# Mutagenesis of the L7 Loop Connecting $\beta$ Strands 12 and 13 of Calcineurin: Evidence for a Structural Role in Activity Changes[†]

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ABSTRACT: Calcineurin is a heterodimer consisting of a catalytic A-subunit and a B-subunit, and is regulated by binding of calmodulin and calcium. The C-terminus of the A-subunit contains an autoinhibitory domain which plays an important role in regulation of calcineurin activity. In this study, we have mutated the L7 loop connecting $\beta$ strands 12 and 13 in calcineurin A. These mutants included two chimeric mutants in which a four amino acid stretch in the cognate L7 loops of the related proteins phosphatase-1 (GEFD) or -2A (YRCG) were substituted for the calcineurin sequence DVYN (313−316), a point mutation (L312C), and a truncated mutant in which the YRG sequence replaced residues 313−316. Examination of the activities of these mutants led to the striking finding that truncation of the loop region by one residue resulted in hyperactivation of the calcineurin A-subunit. That the hyperactivation is due to conformational effects on the catalytic core of the enzyme was established since this effect was maintained in truncation mutants (at residues 456 and 388) in which the calmodulin and autoinhibitory domains were deleted. These studies provide evidence that the L7 loop is an important structural element in the conformation of the active site, and may participate in the conformational transitions of calcineurin between a catalytically repressed state and an activated state under the influence of the B-subunit.

Calcineurin was originally discovered as a calmodulin binding protein in brain extracts, and was later shown to be a calcium/calmodulin-dependent protein phosphatase (Stewart et al., 1982; for reviews, see Pallen et al. (1988), Klee et al. (1988), Klee (1991), and Shenolikar and Nairn (1991). Although calcineurin is present in all mammalian cells, it is most highly abundant in brain tissue (Klee, 1991). While the biological functions of calcineurin in brain have not been completely elucidated, it is most highly concentrated in the hippocampus, and may play a role in regulating neurotransmitter action (Klee, 1991). The enzymology of calcineurin has been extensively studied (Pallen & Wang, 1988; Klee et al., 1988). Calcineurin consists of two subunits: a catalytic A-subunit of 59 kDa, and a regulatory B-subunit of 19 kDa which has sequence similarity to calmodulin. The A-subunit is inactive on protein substrates, but partially active on *p*-nitrophenyl phosphate (PNPP).[1] The B-subunit is essential for the stimulation of the activity of calcineurin A, but activation of the heterodimer requires the release of the effects of a C-terminal autoinhibitory domain. This was discovered by the proteolytic cleavage of the A-subunit which results in calmodulin-independent activation (Tallant & Cheung, 1984; Hubbard & Klee, 1989). Calcineurin is therefore activated by dual processes involving two calcium binding proteins (Perrino et al., 1995; Stemmer & Klee, 1994). In addition, the transition metals $Mn^{2+}$ and $Ni^{2+}$, are activators of calcineurin when assayed on PNPP (Pallen & Wang, 1982, 1986). The use of synthetic peptides (Hashimoto et al., 1990) defined an autoinhibitory sequence (459−482)[2] which was extended in the N-terminal direction to residue 420 by deletion mutagenesis (Perrino et al., 1995). The expectation that calcineurin may play a considerable role in calcium-mediated signal transduction pathways has been realized by the discovery that it is the target of the immunosuppressant drugs FK506 and cyclosporin A, both of these being inhibitory as their complexes with their respective immunophilin proteins (Clipstone & Crabtree, 1993). The immunosuppressive functions of FK506 involve a requirement for calcineurin in the dephosphorylation of the transcription factor NF-AT, and may be part of a general role for calcineurin in the $Ca^{2+}$-dependent regulation of gene transcription (Liu et al., 1991, 1992; Enslen & Soderling, 1994).

The crystal structure of calcineurin has been determined, as the FK506/FKBP12 complexes of a C-terminal truncated bovine calcineurin (Griffith et al., 1995), and human calcineurin (Kissinger et al., 1995). The catalytic cores of calcineurin A (40−340) and PP1 have ca. 40% sequence identity, and their structural architecture is highly conserved. In this work, we report a study of the regulation of calcineurin A by mutagenesis of the L7 loop which connects $\beta$ strands 12 and 13; double mutations in which the C-terminal autoinhibitory domain was deleted were also studied to distinguish between effects involving activation of the catalytic core and those arising from autoinhibition.

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[1] Abbreviations: PP1 and PP2A, catalytic subunits of protein phosphatase-1 and -2A, respectively; PCR, polymerase chain reaction; AI, autoinhibitory; PNPP, *p*-nitrophenyl phosphate.

[2] Numbering of numbering of the residues of the A-subunit is based on that for the $\alpha\delta$ isoform (Perrino et al., 1992).

