A Hydrophobic Domain of Ca\(^{2+}\)-modulating Cyclophilin Ligand Modulates Calcium Influx Signaling in T Lymphocytes*

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The complex of cyclophilin A or cyclophilin B with the immunosuppressant drug cyclosporin A has been shown to bind and inhibit the calcium-dependent phosphatase calcineurin (1–4). Because weak binding of cyclophilins to calcineurin can be detected even in the absence of cyclosporin A, it has been proposed that the cyclophilins normally participate in the regulation of calcineurin (5).

Ca\(^{2+}\)-modulating cyclophilin ligand (CAML) was originally described as a cyclophilin B-binding protein whose overexpression in T cells causes a rise in intracellular calcium, thus activating transcription factors responsible for the early immune response. As reported here, structure-function analysis of the CAML gene in Jurkat T cells indicates that two of CAML's putative membrane-spanning domains are necessary and sufficient for the modulation of intracellular calcium. We propose that the hydrophobic C-terminal tail of CAML forms its effector domain, thus implicating the N-terminal hydrophilic domain in a regulatory role. These findings define a novel protein motif that functions in intracellular calcium signaling.

The complex of cyclophilin A or cyclophilin B with the immunosuppressant drug cyclosporin A has been shown to bind and inhibit the calcium-dependent phosphatase calcineurin (1–4). Because weak binding of cyclophilins to calcineurin can be detected even in the absence of cyclosporin A, it has been proposed that the cyclophilins normally participate in the regulation of calcineurin (5).

Ca\(^{2+}\)-modulating cyclophilin ligand (CAML) was isolated by a yeast two-hybrid screen of a B lymphocyte cDNA library using cyclophilin B as bait for endogenous cyclophilin ligands (6). When overexpressed in Jurkat T cells, this cyclophilin-binding protein causes the calcineurin-dependent activation of NFAT, a T cell-specific transcription factor responsible for inducing interleukin-2 expression (1). The site of action of CAML was determined to be downstream of the T cell receptor and upstream of calcineurin. Further support of a role for CAML in calcium signaling was provided by observations that CAML overexpression relieved the requirement for increased Ca\(^{2+}\), for activation of NFAT but does not relieve the requirement for protein kinase C activation.

The sequence analysis of human CAML failed to show significant homology to other proteins by BLAST and TFASTA (2, 8), but two distinct domains are apparent in hydropathy plots. The Goldman, Engelman, and Steitz hydropathy scale method (9) predicted three membrane-spanning segments in the C-terminal end of CAML and a charged hydrophilic N-terminal helix (6).

Functional analysis of the CAML gene has allowed us to identify domains that modulate calcium flux. Here we show that the residues encompassing the last two putative membrane-spanning segments are necessary and sufficient for inducing NFAT activity in T cells.

**MATERIALS AND METHODS**

Plasmids and Deletions—The pFLEX expression vector was constructed by inserting 27 base pairs that encode the FLAG epitope into the pB7.5 mammalian expression vector polylinker (10). CLX31 was constructed by inserting CAML cDNA into pFLEX, as described previously (6). Plasmids directing expression of truncated forms of CAML were made by standard protocols that included exonuclease III digestion and polymerase chain reaction (11). Fragments of the CAML coding sequence generated by polymerase chain reaction and use of Pfu polymerase (Stratagene) were ligated into the Xhol and EcoRI restriction endonuclease sites of pFLEX. Plasmids were generated by the amino acid residues included in the product protein, with 1–296 being the full-length native protein. The primer sequences are listed in Table I. The expression plasmid was described elsewhere (12). Briefly, it contains three copies of the NFAT enhancer element and the interleukin-2 minimal promoter to direct expression of the secreted alkaline phosphatase (SEAP) gene.

Cell Culture—Tag Jurkat T cells, stably transfected with the SV40 large T antigen, were obtained from J. Northrop (Stanford Medical Center) and maintained in RPMI 1640 (Life Technologies, Inc.) plus 10% fetal calf serum (Gemini Bioproducts) at 37 °C in 5% CO\(_2\) at a density of 10\(^7\)–10\(^8\) cells/ml. COS cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) plus 10% fetal calf serum.

NFAT Functional Assay—Mutant CAML expression was assayed by transient co-transfection with the NFAT reporter plasmid, SXXNFAT, using 6 μg of pFLEX or CAML expression construct, 6 μg of SXXNFAT, and 2 μg of a vector expressing luciferase as a transfection control. Electroporation into Tag Jurkat cells and measurement of NFAT-SEAP were performed as described previously (12). Treatment of the transfected cells with 32 mM PMA was used to activate the nuclear subunit of NFAT, API1 (13). Luciferase activity was measured by standard protocols. Western blot assays of FLAG-labeled CAML constructs indicated that the deletion mutants were correctly expressed in transiently transfected COS cells. Monoclonal FLAG-M2 antibody was obtained from Kodak.

