CTLA-2α (WT). (A) Single alanine substituted. (B) Opposite charged amino acids substituted. (C) Multiple alanines substituted. *X*-axis denotes mutant's names, $p < 0.001$(**), $p < 0.05$(*).

than that of the WT (Fig. 3A). On the other hand, replacing Cys75 with Ala caused a significant increase in the K_i value (Table 2) to more than 60-fold higher than that of WT (Fig. 3A), indicating that the mutant had lost almost all inhibitory activity. Replacement of all other selected amino acids with a single Ala residue had no significant effect on the inhibitory activity or K_i values (Table 2, Fig. 3A). However, replacing some

Table 3. The K_i values and amino acids sequences of multiple amino acid replacements/ changed to amino acids with the opposite charge.

Mutant	Amino acid sequence						K_i (nM)
	20	30	40	50	60	70	
Wild type	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	15 ± 4.2
W12A/W15A/W35A	NE**A**KE**A**KTKFA	KAYNLNEERH	RRLV**A**EENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	>1000
W15A/W35A	NEWKE**A**KTKFA	KAYNLNEERH	RRLV**A**EENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	>1000
W12A/W35A	NE**A**KEWKTKFA	KAYNLNEERH	RRLV**A**EENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	>1000
W12A/W15A	NE**A**KE**A**KTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	500 ± 0.1
G59A/N61A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYM**A**L	**A**QFSDLTPEE	22 ± 3
N61A/F63A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	**A**Q**A**SDLTPEE	70 ± 14
F63A/D65A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQ**A**S**A**LTPEE	30 ± 1.2
T67A/E69A	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDL**A**P**A**E	17 ± 0.3
E14K	NEWK**K**WKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	42 ± 0.7
R31E	NEWKEWKTKFA	KAYNLNEERH	**E**RLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	35 ± 2
N38D	NEWKEWKTKFA	KAYNLNEERH	RRLVWEE**D**KK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPEE	20 ± 3.5
N61D	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	**D**QFSDLTPEE	23 ± 0.8
E69K	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTP**K**E	17 ± 2.8
E70K	NEWKEWKTKFA	KAYNLNEERH	RRLVWEENKK	KIEAHNADYE	QGKTSFYMGL	NQFSDLTPE**K**	22 ± 2.1

The mutants are named base on positions that were replaced by alanine or other amino acids. The replaced amino acids are bold. K_i values are indicated as mean ± SD.

charged amino acids with opposite charged amino acids caused a discernible change in the inhibitory activity. Notably, replacing Glu14, Arg31, with Lys, Glu, respectively, resulted a significant increase of K_i values of 2–3-fold higher than the WT (Table 3, Fig. 3B). Subsequently, replacement of two or three amino acids with Ala significantly changed the inhibitory activity, especially with respect to Trp replacement. When all three Trp were replaced at once (W12A/W15A/W35A), the K_i values indicated that the mutant had lost almost all inhibitory activity (Table 3, Fig. 3C). When two Trp were replaced with Ala (W15A/W35A, W12A/W35A, W12A/W15A), the K_i values significantly increased in comparison to the WT (Fig. 3C). In addition, when Gly59, Asn61, Phe63 and Asp65 (G59/N61A, N61A/F63A, F63/D65A) were replaced with Ala, the K_i values were moderately enhanced (Table 3), showing 1.4–5-fold increases compared with the WT (Fig. 3C). Overall, results of Ala scanning highlight the importance of the Trp stack and Cys75 at functional level. The findings with respect to Trp are in agreement with similar findings of cathepsin L-like propeptides. However, the exiting information given by C75A mutant indicates the critical role of Cys75 for enzyme/Inhibitor interaction. Therefore for further assessment of its role, we attempted to see the hydrogen-bonding interaction between residues C75 and $C25^E$ by replacing Cys75 with Ser (C75S). Interestingly the mutant showed almost similar results to that of C75A mutant, suggesting that the hydrogen-bonding interaction is not involved in the inhibitory activity (Table 2, Fig. 3A). Thus, it is obvious that Cys itself at position 75 is the most critical for CTLA-2α in terms inhibition.

