# Preference of calcium-dependent interactions between calmodulin-like domains of calpain and calpastatin subdomains

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**Abstract Calpastatin molecule contains four repeated inhibition domains, each having highly conserved internal regions A, B and C. The synthetic oligopeptides of regions A and C had no calpain inhibition activity while region B oligopeptide showed weak inhibition activity. Real-time biomolecular interaction analysis using a BIAcore instrument revealed that the bacterially expressed calmodulin-like domain of the calpain large subunit (L-CaMLD) and that of the small subunit (S-CaMLD) interacted, in a $Ca^{2+}$-dependent fashion, preferentially with the immobilized synthetic oligopeptide of region A and that of region C, respectively. Calmodulin showed no specific binding to these oligopeptides. The tripartite structure of the calpastatin functional domain may confer the specific interactions with the protease domain and the two CaMLDs of calpain.**

*Key words:* Biosensor; Calcium-dependent; Calmodulin-like; Calpain; Calpastatin; Protease inhibitor

## 1. Introduction

Both calpain (EC 3.4.22.17, $Ca^{2+}$-dependent cysteine protease) and its endogenous inhibitor protein calpastatin are known to be widely distributed in animal cells (see [1–4] for a review). A calpastatin molecule contains four internally repeated domains (140 amino acid residues, domains 1–4) and a unique domain on its N-terminal side (domain L) [5,6]. Calpain inhibitory activity has been detected in each repeated domain but not for domain L [7]. Conserved residues among the four repeated domains are not randomly distributed but are clustered in three restricted regions designated as A, B and C, respectively, within each domain. Structure–function analyses of recombinant calpastatins with various deletions in their inhibitory domains revealed that region B was essential for inhibition [8–10]. Moreover, synthetic oligopeptides of 16–34 residues containing region B of domain 1 retained inhibition activity, though with less than one-tenth of the inhibition activity of the whole domain [9–11]. The kinetic analyses using calpastatin fragments containing region B have revealed that calpastatin inhibits calpain by virtue of competition with substrates [8,9]. Although regions A and C were suggested to play roles in potentiating the inhibition activity, the mechanism remains unknown.

$\mu$-Calpain (low $Ca^{2+}$-requiring form, calpain I) and m-calpain (high $Ca^{2+}$-requiring form, calpain II) are heterodimers of a small subunit (30 kDa) and a large subunit (80 kDa) [1–4]. The large subunit has four structurally distinct domains, including a catalytic domain with a high degree of sequence homology with cysteine proteases and a calmodulin-like domain (CaMLD) [12]. The small subunit is a regulatory protein which contains a sequence similar to the CaMLD of the large subunit [13]. Physical association of calpain and calpastatin occurs only in the presence of $Ca^{2+}$ [14,15]. The role of $Ca^{2+}$ in the complex formation might be explained by requirement of the ion to induce conformational change of calpain into a form that is able to bind to the inhibitor near the catalytic center. Surprisingly, however, Nishimura and Goll reported that the fragments containing CaMLDs generated on calpain autolysis bound to a calpastatin-immobilized column in the presence of $Ca^{2+}$ and were eluted with a chelating reagent [16]. The mechanism of the $Ca^{2+}$-dependent interaction between calpain and calpastatin, however, has not yet been clarified. Here we show that the CaMLD of the large subunit (L-CaMLD) and that of the small subunit (S-CaMLD) expressed in *E. coli* bind preferentially to oligopeptides of regions A and C, respectively, in a $Ca^{2+}$-dependent fashion.

## 2. Materials and methods

### 2.1. Materials

Human $\mu$-calpain was purified from erythrocytes as described previously [17]. Recombinant human calpastatin domain 1 (hCSD1, residues 137–277) and L-CaMLD of $\mu$-calpain were prepared and purified as described [18]. Synthetic oligopeptides were custom-ordered from Sawaday Technology Inc., Takara Shuzo, or synthesized as described previously [18,19]. Oligopeptides used in the present study are: D1A19 (SGKSGMDAALDDLIDTLGG); D1C19 (SKPIGPDDAIDALSSDFTS); D1B27cys25(CSSTYIEELGKREVTIPPKYRELLAKK) and CNApep25 (CARKEIIRNKIRAIGKMARVFSVLR). Sensor chips CM5, amine- and thiol-coupling kits, and Tween 20 for BIAcore were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). CaM purified from bovine brain was obtained from Sigma Chemical Co. (St. Louis, USA).