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## MATERIALS AND METHODS

*Expression and Purification of Calcineurin Subunits.* The cDNAs for the rat $\alpha\delta$ isoform of calcineurin A (Saitoh et al., 1991; Perrino et al., 1992) and rat calcineurin B (Perrino et al. 1995) were those isolated from rat brain cDNA libraries, and were generous gifts of Drs. B. Perrino and T. R. Soderling, Vollum Institute, Portland, OR. The rat A$\alpha\delta$ protein sequence consists of 511 residues and is identical to that of the rat A$\alpha$ isoform (521 residues) except for the deletion of 10 residues (448−477) (Ito et al., 1989). The coding sequences for the A- and B-subunits were inserted into the pET21a expression vector and expressed in *E. coli* strain HMS174($DE_3$) and $BL_{21}$($DE_3$)PlysS host cells, respectively (Wei and Lee, submitted for publication). The yields of protein were about 10 mg and 70 mg of protein per liter of cell culture after purification for the A- and B-subunits, respectively. Mutants in the L7 loop were constructed using the Altered Site II mutagenesis system (Promega). These were replacements of residues 313−316 (DVYN) with the sequences YRCG, GEFD, and a point mutation (L312C). The following oligonucleotides were used for the construction of the YRCG, GEFD, and L312C mutants, respectively: 5′-CGGCACCAAATTACTTATATCGGGGTAA-TAAAGCTGCAGTGTTGAAGTACG-3′; 5′-CGGCACC-AAATTACTTATATCGGTGCGGTAAAGCTG-CAGTTGAAGAAGTACG-3′; 5′-CGGCACCAAATTACT-TAGGTGAGTTCGATAATAAAGATGCAGT-GTTGTAAGTACG-3′; and 5′-CCAAATTACTGCGATGTG-TAC-3′. The correctness of the mutants was checked by DNA sequencing. In the course of construction of the YRCG mutant, a mutant with the YRG sequence inserted instead of the YRCG sequence was isolated, possibly due to an error in the synthesis of the oligonucleotide primer. A second primer was synthesized, and the correct sequence was obtained. Two series of truncation mutants of the wild-type, the YRG, the GEFD, and the L312C mutants which were terminated at residues 456 and 388, respectively, were constructed by PCR amplification using the primers 5′-CGCGAAGCTTTCACTTATGTTGTTGTGAGAATCC-3′ (at 456) and 5′-CGCGAAGCTTTCACGTGGCTCCGT-CAAATCCATCTTC-3′ (at 388). The YRG, YRCG, GEFD, and L312C mutants and their respective C-terminal deletion mutants at residue 456 were purified by calmodulin−Sepharose 4B affinity chromatography followed by Sephacryl S-200 chromatography (Wei & Lee, 1977). The truncation mutants at residue 388 were purified on calcineurin B−Sepharose 4B affinity chromatography. Preparation of the calmodulin−Sepharose and B-subunit−Sepharose columns was as described by Wei and Lee (1997). Recoveries of protein were about 8−10 mg for the full-length A-subunits, 3−4 mg for the A456 truncations, and 1−2 mg for the A388 mutants from 1 L cell cultures. Protein determinations were performed by the procedure of Bradford (1976).

*Assay of Phosphatase Activity Using p-Nitrophenyl Phosphate.* Assays for activity toward *p*-nitrophenyl phosphate (PNPP) using $Mn^{2+}$ as the activator were performed in 50 mM TrisHCl, pH 7.4, 0.5 mM $MnCl_2$, 0.5 mM dithiothreitol, 0.2 mg/mL bovine serum albumin, and 20 mM PNPP. When $Ca^{2+}$ was used as the activator, $MnCl_2$ was replaced with 6 mM $MgCl_2$ and 0.2 mM $CaCl_2$. Reactions were performed in a volume of 0.2 mL at 30 °C for 20 min and terminated by the addition of 1.8 mL of 0.5 M sodium carbonate. The absorbance was read at 410 nm. Units of activity were defined as nanomoles of PNPP hydrolyzed per minute. Calmodulin and B-subunit, where added, were 0.3 $\mu$M and 0.1 $\mu$M, respectively. Kinetic analyses were performed in the presence of $Mn^{2+}$ at PNPP concentrations of 5, 10, 15, 20, 30, and 40 mM. Data were analyzed using SigmaPlot.