Digestion of mutant proteins by cathepsin L

Bombyx cysteine proteinase inhibitor (BCPI) is a potent inhibitor of its cognate enzyme, *Bombyx* cysteine proteinase (BCP), and is resistant to enzymatic digestion by the enzyme (*22*). CTLA-2α was also shown to be resistant to digestion by rabbit cathepsin L through continuous incubation overnight (Fig. 4A). To assess whether the mutant proteins are still capable of resistance and whether this is related to their inhibitory potency, we incubated each mutant protein with rabbit cathepsin L. Similarly, the proteins of mutant C75A were resistant to digestion even though it is not inhibitory (Fig. 4B). Contrary to these observations, the mutant W12A/W15A (Fig. 4C) exhibited onset of digestion when incubated with cathepsin L. The digestion profiles of some important mutants are illustrated in Fig. 5. The mutant proteins that exhibited an inhibitory activity in the nanomolar concentration range were resistant to digestion. The mutants N61A/F63A and W35A were resistant to digestion for up to 4 h of incubation, whereas the mutant Δ45–49 was resistant for up to 1 h of incubation. The mutants W12A and W15A were shown to be resistant for only 3 min. In contrast, all other Trp replaced mutants and five amino acid deleted mutants that are shown in Fig. 5 were susceptible to digestion and showed a rapid onset of digestion. Taken together, it is suggested that the CTLA-2α protein is vulnerable to digestion by cathepsin L instead of inhibition when functionally essential sequences or residues are deleted or replaced, except in the case of mutant C75A when no digestion was observed.

Identification of cathepsin L/CTLA-2α complex

Our overall results point to the dominant role of the C75. The K_i values of both mutant C75A and C75S indicated that C75 itself is needed for the proper function of the inhibitor. Therefore, our interest was developed to determine the formation of complex of cathepsin L /CTLA-2α**.** To achieve this, cathepsin L /CTLA-2α, cathepsin L /mutant C75S, CTLA-2α and cathepsin L were incubated and subjected to SDS–PAGE with or without β-mercaptoethanol in the loading buffer and analysed by western blot using anti-CTLA-2α antibody and anti-cathepsin L antibody. The analysed western blots are indicated in Fig. 6. When the western blot was probed with anti-CTLA-2α antibody, prominent bands corresponding to CTLA-2α could be detected in lanes 3, 4 and 5.