### 2.2. Assay of calpain inhibition

Digestion of casein, 2 mg/ml, was performed using 8 $\mu$g $\mu$-calpain in a reaction volume of 50 $\mu$l in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM $Ca^{2+}$, 5 mM 2-mercaptoethanol, in the presence or absence of calpastatin peptides, at 25°C for 30 min. Reactions were terminated by adding SDS-containing sample loading buffer for SDS-PAGE, and boiled for 2 min. SDS-PAGE was performed on 15% slab gels, followed by staining with Coomassie brilliant blue R250.

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*Abbreviations:* CaMLD, calmodulin-like domain; L-CaMLD, CaMLD of $\mu$-calpain large subunit; S-CaMLD, CaMLD of calpain small subunit; CaM, calmodulin; NHS, *N*-hydroxysuccinimide; hCSD1, human calpastatin domain 1; PAGE, polyacrylamide gel electrophoresis. SPR, surface plasmon resonance.


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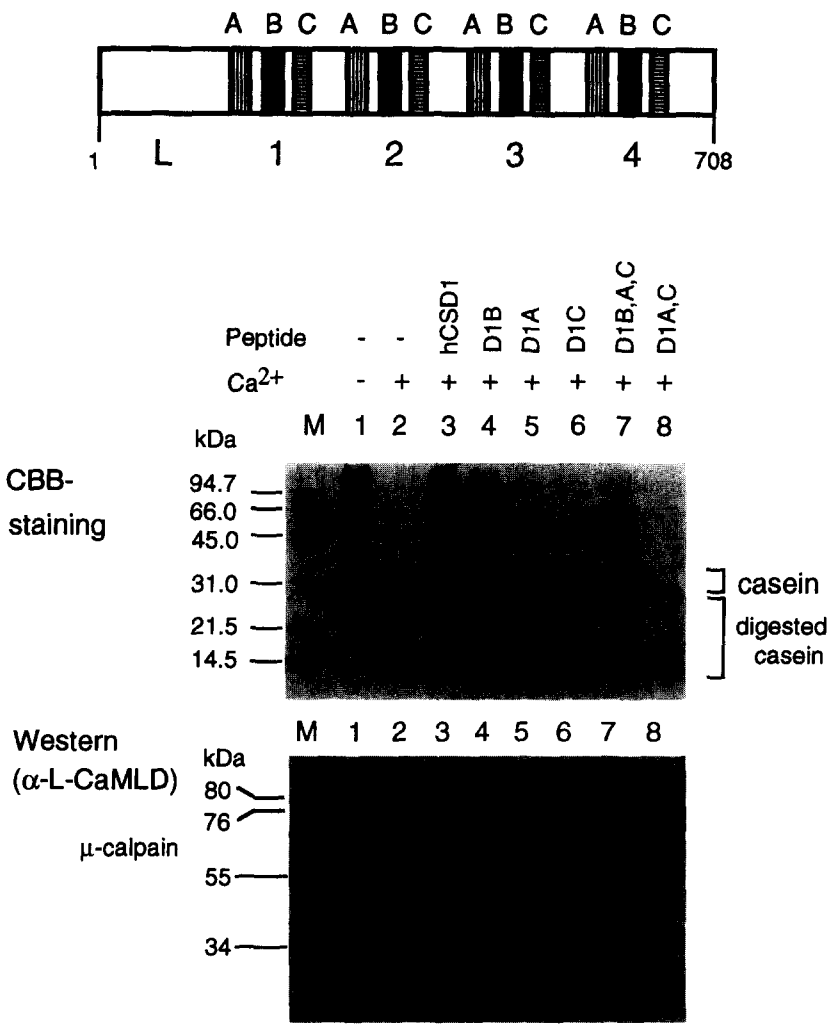