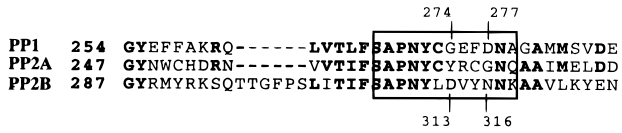


FIGURE 1: Alignment of the L7 loop regions connecting $\beta$ strands 12 and 13 in PP1, PP2A, and calcineurin. The diagram shows the alignment of the loop regions of rabbit muscle PP1, PP2A, and calcineurin A. This loop region is conserved (boldface residues) except for the residues between 274−277 (GEFD) of PP1, 267−270 (YRCG) of PP2A, 312−316 (LDVYN) of calcineurin (PP2B), and the last residue in the loop. Numbering of calcineurin residues is for the rat calcineurin A$\alpha\delta$ isoform (Perrino et al., 1992). Assignment of the loop regions connecting $\beta$ strands 12 and 13 is based on the crystal structures of PP1 (Egloff et al., 1995) and calcineurin (Griffith et al., 1995). The sequence of PP2A is from da Silva et al. (1987).

*Assay of Phosphatase Activity Using RII Peptide.* RII peptide was obtained from Biomol Research Laboratories Inc. and labeled with [$\gamma$-$^{32}$P]ATP using the catalytic subunit of cAMP-dependent protein kinase to a specific a radioactivity of 500−1000 dpm/pmol as described by Perrino et al. (1992, 1995). Assays were performed as described by Perrino et al. (1995). For the assay in the presence of $Mn^{2+}$, the reaction mixture (20 $\mu$L) contained 50 mM TrisHCl, pH 7.4, 0.5 mM $MnCl_2$, 0.5 mM DTT, 0.1 mg/mL bovine serum albumin, and 30 $\mu$M phosphorylated RII peptide. For assays in the presence of calcium ion, the reaction mixtures contained 50 mM TrisHCl, pH 8.5, 20 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.1 mg/mL bovine serum albumin, and 30 $\mu$M phosphorylated RII peptide. Units of activity were defined as nanomoles of [$^{32}$P]$P_i$ released per minute. Calmodulin and B-subunit, where added, were 0.3 $\mu$M and 0.1 $\mu$M, respectively.

## RESULTS

*Design, Expression, and Characterization of Mutants of Calcineurin A.* The rationale for mutation of the A-subunit of calcineurin at the L7 loop came from our studies of the rabbit muscle protein phosphatase-1 (PP1) catalytic subunit. Mutation of this loop region in PP1 affects its response to several xenobiotic inhibitors including okadaic acid, calyculin A, and microcystin (Zhang et al., 1994, 1996). In particular, we have been able to increase the sensitivity of PP1 to okadaic acid by a chimeric mutation in which four residues of the PP2A loop sequence were introduced into PP1. Since okadaic acid is also known to weakly inhibit calcineurin (Biajolan & Takai, 1988), chimeric mutations of calcineurin were made by insertion of a four amino acid sequence corresponding to the PP1 and PP2A sequences. The sequence alignment of the L7 loop region in question for PP1, PP2A, and calcineurin is shown in Figure 1. The region consists of 12 residues, and has 6 of the 42 residues that are invariant in a multiple sequence alignment of the eukaryotic Ser/Thr protein phosphatase family (Barton et al., 1994). This includes a stretch of five invariant residues (SAPNY) followed by five variant residues. The mutants constructed

in this study all fall in these five variant residues (Figure 1). For the purposes of this study, the YRCG, GEFD, and L312C mutants were constructed. In addition to these three, a fourth mutant, YRG, was studied. The latter was a mutant containing a deletion in the YRCG sequence which was isolated during the process of construction of the YRCG mutant (Materials and Methods). In the course of characterization of these mutants, it was observed that the free A-subunits exhibited unusually high activities in several cases. In order to assess the basis for these increased activities, additional mutants of the wild-type, YRG, GEFD, and L312C mutants were made in which the A-subunits (511 residues) were truncated at residues 388 or 456. The A456 mutants are ones in which part of the autoinhibitory region (459−476) that is shown to be in contact with the catalytic core (Kissinger et al., 1995) is removed, and the A388 mutants are ones in which the calmodulin binding region (390−414) (Kincaid et al., 1988) as well as the extension of the autoinhibitory region (420−457) described by Perrino et al. (1995) was removed. These two sections of the autoinhibitory region from 459−476 and 420−457 will be referred to as AI-1 and AI-2 for convenience.