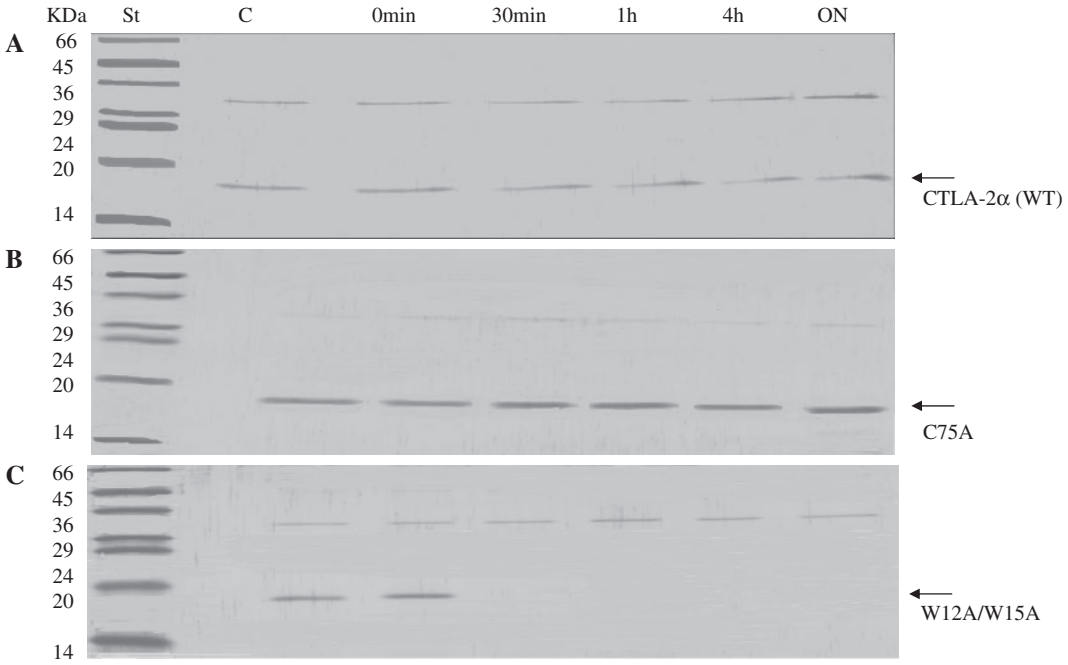

Fig. 4 Digestion of the CTLA-2α recombinant proteins by cathepsin L. The digested proteins were separated by 15% SDS–PAGE and visualized by silver staining in the presence of a standard marker (St). A control sample (C) was incubated overnight in the presence of E-64. Samples were taken just after the start of incubation (0 min) and after 30 min, 1 h, 4 h and overnight (ON). (A) WT CTLA-2α protein. (B) Mutant C75A C: mutant W12A/W15A.

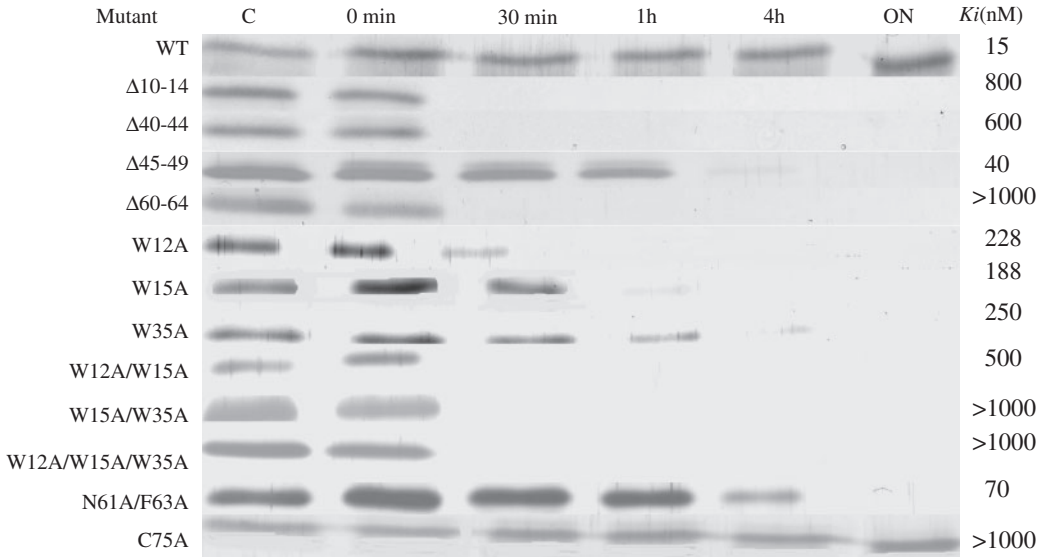

Fig. 5 Digestion profiles of the various CTLA-2α mutant proteins. Digestion was carried out as shown in Figure 4, and only bands corresponding to CTLA-2α are presented. The names of the mutants (right) and their respective K_i values (left) are indicated.

In lane 4, an additional band that is thought to be complex of cathepsin L/CTLA-2α could be seen. The absence of such band in lane 5, which was loaded with the same sample that of in lane 4 but treated with β-mercaptoethanol, indicates that the complex disappear under reducing condition, suggesting that complex formation involve disulfide bonding. Further, lack of band corresponding to such a complex in lane 6, which was loaded with incubated cathepsin L/mutant C75S protein confirms that the complex is formed by disulfide bonding. Moreover, a faint but distinct band was seen in lane 7 with anti-cathepsin L antibody which further indicate that the band in lane 4 is complex of cathepsin L/CTLA-2α.