Fig. 1. The inhibition potency of calpastatin domain 1 and oligopeptides of regions A, B and C. (Top panel) Repeat domain structure of human calpastatin. Regions A, B and C, which are highly conserved among the four internally repeated domains 1–4 are marked. (Middle panel) Casein was digested with $\mu$-calpain with or without calpastatin domain 1 peptides as described in section 2. The mixture containing no $Ca^{2+}$ was processed as a control (lane 1). After SDS-PAGE using a 15% slab gel, the gel was stained with Coomassie brilliant blue R250 (CBB-staining). Lane 1, control; lane 2, without calpastatin peptide; lane 3, 1 $\mu$M hCSD1; lane 4, 1 $\mu$M D1B27cys ; lane 5, 1 $\mu$M D1A19; lane 6, 1 $\mu$M D1C19; lane 7, 1 $\mu$M each D1B27cys, D1A19 and D1C19; lane 8, 1 $\mu$M each D1A19 and D1C19; lane M, SDS-PAGE molecular mass standards, Low range (Bio-Rad). (Bottom panel) Aliquots of the mixtures shown in the middle panel were analyzed by Western blotting. After SDS-PAGE using a 10% slab gel, samples were transferred to a nylon membrane filter (Immobilon-P, Millipore) and detected with anti-L-CaMLD antibody. Prestained SDS-PAGE standards, Low range (Bio-Rad), were used for molecular mass calibration (Lane M). Unprocessed (80 kDa) and processed (76 kDa, 55 kDa and 34 kDa) forms of $\mu$-calpain are indicated beside the marker lane.

*2.3. Purification of S-CaMLD expressed in* E. coli

The expression plasmid of S-CaMLD (pET-S-CaMLD) was constructed similarly to that of L-CaMLD (pET-$\mu$-L-CaMLD) [18,20]. Briefly, the cDNA fragment encoding CaMLD of the pig calpain small subunit (S-CaMLD) were prepared by the PCR method using the previously isolated cDNA clone as a template [13], and inserted into the *Bam*HI site of plasmid pET-3d (a vector for expression by T7 RNA polymerase; from Novagen, Madison, USA). The S-CaMLD was expressed in *E. coli* strain BL21(DE3)pLysE as described before [18]. The supernatant cell lysate was filtered through a 0.45-$\mu$m cellulose acetate membrane filter and purified using oligopeptide of D1C19 immobilized on a NHS-activated HiTrap Sepharose column (Pharmacia). The column was equilibrated with buffer B (20 mM Tris HCl, pH 7.5, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 50 mM NaCl) containing 1% Triton X-100, and 0.5 mM $CaCl_2$. The crude S-CaMLD was added with Triton X-100, NaCl, $CaCl_2$ to a final concentration of 1%, 50 mM, and 0.5 mM, respectively, and then applied to the column. The column was washed with 10 vols. of buffer B, containing 0.1% Triton X-100, and 0.5 mM $CaCl_2$, further washed with 20 vols. of buffer B, containing 0.5 mM $CaCl_2$. The adsorbed CaMLD was eluted from the column with buffer B containing 1 mM EGTA.

*2.4. Real-time biomolecular interaction analysis*

Real-time analyses of the $Ca^{2+}$-dependent interaction between CaMLDs and calpastatin oligopeptides were performed using a BIAcore instrument (Pharmacia Biosensor). The principle and application of the system employing surface plasmon resonance (SPR) detection was described by Karlsson et al. [21]. Coupling of D1A19 (calculated pI, 4.37) and D1C19 (calculated pI, 3.45) to the sensor chips CM5 were performed by the EDC/NHS method under high salt conditions at pH 8.5 as described [18]. Calcineurin oligopeptide (CNApep25) was immobilized to a sensor chip CM5 by the ligand thiol method [19]. The SPR measurements for the binding of CaMLDs and CaM to oligopeptides were performed at 2 $\mu$l/ml in buffer F (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1.5 mM $CaCl_2$, 0.005% Tween 20). The sensor chips were regenerated after each analysis cycle by injecting 6 ml of buffer F without $CaCl_2$, and then initialized with continuous flow of buffer F.