The expression system used for these studies was the pET21a vector which we have used for the expression of the rat brain calcineurin A$\alpha\delta$ isoform (Wei & Lee, 1997). This system allows for the isolation of 5−10 mg of soluble A-subunit per liter of cell culture after purification on calmodulin A−Sepharose and gel filtration. In the case of the A388 series of mutants, isolation was performed on a B-subunit−Sepharose affinity column instead of calmodulin−Sepharose because of the deletion of the calmodulin binding region. We have shown that reconstitution of the recombinant A- and B-subunits is immediate, and gives rise to an enzyme that is functionally indistinguishable from the protein isolated from brain tissue (Wei & Lee, 1997). All the mutants made in these studies were found to be expressed as soluble proteins similar to the wild-type enzyme, and all were purified to near-homogeneity. Yields of the calcineurin A proteins ranged from 1 to 10 mg per liter of culture (Materials and Methods). A representative SDS−PAGE gel stained for protein for the wild-type calcineurin A and the A456 and A366 truncations is shown in Figure 2. The A456 and A366 mutants migrated at the positions expected for their estimated molecular masses of 53 and 45 kDa. All the mutants in this study readily formed functional heterodimers on addition of the B-subunit.

Two substrates, *p*-nitrophenyl phosphate (PNPP) and a phosphorylated 19 residue peptide (RII peptide) based on the sequence of the RII regulatory subunit of protein kinase A, were used (Perrino et al., 1992). This was done to evaluate the behavior toward PNPP, which is a small molecule whose binding is restricted to the active site, and which might be expected to be a reporter for local structural changes involving the active site, and a larger peptide substrate which more closely resembles the behavior of calcineurin on protein substrates. In addition, the activities of the free calcineurin A-subunits, the reconstituted AB heterodimers, A-subunit plus calmodulin, and the heterodimers plus calmodulin were determined. The results were expressed as specific activities (units of activity per milligram of protein) based on the A-subunit in order to provide comparisons of the free A-subunit with the heterodimer. Values for the truncated enzymes were normalized

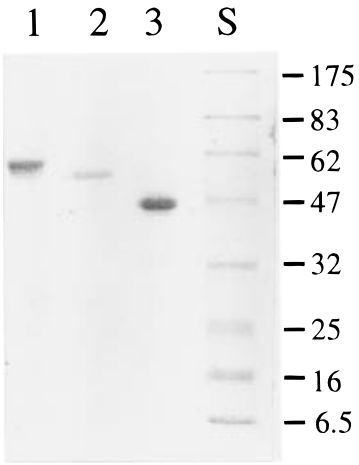


FIGURE 2: Purification of recombinant calcineurin A and the A456 and A388 truncation mutants. Preparations of wild-type calcineurin A and the A456 and A388 mutants were expressed in *E. coli* and purified as described under Materials and Methods. The proteins were run on 12% acrylamide gels and stained with Coomassie blue. Lane 1, full-length A-subunit; lane 2, A456 mutant; lane 3, A388 mutant; lane S, prestained protein standards.

to account for the loss of size by the factors 0.892 and 0.759 for the A456 and A388 enzymes, respectively.

*Functional Characterization of the L7 Loop/AI Mutants Assayed Using PNPP*. The activities of the purified mutants were first assayed with PNPP in the presence of $Mn^{2+}$, which has been shown to activate calcineurin without a requirement for calcium/calmodulin (Pallen et al., 1988). The results (Table 1) provided the striking and immediate observation that three of the mutants (YRG, GEFD, L312C) were more active than the wild-type enzyme, both in the case of the free A-subunits and for the heterodimers. Furthermore, it is readily apparent that the YRG mutant represents an apparently hyperactivated form of the enzyme (Table 1). The YRG heterodimer had a specific activity of about 2800 units/mg, representing an 8-fold increase in activity over the recombinant wild-type heterodimer. The parent YRCG mutant was found to have a lower activity than the wild-type, showing that the deletion of a single residue in the L7 loop is responsible for the activation observed for the YRG mutant. The free YRG A-subunit is highly active in comparison to the free wild-type A-subunit. The YRG A-subunit, compared to both the parent YRCG mutant and the wild-type enzyme, is essentially deregulated and independent of both the B-subunit and calmodulin when assayed using PNPP in the presence of $Mn^{2+}$. Smaller but significant increases were also observed for the GEFD and L312C mutants (ca. 2- fold).

Two possible explanations can be offered for the observed effects discussed above, these being that the modification of the L7 loop resulted in a conformational change that affects catalytic efficiency, or that the modification resulted in relief of the negative control exerted by the autoinhibitory region of the C-terminus. Examination of the activities of the truncated enzymes, specifically the A388 series, showed that the YRG mutant consistently was more active than the wild-type, establishing that the activation observed was attributable to the mutation of the L7 loop. Two effects of deletion of the AI-1 region are observed when assayed in the presence of $Mn^{2+}$. The truncated free A-subunits of the wild-type and the other three L7 loop mutants (Table 1) were now as active as the heterodimers. The activity of the