Discussion

For the first time, we report the findings of a comprehensive study analyzing the inhibitory mechanisms of the propeptide-like cysteine proteinase inhibitor CTLA-2α. Since CTLA-2α is efficiently expressed in *E. coli* and its active inhibitory proteins are recovered in their soluble form (*26*), the bacterial expression system can be used as an efficient system for *in vitro*

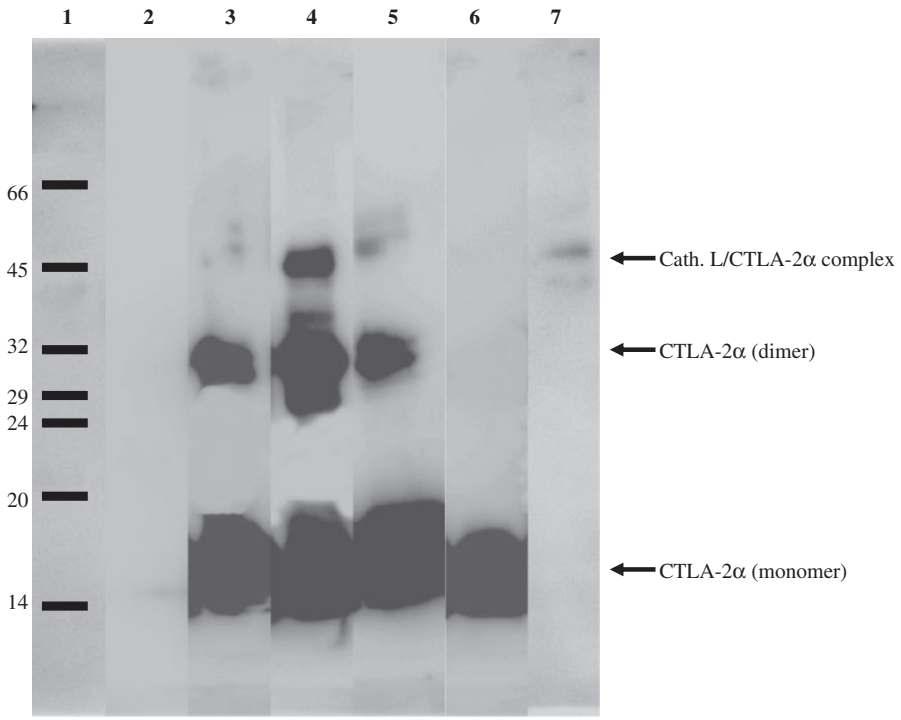

Fig. 6 Western blots analysis for cathepsin L/CTLA-2α Complex. Lane 1, a molecular marker showing particular sites of the marker protein; lane 2, cathepsin L; lane 3, CTLA-2α; lane 4,5,7, cathepsin L/CTLA-2α; lane 6, cathepsin L/mutantC75S. Probing was done with anti-CTLA-2α antibody for lanes 1–6 whereas lane 7 was probed with anti-cathespin L antibody. The electrophoresis was done under non-reducing conditions except for lane 5 where the sample buffer contained 5% β-mercaptoethanol.

mutagenesis studies of CTLA-2α. The proteins were expressed as fusion proteins with an N-terminal His-tag sequence, and kinetic studies were performed with these extra amino acid terminal sequences. Our primary observation with respect to the BCPI-β revealed that the His-tag has little influence on the inhibitory activity. In this study, WT CTLA-2α and its mutants obeyed classical binding kinetic toward rabbit cathepsin L. However, WT CTLA-2α obeyed slow-binding kinetics towards human cathepsin L as we reported previously (*26*). Even though, the reasons for the discrepancy in the kinetic mechanism are beyond the scope of this study. It could be reasonably explained that even the same inhibitor could obey different inhibition mechanism depending on the source of the target enzyme as we have shown in our previous study (*31*).