### 3. Results and discussion

Calpastatin is a specific inhibitor of calpain, and has not been known to inhibit any other proteases [1–3]. Absence of significant homology at the primary structure level with any other protease inhibitors, including the cystatin superfamily (a class of inhibitors of cysteine proteases), may account for this specificity [6]. In other words, the inhibition mechanism of calpastatin could not be readily inferred from other protease inhibitor systems. Fig. 1 (top panel) shows the schematic structure of human calpastatin. The highly conserved internal repeat regions A, B and C are located on separate exons in the human calpastatin gene, suggesting distinct roles in inhibitory function [11,22]. Moreover, regions A and C have similar sequences [6,20]. Recently we showed that the L-CaMLD of $\mu$-calpain bound in a $Ca^{2+}$-dependent fashion to calpastatin domain 1 but not to the region B oligopeptide, by using a Sepharose column immobilizing the L-CaMLD fused with glutathione-*S*-transferase [20]. Furthermore, the oligopeptide of D1A19 bound specifically to the L-CaMLD [18]. The presence of the S-CaMLD in the small subunit of calpain, as well as the finding of similar motifs between regions A and C, motivated us to examine recognition specificity between the CaMLDs. We also investigated the roles of regions A, B and C in calpain inhibition and discussed the functional significance of the presence of these three regions in the calpastatin molecule.

In agreement with previous reports [9–11], the region B oligopeptide D1B27cys had calpain inhibition activity with a weaker potency than the full-size domain 1 (Fig. 1, middle panel, lanes 3 and 4). Although regions A and C are known to be important for potentiation of the inhibition activity of calpastatin [8–10,23], the oligopeptides corresponding to these regions showed neither inhibition activity by themselves (lanes 5, 6 and 8) (even up to 100 $\mu$M; data not shown) nor potentiated the activity of D1B27cys when mixed together (lane 7). These data indicate that the tethered regions of A, B and C are required to exert full inhibition activity.

Several reports have indicated that $Ca^{2+}$-dependent autolytic conversion of $\mu$-calpain from the 80 kDa form to the 76 kDa form precedes before it exerts proteolytic activity toward casein and other substrates, hence calling the process 'activation' of $\mu$-calpain [3,4,24]. In agreement with the above notion, Western blot analysis using anti-L-CaMLD antibody, which was raised in rabbits using purified L-CaMLD, revealed that the 80 kDa form of $\mu$-calpain was autolyzed and converted to the 76 kDa form during caseinolysis (Fig. 1, bottom panel). Faint bands corresponding to 55 kDa and 34 kDa were also detected. The recombinant human calpastatin domain 1 (hCSD1) blocked the autolysis (lane 3), but the region B oligopeptide did not (lane 4).

Previously we reported the expression of the recombinant L-CaMLD in *E. coli* and $Ca^{2+}$-dependent affinity purification of the protein using a D1A19-Sepharose column [18]. A similar approach was taken to obtain recombinant S-CaMLD from *E. coli*. As illustrated in Fig. 2 (upper panel), the cDNA fragment containing the region corresponding to amino acid residues 84–266 of the small subunit of pig calpain was inserted into the *Bam*HI site of pET-3d and expressed by T7 RNA polymerase. A high level of S-CaMLD expression was observed (Fig. 2, lower panel, lane 1) and the protein was mostly recovered in the soluble fraction (lane 2). When the crude S-CaMLD was applied to the D1A19-Sepharose column in the buffer contain-