Table 1: Specific Activities of Calcineurin Mutants Assayed with PNPP as the Substrate[a]

| | assayed with $Mn^{2+}$ | | | | assayed with $Ca^{2+}$ | | | |
|---|---|---|---|---|---|---|---|---|
| variant | A | A+C | A+B | ABC | A | A+C | A+B | ABC |
| A511 | | | | | | | | |
| WT | 77 | 220 | 115 | 350 | 40 | 140 | 86 | 380 |
| YRCG | 86 | 140 | 90 | 130 | 9 | 9 | 9 | 9 |
| YRG | 2380 | 2800 | 2000 | 2770 | 570 | 790 | 770 | 1190 |
| GEFD | 200 | 720 | 180 | 700 | 50 | 140 | 80 | 240 |
| L312C | 230 | 520 | 300 | 740 | 64 | 320 | 130 | 530 |
| A456 | | | | | | | | |
| WT | 400 | 370 | 450 | 450 | 48 | 66 | 186 | 190 |
| YRG | 2500 | 2500 | 2600 | 2500 | 450 | 600 | 1130 | 1010 |
| GEFD | 590 | 625 | 590 | 560 | 120 | 200 | 160 | 240 |
| L312C | 340 | 300 | 400 | 350 | 30 | 60 | 100 | 130 |
| A388 | | | | | | | | |
| WT | 1100 | − | 1300 | − | 200 | − | 510 | − |
| YRG | 3600 | − | 3700 | − | 1130 | − | 2260 | − |
| GEFD | 1540 | − | 1460 | − | 130 | − | 380 | − |
| L312C | 1600 | − | 2550 | − | 230 | − | 1160 | − |

[a] Activities were assayed as described under Materials and Methods. Values are presented as units/mg of A-subunit protein. "A", A-subunit; "C", calmodulin; "B", B-subunit. A511, full-length A-subunits; A456, truncated at residue 456; A388, truncated at residue 388. The data for the A466 and A388 series were normalized for the loss of size to permit comparison on a relative molar basis. For the A388 series, assays in the presence of calmodulin were performed, but these were essentially the same as those in the absence of calmodulin and are not shown.

truncated A456 wild-type AB heterodimer was increased by ca. 50%, while those of the YRG−A456 and GEFD−A456 heterodimers were slightly lower than the parent full-length enzymes. The L312C−A456 mutant had a significantly lower activity than the parent heterodimer. Thus, removal of the AI-1 domain surprisingly did not result in an increase in activity toward PNPP.

When the activities of the double mutants truncated at residue 388 assayed with PNPP/$Mn^{2+}$ are examined, it is seen that the truncated free A-subunits were as active as their heterodimers, with the exception of the L312C−A388 catalytic subunit, which retained a requirement for the B-subunit for full activation. Truncation at residue 388 also led to highly significant increases in activity by factors of 3-, 1.3-, 2-, and 3.2-fold over the parent full-length heterodimers, for the wild-type, YRG, GEFD, and L312C enzymes, respectively. The highest activity was exhibited by YRG−A388 which had a specific activity of 3700 units/mg, which is 11-fold higher than that of the wild-type heterodimer, and 3 times that of the wild-type A388 heterodimer. Comparison with the data for the A456 truncated enzymes shows that the AI-2 region exerts a much greater inhibitory effect than the AI-1 region.

When the activities on PNPP were measured in the presence of $Ca^{2+}$, similar findings were obtained regarding the activation of the enzyme by mutation of the L7 loop. However, the regulatory effects of the B-subunit and calmodulin are now evident (Table 1). The activation of the YRG mutant is evident when the activities of the free subunits or the heterodimers are examined, although its activity (1190 units/mg) is less than the activity in the presence of $Mn^{2+}$ (2800 units/mg). Also striking is the very low activity of the YRCG mutant. This mutant, which exhibited about 33% of the wild-type activity in the presence of $Mn^{2+}$, had less than 3% of the activity of the wild-type in the presence of $Ca^{2+}$. When the A456 series is examined, it is seen that the loss of the AI-1 region did not lead to increases in activity, but in lower activities for the wild-type and L312C mutant. A second observation that can be made is that the free A456 truncated A-subunits are not fully active (as when assayed in the presence of $Mn^{2+}$) and retain a requirement for calmodulin and the B-subunit.

When the activities of the A388 series of mutants are examined, the following observations can be made. The activities of the free A388 subunits are increased over their respective full-length A-subunits, and are further increased by the B-subunit. In all cases, these are higher than those of the full-length heterodimers, except for the GEFD mutant. The loss of the entire autoinhibitory region plus the calmodulin binding region in all cases leads to increase in activity over the heterodimer assayed in the presence of calmodulin and calcium or $Mn^{2+}$. This means that under optimal conditions of activation, the wild-type heterodimers do not reach the same activity as when the AI region is completely deleted. In the case of the A388 mutants, in which the calmodulin binding region and the entire autoinhibitory region is deleted, a comparison of the activities in the presence of $Mn^{2+}$ and in the presence of $Ca^{2+}$ shows that $Mn^{2+}$ is able to mimic the B-subunit activation of the calcineurin A-subunit except in the case of the L312C mutant. To summarize these findings, it is clear that calcineurin A can be activated by two means: modification of the L7 loop and also by truncation of the autoinhibitory regions, of which the AI-2 region appears to be the dominant factor.