By truncation study it is found that extended C-terminal region (Leu80–Glu109) in CTLA-2α is not functionally important. This is similar to other reported propeptides such as that for human cathepsin L, in which deletion of 15 C-terminal residues do not affect inhibition (*14*), and in rat cathepsin B, the last 10 residues are reported to be functionally non-essential, suggesting little contact with the cognate enzymes (*15*). However, BCPI, in which the C-terminal end is shorter than that in CTLA-2α (Fig. 1A), is still a potent inhibitor, suggesting that the C-terminal end interacts with the enzyme active site (*19*, *22*). This indicates that longer C-terminals are not essential for propeptides or propeptide-like inhibitors, but rather only the sequence up to the enzyme-contacting region is essential.

Central region (Asn10–Ser79) is the functional region of CTLA-2α. Within this region, three essential regions (Asn10–Phe19, His30–Ala44 and Ser55–Ser79) were identified (Fig. 1B). The regions Asn10–Phe19 and His30–Ala44 mostly relate to the α1 and α2 helices, respectively; whereas, the Ser55–Asn79 region contains a sequence that interacts with prosegment binding loops (PBL), the α3-helix, and a sequence that interacts with active site of the enzyme. Also, it must be noted that within these regions, several highly conserved residues among propeptide-like inhibitors are located, including GNFD and TPEE. In contrast, a cathepsin L proregion that had 20 N-terminal residues deleted and lacked its α1-helix, still retained significant inhibitory activity (*14*). However, removal of the sequence corresponding to the α2-helix resulted in a dramatic reduction in potency (*14*). It has also been demonstrated that in cathepsin L, the α2-helix contributes to the conformational stabilization of the adjacent structures, the β1-strand and α3-helix, which act as anchors in the mature enzyme (*47*). In addition, systematic deletion of the N-terminal 20 residues, which resemble the α2-helix, in a synthetic peptide corresponding to cathepsin B propeptide exhibited a progressive increase in K_i value (*15*).

Alanine scanning experiments revealed that three Trp in the α1/α2-helices contribute significantly to inhibition because W12A, W15A and W35A mutants decreased inhibitory activity significantly. These Trp residues form the hydrophobic core between the first and second α-helices, not contacting directly with the cathepsin L moiety. They are thought to be necessary

for the correct conformation of two α-helices in formation of globular domain. In addition, analysis of circular dichroism spectra revealed destabilization of α-helices when compared to WT as indicated in Supplementary Fig. S1. Our findings with regards to Trp replaced mutants of CTLA-2α reveal a structural form similar to that of well-established cathepsin L like propeptides (*47–49*). However, it is interesting to note that CTLA-2α has only 42% sequence homology with mouse cathepsin L proregion. Similarly, Kreusch *et al.* (*34*) described that human cathepsin S propeptide mutants in which one of its three Trp residues has been replaced are 3-fold less effective as inhibitors than mature cathepsin S. Also, they suggest that Trp residues play an essential role in the post-translational events such as proper folding, transport, phosphorylation, secretion and maturation of procathepsin S. Also, Alanine scanning for functionally important motifs of rat cathepsin B propeptide revealed that the aromatic side chain of Trp24, which is located in a depression on the surface of the protein, contributes decisively to inhibition (*15*). In this context, it is obvious that N-terminal globular domain of CTLA-2α decisively and strongly influence the affinity of the protein to the target enzyme even though it has no direct contact with the occlusion of the active site cleft or other region of the enzyme. Moreover, although two α-helices do not contact directly with cathepsin L, they might be essential for maintaining the correct geometry of the succeeding Phe56–Asn61 loop that contacts with cathepsin L. Therefore any alteration in the structural geometry of α-helices causes loss of inhibition. The results from the digestion experiments in which mutants that had their Trp residues replaced were rapidly digested by the enzyme (Figs. 4 and 5). This further explains that any changes that affect the N-terminal structural geometry cause drastic loss of inhibition and protein become susceptible to proteolytic degradation.