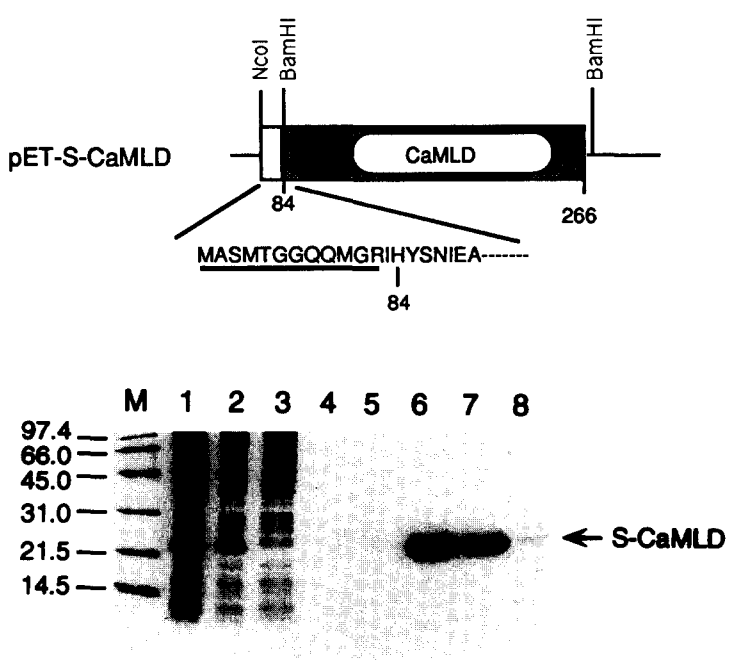


Fig. 2. Expression and purification of the recombinant S-CaMLD from *E. coli*. (Upper panel) Schematic representation of the expression plasmid of S-CaMLD. The cDNA fragment corresponding to the CaMLD of the small subunit (S-CaMLD) was inserted into the *Bam*HI site of pET-3d, an expression vector for T7 RNA polymerase. The resultant plasmid was designated pET-S-CaMLD. The N-terminal peptide sequence encoding T7 gene 10 is underlined. (Lower panel) SDS-PAGE analysis of the S-CaMLD prepared from *E. coli*. The S-CaMLD was purified by affinity chromatography using an oligopeptide column (D1C19). The supernatant of the *E. coli* lysate was applied to the column in buffer containing $Ca^{2+}$ and the adsorbed S-CaMLD was eluted with buffer containing EGTA as described in section 2. Samples were analyzed by SDS-PAGE on a 15% slab gel. Lanes 1, total lysate; 2, supernatant; 3, flow-through fraction; 4 and 5, wash fractions; 6–8, elution fractions. M, molecular mass SDS-PAGE standards, Low range (Bio-Rad). Arrows indicate the S-CaMLD.