*Kinetic Analysis of the YRG Mutant.* The kinetic parameters for the YRG A-subunits and their heterodimers assayed with PNPP in the presence of $Mn^{2+}$ were determined. No significant effects on $V_{max}$ were found. The $V_{max}$ values for the free YRG A-subunits and their heterodimers were similar. However, the significant findings were that there are changes in $K_m$, which ranged from 18 mM for the free A-subunit to 8 mM for its heterodimer. The effects of truncation were reductions of $K_m$ to values of 5−6 mM for the YRG A456 mutant , and 1−3 mM for the YRG A388 mutant. These results show that the apparent activation of the free YRG A-subunit in the presence of $Mn^{2+}$ is due to a reduction in $K_m$, since the enzyme is normally assayed in 20 mM PNPP. For the wild-type enzyme, the free A-subunit is weakly active, and the $K_m$ was not determined. Examination of the kinetic parameters of the wild-type truncation mutants showed that there were no significant changes in $K_m$ values.

Table 2: Specific Activities of Calcineurin Mutants Assayed with RII as the Substrate[a]

| | assayed with $Mn^{2+}$ | | | | assayed with $Ca^{2+}$ | | | |
|---|---|---|---|---|---|---|---|---|
| variant | A | AC | AB | ABC | A | AC | AB | ABC |
| A511 | | | | | | | | |
| WT | 5.2 | 16 | 90 | 250 | 0.02 | 1 | 18 | 76 |
| YRCG | 2.3 | 6 | 8 | 13 | 0 | 0.07 | 0.8 | 10 |
| YRG | 27 | 170 | 365 | 990 | 1.4 | 13 | 38 | 220 |
| GEFD | 2.7 | 27 | 56 | 320 | 0.3 | 3.2 | 1 | 70 |
| L312C | 5.4 | 20 | 80 | 306 | 0.2 | 5 | 50 | 130 |
| A456 | | | | | | | | |
| WT | 4.7 | 30 | 160 | 360 | 0.3 | 2 | 15 | 73 |
| YRG | 55 | 170 | 220 | 550 | 1.6 | 7 | 40 | 190 |
| GEFD | 17 | 75 | 200 | 450 | 0.9 | 3 | 16 | 80 |
| L312C | 4.5 | 24 | 105 | 280 | 0.4 | 3 | 18 | 95 |
| A388 | | | | | | | | |
| WT | 160 | − | 630 | − | 20 | − | 130 | − |
| YRG | 460 | − | 800 | − | 40 | − | 340 | − |
| GEFD | 90 | − | 540 | − | 4 | − | 80 | − |
| L312C | 180 | − | 950 | − | 10 | − | 200 | − |

[a] Activities were assayed as described under Materials and Methods. Values are presented as units/mg of A-subunit protein. "A", calcineurin A-subunit; "C", calmodulin; "B", B-subunit. A511, full-length A-subunits; A456; truncated at residue 456, A388, truncated at residue 388. The data for the A466 and A388 series were normalized for the loss of size to permit comparison on a relative molar basis. For the A388 series, assays in the presence of calmodulin were performed, but these were essentially the same as those in the absence of calmodulin and are not shown.

Overall, the YRG mutation and its truncation mutations exhibited improved catalytic efficiencies that range from 8 to 60 times greater than that of the wild-type enzyme.

*Effects of Mutation on Activities Assayed Using RII Peptide*. When assayed with the RII peptide (Table 2), a striking contrast with the PNPP activities is seen in that all the free A-subunits for both the full-length and A456 truncations have very little activity, <10% and <1% of their respective heterodimers when assayed in the presence of either $Mn^{2+}$ or $Ca^{2+}$, respectively. However, similar effects of L7 loop modification as noted for the assays with PNPP still hold in regard to the activation of the YRG mutation. The observation that loss of the AI-1 region does not lead to activation, but that it is deletion of AI-2 that results in release of autoinhibition, also holds with RII substrate as seen by examination of the activities with the A456 and A388 mutants. $Mn^{2+}$ did not elicit the full activation of the free truncated subunits that was observed with PNPP as the substrate, and they all required the addition either of the B-subunit (A388 series) or of both the B-subunit and calmodulin (A456 series). In the case of the A456 series, the B-subunit was unable to elicit the full activity as when PNPP is the substrate. The A388 mutants, in which all of the autoinhibitory region is removed, showed an increase in activity of the free A-subunits, and were fully activated by the B-subunit (Table 2).