The most important and striking finding in our study was the elucidation of the contribution of C75. In the current study, replacement of Cys75 with Ala or Ser caused drastic loss of inhibition. The results of C75S mutant indicate lack of involvement of hydrogen bond formation at the active site. This suggests that the thiol side chain of Cys75 is essential for the inhibition. Since equivalent Cys is not found in the propeptide of cathepsin L and related enzymes, the residue might be specific for CTLA-2α. Cys75 is located close to the catalytic $Cys25^E$ of cathepsin L, suggesting the interaction of both thiol residues to generate disulfide bond resulting the inhibition. For further clarification of this exiting point we tried to determine the formation of complex of cathepsin L/ CTLA-2α. Initially we attempted to detect the complex by incubating cathepsin L/ CTLA-2α in buffer that contained cysteine because it is known that reducing reagent is essential for activation of enzyme. But it was not possible to detect a complex formation. Therefore, at first cathepsin L/ CTLA-2α was incubated with cysteine containing buffer and quickly exchanged the buffer by which the complex is stabilized. By preliminary western blot analysis, we found three subunits for rabbit cathepsin L with approximate molecular masses of 31, 25, 20 kDa. As indicated in (Fig. 6), the appearance of a distinguish band with approximate molecular mass of 45 kDa suggests that cathepsin L (31 or 25 kDa)/ CTLA-2α (16 kDa) interaction makes a complex. Further its disappearance in reducing condition and with mutant C75S in which Cys75 is replaced confirms that complex is formed by disulfide bonding. In this context, it could be proposed that the formation of disulfide bond between $C75–C25^E$ is essential for the cathepsin L/ CTLA-2α interaction. In contrast, replacement of Cys42 with Ala in the active site binding region of the propeptide of rat cathepsin B causes a 140-fold increase in K_i, where it is suggested that the side chains of residue Cys42 occupy the S_1 subsite and interact with many hydrophobic residues (*15*).

Since CTLA-2α has high sequence similarity to cathepsin L-like propeptides, using crystal structures of human procathepsins L, S and K as template, a typical model of the complex of CTLA-2α and cathepsin L was constructed by molecular modelling. The tertiary structure of the complex thus constructed resembled well with the structure of procathepsins (Fig. 7A). There are three α-helices in the N-terminal half of CTLA-2α, namely α1(Ser7–Phe19) α2(Glu28–Gln51) and α3 (Thr67–Phe71) (Fig. 7A). Between second seven-turn α-helix and third two-turn α-helix, a loop structure is obvious which is thought to be important to the affinity of CTLA-2α with cathepsin L. The part following to the third helix dips into the substrate-binding cleft of cathepsin L. C-terminal part of CTLA-2α might form an extended loop structure, although the molecular modelling of this part is rather difficult due to the lack of templates. Mouse cathepsin L has been shown to be two-chain structure consisting from heavy and light chains, which is generated by the proteolytic deletion of the Asp–Ser dipeptide localizing between $Thr175^E$ and $Asn176^E$ in the initial transcript of the enzyme. The tertiary structure of mouse cathepsin L resembled well with the human enzyme. Although human enzyme has shown to have three disulfide bonds ($Cys22^E–65^E$, $56^E–98^E$, $156^E–207^E$), additional fourth disulfide bond ($Cys12^E–33^E$) is expected in the mouse enzyme. It is notable that catalytic $Cys25^E$ locates close to Cys75 of CTLA-2α. The Phe56–Asn61 loop contacts with the $Pro141^E–Ile150^E$ loop of cathepsin L. The interactions between these two loops are hydrogen bonds formed between the main-chain atoms of these two antiparallel loops, and also hydrophobic contacts between side chains including Phe56, Tyr57, Met58, Phe71, $Pro141^E$, $Leu143^E$, $Phe145^E$, $Tyr146^E$ and $Ile150^E$. Apart from these direct contacting areas, a major hydrophobic core formed by Trp12, Trp15 and Trp35 is obvious between the first and second α-helices, probably contributing to the stability of the N-terminal helical structures of CTLA-2α (Fig. 7B). The sequence that is thought to interact with PBL is Phe56–Asn61 that located in the third essential region as identified in this study. The Phe56–Asn61 loop is thought to be important for the affinity of CTLA-2α with cathepsin L, contacting directly with the enzyme moiety. Although a single Ala mutation of a residue in