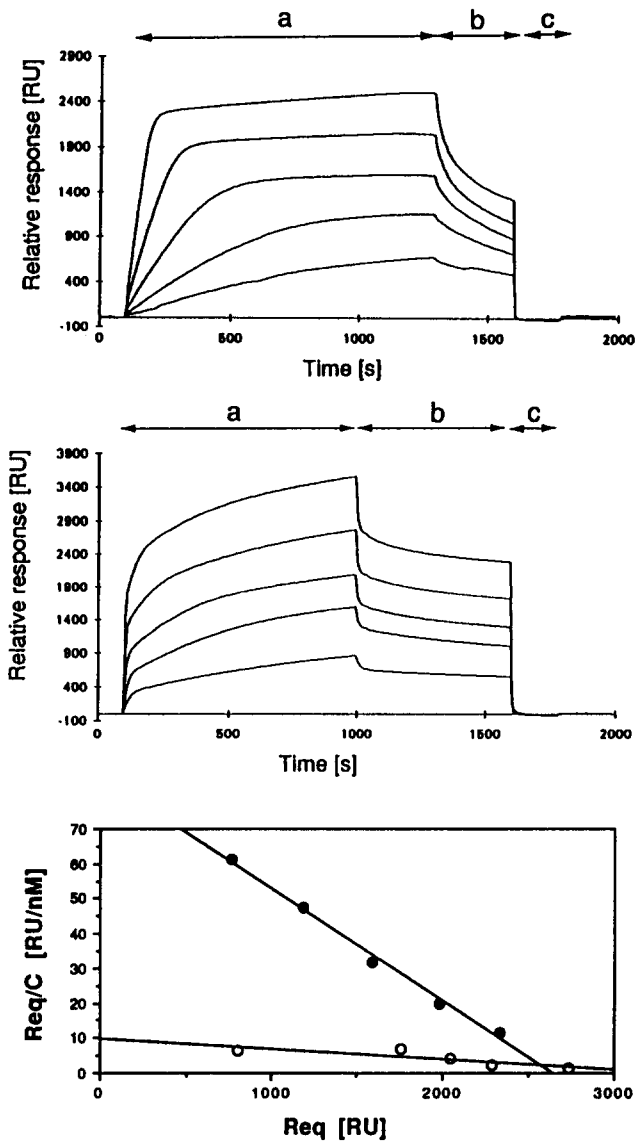


Fig. 3. BIAcore analysis of the $Ca^{2+}$-dependent interaction of CaMLDs with D1C19. (Top and middle panels) Representative overlaid sensorgrams illustrating the real-time binding of S-CaMLD (panel A) at 12.5, 25, 50, 100 and 200 nM, from bottom to top, and L-CaMLD (panel B) at 125, 250, 500, 1000 and 2000 nM, from bottom to top, to the oligopeptide D1C19 immobilized on the biosensor chip. Analysis was performed with buffer F. Arrows indicate phases of (a) association, (b) dissociation and (c) regeneration. Regeneration was performed by washing the sensor chip with buffer F without $Ca^{2+}$. (Bottom panel) Scatchard plot analysis. Relative response values expressed as steady-state binding ($R_{eq}$) at each protein concentration in panels A and B were estimated by extrapolating $dR/dt$ (time derivative of relative response, binding rate) to zero using the kinetics evaluation software installed in BIAcore. The reciprocal of the slope gives a value for the dissociation constant ($K_D$) from the following equation: $R_{eq}/C = R_{max}/K_D - R_{eq}/K_D$. $R_{eq}$, steady-state binding value; $C$, CaMLD concentration; $R_{max}$, maximum binding capacity of the immobilized oligopeptide. S-CaMLD (●); L-CaMLD (○).

ing $Ca^{2+}$, most S-CaMLD was recovered in flow-through fractions (data not shown). In contrast, most S-CaMLD was absorbed to the D1C19 immobilized-Sepharose column and little S-CaMLD was observed in the flow-through fraction (lane 3). After extensive washing, the adsorbed S-CaMLD was eluted from the column with buffer containing 1 mM EGTA and no $Ca^{2+}$ (lanes 6 and 7).

The $Ca^{2+}$-dependent binding of S-CaMLD to D1C19 was further examined by using a biomolecular interaction analysis biosensor instrument, BIAcore. Solutions of the purified S-CaMLD were passed over the D1C19-immobilized biosensor chip in the presence of $Ca^{2+}$. Fig. 3 (top panel) displays an example of overlaid sensorgrams using five different concentrations of S-CaLMD (12.5–200 nM). Rates of association ($dR/dt$) were analyzed with kinetics evaluation software installed in BIAcore. The plots of $dR/dt$ vs. relative response gave linear relationships (data not shown), and relative response values expressed as steady-state binding ($R_{eq}$) were estimated by extrapolating $dR/dt$ to zero. The reciprocal of the slope in a Scatchard plot gave a value of the dissociation constant ($K_D$) of $3.1 \times 10^{-8}$ M for S-CaMLD (Fig. 3, bottom panel). The binding of L-CaMLD to the D1C19-immobilized sensorchip was weak and required higher concentrations of the protein for the analysis (125–2000 nM) (Fig. 3, middle panel). Scatchard analysis gave a value of $K_D = 3.5 \times 10^{-7}$ M (Fig. 3, bottom panel), indicating that D1C has approximately 10 fold higher affinity to S-CaMLD than to L-CaMLD.

The binding specificity of CaMLDs were further clarified by using sensorchips immobilizing different oligopeptides. Fig. 4 displays sensorgrams using 50 nM of L-, S-CaMLD or CaM. The L-CaMLD bound preferentially to D1A19 (top panel) while S-CaMLD bound to D1C19 (middle panel). Calmodulin (CaM) did not show significant binding to these oligopeptides while didi show specific binding to a 25-residue oligopeptide (CNApep25) of calcineurin ($Ca^{2+}$/CaM-dependent protein phosphatase) (bottom panel) [19].

Interaction of CaM and its target $Ca^{2+}$-dependent binding peptides have been well studied (see [25] for a review). A comparison of these CaM-binding sequences reveals little homology in primary structures, but the conspicuous feature common to the sequences is the presence of several basic residues and no or only a few acidic residues, as in the case of the CaM-binding site in calcineurin [19,25]. Furthermore, hydrophobic residues frequently repeat in the sequences over a three to four residue period. The above feature conforms to a basic amphiphilic α-helical motif where hydrophobic and basic residues are localized on each side of the helical cylinder [25]. Likewise, although calpastatin shows little secondary structure in solution [10], the potential α-helix regions found in the conserved sequences of regions A and C [8,20] may form amphiphilic α-helical structures upon complexing with the CaMLDs. Indeed, the calpastatin domain I mutant, which had a substitution of one of the conserved hydrophobic Leu residues in region A with a helix breaker residue Pro, demonstrated a striking decrease in the binding ability of the L-CaMLD, and also showed reduced calpain inhibition activity [23].