When assayed in the presence of calcium, the activities with the RII peptide reflected a much tighter regulation of the enzyme activity by calcium and calmodulin. The free full-length and A456 A-subunits exhibited very little activity until removal of the entire autoinhibitory region (A388 series), and even then these were a much smaller fraction of the heterodimer activities than seen under any other assay conditions, the most being about 15% of the full activity. The A456 mutants were hardly activated by calmodulin and poorly activated by the B-subunit. In the case of the A388 mutants, it can be estimated that for a peptide substrate less than 15% of the maximal activity is expressed by the free A-subunits. These data support the concept that calcineurin A is largely in an inactive state, and activation of the catalytic core is dependent mainly on the B-subunit. Previous studies have shown that the effect of the B-subunit on the wild-type A-subunit is a reduction in $K_m$ with PNPP (Stemmer & Klee, 1994). Using RII peptide, truncation of the C-terminus at residue 457 led to a roughly 2-fold increase in $V_{max}$ and a 4-fold reduction in $K_m$ (Perrino et al., 1995).

## DISCUSSION

In this study, we have shown that mutations of the L7 loop region connecting $\beta$ strands 12 and 13 have significant effects on calcineurin A activity, and have also examined the behavior of the corresponding truncated mutants in which the C-terminal AI-1 region (A456 series) and the AI-1, AI-2, and calmodulin binding regions were deleted (A388 series). The findings provide some novel observations that are relevant to an understanding of the regulation of calcineurin. The major findings are that both modification of the L7 loop and truncation of the C-terminus lead to the hyperactivation of calcineurin. The double truncation/L7 loop mutants also establish that the effects of mutation of the L7 loop are separable from the effects of deletion of the autoinhibitory region; i.e., the two effects are relatively independent.

The crystal structures of human calcineurin, the human calcineurin FK506/FKBP12 complex, and the bovine brain calcineurin/FKBP12/506 complex have been reported (Griffith et al., 1995; Kissinger et al., 1995). The architecture of the enzyme provides a basis for the interpretation of the effects observed in our studies. A diagram of the calcineurin structure is shown in Figure 3 to represent the key regulatory elements. The structures of the C-terminal extension of calcineurin, which contains the B-subunit binding region, the calmodulin binding region, and an autoinhibitory region, in that order, are of particular interest. A portion of the previously described C-terminal autoinhibitory peptide (Hashimoto et al., 1990) from residue 459 to residue 476 is shown to be in close contact with the active site. The FKBP12/FK506 complex makes an extended contact with the B-subunit/A-subunit interface, and also has a secondary contact with residues 310−314 in the L7 loop, displacing the entire C-terminal autoinhibitory region (Griffith et al., 1995;

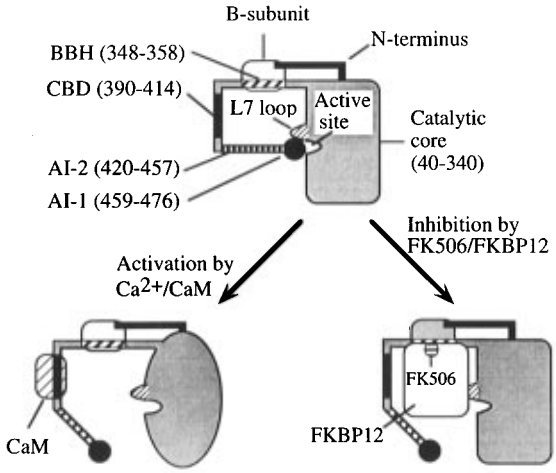


FIGURE 3: Diagrammatic representation of the regulatory regions of calcineurin. The diagram shows the catalytic domain (40−340) and the C-terminal extension of calcineurin, and is based on the crystal structure (Kissinger et al., 1995). The C-terminus contains the amphipathic helix (348−358) that is the B-subunit binding helix (BBH), the calmodulin binding domain (CBD) (Kincaid et al., 1988), and the autoinhibitory region (Hashimoto et al., 1990; Perrino et al., 1995). The region beyond the B-subunit binding region (from 360−511) is disordered and is not present in the crystal structure (Kissinger et al., 1995), except for the AI-1 sequence (459−476). Shown in the diagram are the two mechanisms for the regulation of calcineurin A, through dislocation of the AI regions (left), and a B-subunit-mediated conformational change in the catalytic core. A diagrammatic representation of the structure of the FK506/FKBP12 complex with calcineurin is shown on the right.

Kissinger et al., 1995). The FKBP12 protein occludes, but does not entirely block, the active site.