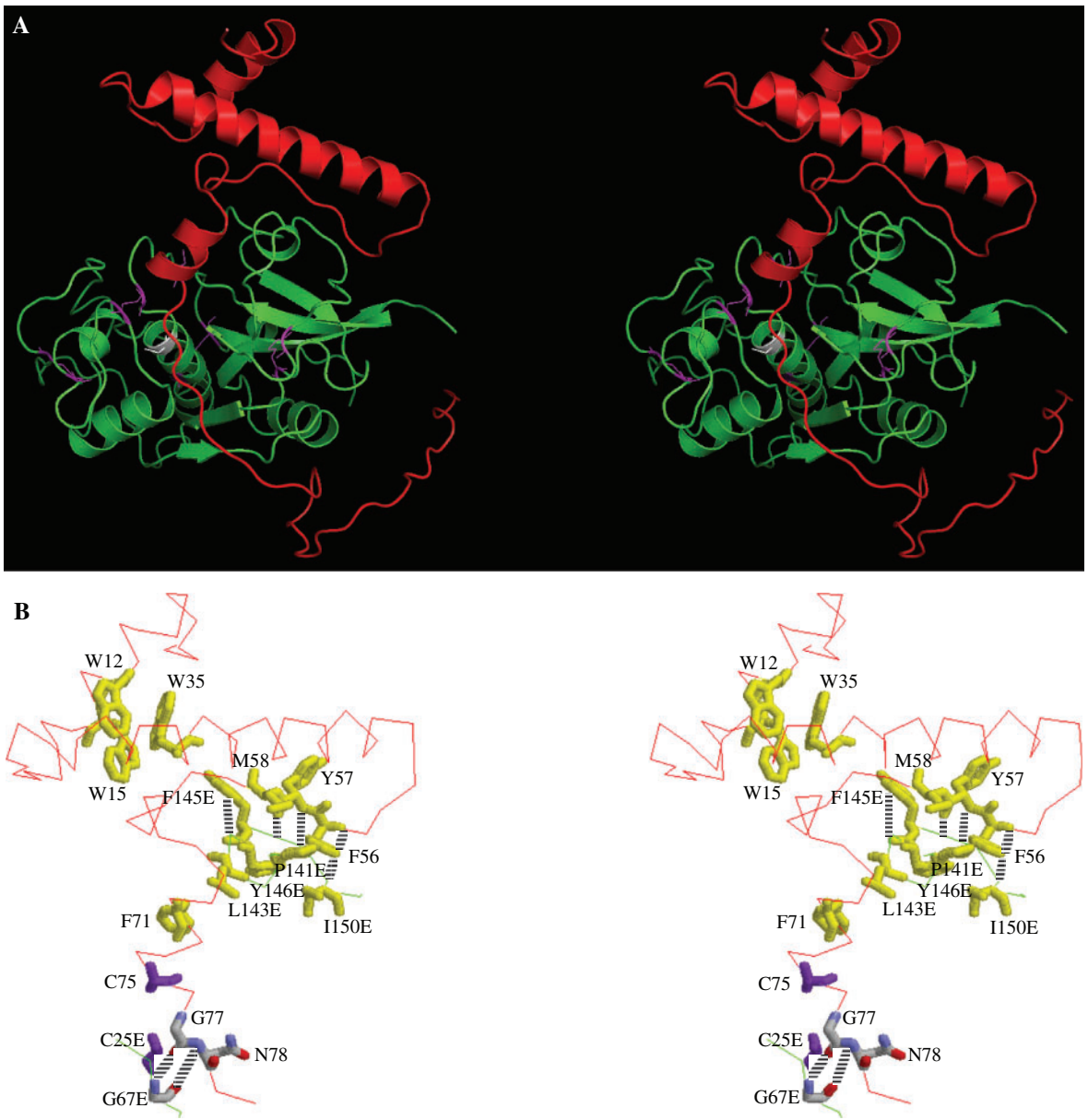

Fig. 7 Stereoview of the tertiary structure of the complex of mouse CTLA-2α and mouse cathepsin L. (A) The structures of CTLA-2α and cathepsin L are shown with red and green, respectively. Cys residues are shown with purple wireframes except for Cys25 ^{E}, a catalytic Cys, which is shown with white wireframes. The figure was crated with Pymol. (B) Highlights the residues being involved in the interactions between two polypeptides. Backbones of CTLA-2α and cathepsin L are shown with red and green lines, respectively. Hydrophobic residues and Tyr are shown with thick yellow wireframes, Cys75 and Cys25^{E} with purple wireframes, Gly77, Asn78 and Gly176 with wireframes of carbon (gray), nitrogen (blue) and oxygen (red), respectively. Possible hydrogen bonds are given with dashed lines. The figure was crated with Rasmol.

this loop did not affect the K_i value significantly, this is not inconsistent with the essential role of the loop. Since the CTLA-2α loop interacts with cathepsin L through various forces including the hydrogen bonds of antiparallel main chains (Phe56N–Gly149^{E} O, Tyr57N–Ser148^{E} O, Tyr57 O–Tyr146^{E} N and Gly59N–Gln144^{E}O) and hydrophobic forces between Phe56, Tyr57, Met58, Phe71, Pro141^{E}, Leu143^{E}, Phe145^{E}, Tyr146^{E} and Ile150^{E}, similarly to the proseg-ment of cathepsin L, it is anticipated that a single Ala mutation is insufficient to change the affinity of CTLA-2α. It is shown that the deletion of the loop decreased the inhibitory activity significantly (Table 1). The assumption of main-chain hydrogen-bonding interactions was supported by the facts that the change of side chains of Tyr76, Gly77, Asn78 and Ser79 with Ala mutations did not affect the inhibitory activity. Although the side chains of Asn78 and Ser79 seem to be located close to the Asn160^{E} N$^{\delta}$2 and Gly67^{E} O/Asn66^{E} N$^{\delta}$2, respectively, their interactions are thought to