The unfolded nature of the calpastatin molecule may afford the unique physicochemical properties such as heat and acid resistance [1,26], but the inhibitor protein may retain potential specific recognition signals toward the CaMLDs. By forming acidic amphiphilic α-helical structures in regions A and C, calpastatin may be recognized by the CaMLDs of calpain in the presence of $Ca^{2+}$. As revealed by the present study, there is a preference among the binding sequences among the CaMLDs and CaM (Fig. 4). The recognition specificity may reside in the difference in the amphiphilic α-helical motifs: acidic or basic

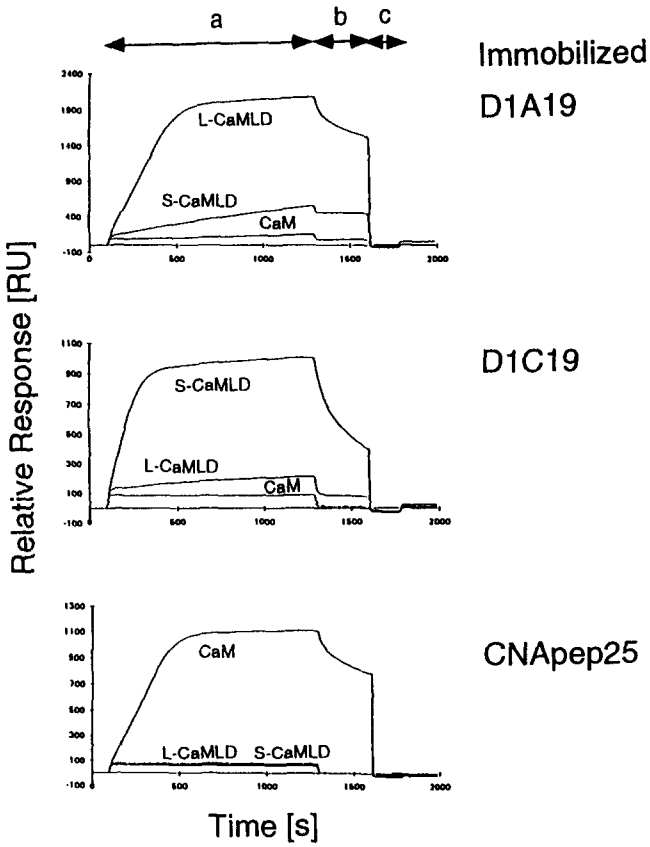


Fig. 4. Analysis of binding specificities of oligopeptides to CaMLDs and CaM with BIAcore. The oligopeptides immobilized on the biosensor chips were: top panel, D1A19 (region A); middle panel, D1C19 (region C); bottom panel, CNApep25 (CaM-binding region in human calcineurin A-2). Analysis was performed using L-CaMLD, S-CaMLD or CaM at 50 nM as described in the legend to Fig. 3, and sensorgrams were overlaid. Arrows indicate phases of (a) association, (b) dissociation and (c) regeneration.

(CaMLD-binding peptide vs. CaM-binding peptide); conservation of particular residues at certain positions (region A vs. region C).

Binding of $Ca^{2+}$ to the two CaMLDs may induce conformational change of the enzyme and make the catalytic site (protease domain) accessible to substrates and calpastatin. While regions A and C bind to the L- and S-CaMLDs, respectively, region B occupies the catalytic center of the protease domain. The kinetic analyses using calpastatin fragments containing region B have revealed that calpastatin inhibits competition with substrates [8,9]. Binding of region B near the catalytic center of calpain was further supported by competition experiments using small molecule inhibitors which reacted with the active site cysteine residue [9,15,27]. The tripartite subdomain structure of calpastatin (regions A, B and C) may contribute to the acquisition of stronger inhibition potency by formation of a tighter calpain–calpastatin complex. Verification of the proposed model must await the X-ray crystallographic analysis of calpain–calpastatin complex.

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