The striking observations made in this study relate to mutations of the L7 loop region. Our finding that the L7 loop mutations are able to increase catalytic efficiency toward PNPP provides experimental support for the view that this region is structurally important in mediating the conformational changes at the active site that may underlie the activation process. Consideration of the enzyme structure provides several reasons for this. The active site is formed in part by the positioning of two $\beta$ sheets in a $\beta$ sandwich. These two $\beta$ sheets are connected by the L7 loop which forms a prominent extension that overhangs the active site. Thus, structural modifications in this region are likely to be capable of distortion of the orientation of the two $\beta$ sheets that form the catalytic site. This is consistent with the results of truncation of the L7 loop region as reflected by the behavior of the YRG mutant, and with the observations that mutation of the loop region had less effect than that of its shortening by one residue. Our findings are consistent with the view that the calcineurin A-subunit, while having a similar architecture as PP1, differs in that it is normally in a conformationally constrained form that is not active toward peptide substrates, and that the influences of the B-subunit and calmodulin, which lead to activation of the enzyme, also do not completely remove these conformational constraints. The differences in response of the A-subunits to the B-subunit that are seen with PNPP and RII peptide in the presence of calcium indicates that the conformational effects of the B-subunit also involve the peptide binding site.

The activity of calcineurin on the RII peptide in the presence of calcium/calcineurin may reasonably be regarded as most likely reflecting the physiological behavior of the enzyme. Our studies and those of Perrino et al. (1995) show that removal of the entire autoinhibitory region does not lead to significant activation of the free A-subunit. This implies that the catalytic core is in an inactive conformation, that is regulated by the B-subunit, i.e., calcineurin is regulated by a dual mechanism that involves relief of the effects of the autoinhibitory region, as well as the activation of the catalytic core by calcium binding to the B-subunit. In addition to providing a mechanism for calmodulin regulation, the evolution of a dual inhibitory control may reflect a physiological need for the suppression of calcineurin activity except when needed.

Soderling and colleagues (Hashimoto et al., 1990; Perrino et al., 1995), prior to the solution of the crystal structure, had defined two contiguous autoinhibitory sequences covering the C-terminus from 420 to 482. These are AI-1, based on the use of synthetic peptides (Saitoh et al., 1992), and AI-2, based on deletion mutagenesis at residues 420 and 457 (Perrino et al., 1995). This implied a mechanism whereby the enzyme is autoinhibited, so the activation of calcineurin by calcium could be envisaged as occurring through a mechanism in which the autoinhibition is relieved through the action of calcium/calmodulin (Hubbard & Klee, 1989; Perrino et al., 1995). Our findings regarding the effects of truncation of the wild-type enzyme are similar to, and consistent with, results obtained by Perrino et al. (1995), who examined the properties of the same calcineurin A isoform as in this work assayed using RII peptide with calcium as the activating ion. The work of Soderling and colleagues (Perrino et al., 1995) is confirmed by the crystallographic studies (Kissinger et al., 1995) in that the original autoinhibitory peptide (AI-1) is shown to be in contact with the A-subunit in a manner that could explain its role in autoinhibition, but paradoxically, both their studies and ours show that deletion of this region is less important than deletion of the contiguous AI-2 sequence. Thus, there remain aspects of the function of the autoinhibitory region that are not readily explained by the crystal structure, since AI-2 is in the region of disordered structure (Kissinger et al., 1995). There could be additional interactions of the AI-2 region with the rest of the calcineurin structure which prevent the expression of full activity even in the presence of the B-subunit, as reflected by the failure of the A456 mutants to reach the same levels of activity as the A388 mutants. Since the hydrolysis of PNPP is less affected than the peptide substrate, it suggests either that in some way the AI-2 region is responsible for additional conformational changes that affect peptide binding or that it interacts with the enzyme at the peptide binding regions.

The simplest model for calcineurin regulation is for an inactive catalytic core that requires the binding of calcium to the B-subunit for activation through a conformational change that affects the geometry of the active site, and possibly also the substrate binding site, upon which is superimposed an autoinhibitory system that functions through steric occlusion and which is regulated by calcium binding to calmodulin. However, the finding that the full-length heterodimers do not express the full activity that is available when the AI region is deleted is apparently inconsistent with the operation of a simple steric mechanism for autoinhibition in which there is a total loss of interaction in the presence of calcium/calmodulin. This implies that there are interactions of the AI region with the catalytic core that are maintained even in the presence of calcium and calmodulin,

and Perrino et al. (1995) have suggested that the AI-2 region may interact with the B-subunit.

In summary, our findings show that the L7 loop in calcineurin is an important structural feature that may be involved in conformational changes in the active site of calcineurin. These changes reflect the existence of the calcineurin A-subunit in a catalytically constrained state that is subject to regulation by the B-subunit and calmodulin.

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