be weak/ineffective since mutation of Asn78 and Ser79 side chains did not affect the inhibitory activity. The residues of CTLA-2α that bind to the active-site cleft, namely residues included in the Tyr76–Asn81 loop, were not affected by Ala mutation. The major contacts of CTLA-2α with the residues in the active-site cleft are thought to be the hydrogen bonds between Gly77O and Gly67^{E}N, and between

Asn78N and Gly67^{E}O. A flexible structure of the peptide bond around the residue 77 might be preferred, since G77 is well conserved in CTLA-2α, procathepsin L and BCP proenzyme (Fig. 1A). In contrast, in cathepsin L, the sequence of the active site contacting region has been identified as M^{75}–N–G–F–E^{79} (*47*). Although attempts have been made to make synthetic peptides specifically corresponding to this active site binding sequence, they were shown to exhibit a micro molar range inhibition towards cathepsin L (*50*). For the rat cathepsin B propeptide, C^{82}–G–T–V–L^{86} has been identified as an essential sequence for interaction with the substrate binding cleft (*15*). In BCPI, the sequence L^{82}–G–L–R–L–P–A–K^{89} has been proposed as a region that interacts with the substrate binding cleft (*19*).

In conclusion, the α1/2-helices, which form the backbone of the globular domain of CTLA-2α, do not interact directly with enzymes, but contribute decisively to its inhibitory action. The three highly conserved Trp residues are essential for maintaining the structural integrity through hydrophobic interactions at the intersection of the α1 and α2-helices. It could be proposed that the Cys75–C25^{E} disulfide bonding is essential for cathepsin L/ CTLA-2α interaction. Finally, these findings are helpful in further studies in relation to kinetics and inhibitor design processes.

Supplementary data

Supplementary Data are available at *JB* Online.

Conflict of interest
None declared.

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