FACS Analysis—Tag Jurkat cells were transiently transfected with 6 μg of a CAML expression vector together with 2 μg of co-transfected vector expressing mouse CD8. Cells were stained with rat anti-mouse CD8 conjugated to fluorescein and with 3 μM indol-1-acetylomethyl ester (Molecular Probes). Cytoplasmic calcium was measured in the transfected (CD8 positive) cells using a Becton Dickinson FACS Vantage instrument by determining the ratio of indol-1 fluorescence at 400–480 nm following excitation at 334–364 nm (14). Cells transfected with CD8 plasmid alone served as the control.

**RESULTS AND DISCUSSION**

Activation of the T cell-specific transcription factor NFAT was assayed by co-transfection of CAML expression plasmids with the NFAT-SEAP plasmid SXXNFAT. Expression of SEAP from this reporter depends on cross-linking of the T cell receptor or stimulation of the second messengers protein kinase C...
and Ca\(^{2+}\) (by treatment with PMA plus ionomycin) and is completely blocked by cyclosporin A or FK506. As shown previously (6), overexpression of full-length CAML replaced the usual requirement for Ca\(^{2+}\) influx, while having no effect on the requirement for PMA costimulation, consistent with a selective effect on Ca\(^{2+}\) signaling. To define the degree of activation in transiently transfected T cells more precisely, we developed an assay comparing the effects of CAML and mutant constructs with that of thapsigargin. Unlike ionomycin, which acts at both the endoplasmic reticulum and the cell surface, thapsigargin activates capacitative Ca\(^{2+}\) influx selectively by depleting intracellular Ca\(^{2+}\) stores (15–18). NFAT activation in control cells was characterized by a discrete maximal response at 5 nM thapsigargin (Fig. 1, dotted line) with PMA costimulation. By marked contrast, CAML overexpression not only significantly activated NFAT in the absence of thapsigargin but also shifted the dose-response curve to the left, causing a maximal response at 2.5 nM and a marked enhancement of activation due to thapsigargin concentrations as low as 250 pM (Fig. 1, solid line). A reproducible measure of CAML activity could be obtained by expressing the degree of activation due to CAML plus PMA as a percentage of maximal activation induced by the addition of thapsigargin.

To define the active residues in the CAML protein, we made 10 different deletion mutants and inserted them into the eukaryotic expression plasmid pFLEX, which adds the FLAG epitope to the N terminus of inserted genes. Proteins encoded by these expression plasmids were verified as being correct by transfection into COS cells, followed by extraction and Western

### Table 1

| Primer name | Amino acid base number | Sequence |
|-------------|------------------------|----------|
| 5' MH13     | 189                    | GATATctcagTGGTGGGATGTGCTCTTTG |
| 5' MH14     | 220                    | GAGaatcCcAACTGGCAGCTACTTATGTT |
| 5' MH15a    | 240                    | GAAAgtcgcACAGTTCAAGTGCTTATGTC |
| 5' MH17     | 268                    | GGCAAcgcACAGTTGATCCACTATGTC |
| 3' MH19     | 146                    | GGCGgaattcCCTCTGGACCCAGTTCTTTG |
| 3' MH5      | 201                    | CTGgattcCAGAAGACCATGACGAG |
| 3' MH16     | 247                    | GAAAAAAAAAGACCATGAGAG |
| 3' MH12     | 265                    | GAAAGACATGACATGACATGAC |
| 3' MH7      | 289                    | AGCAAGACATGACATGACATGAC |

**Fig. 1.** The effect of CAML overexpression on the Ca\(^{2+}\) dependence of NFAT transcription activation. Cells were cotransfected with CAML expression plasmid and the SEAP reporter, as described under "Materials and Methods." The transfected cultures were treated with 0.25–12.5 nM thapsigargin and 32 nM PMA for 24 h. The y values presented are the percent of the maximal stimulation for each transfection and are the average of three separate transfections. Assay values were normalized for variations in transfection efficiency with an assay for co-transfected luciferase expression vector. The x axis is log nM thapsigargin. The zero value is superimposed on the graph for illustration. The error bars represent the standard error.

**Fig. 2.** Functional assay of CAML deletions. The bars on the left indicate schematically the residues of the CAML protein from amino acid 1 to 296 encoded by the expression constructs. The three smaller bars on the top represent the locations of the predicted membrane-spanning regions. Values graphed on the right represent the average of six independent assays, with error bars representing the standard error. The assay values represent CAML-induced activation expressed as percent of maximal stimulation following treatment with PMA and thapsigargin.

**Fig. 3.** Thapsigargin titration of transfected Jurkat cells. Cells were cotransfected with CAML deletion mutant expression vectors and the SEAP reporter, as described under "Materials and Methods." The values graphed are as described in Fig. 1.
blotting with the FLAG monoclonal antibody (data not shown). Each protein yielded a band of the correct size on Western blots, thus demonstrating roughly similar levels of accumulation.

Transfection-titration experiments in Tag J urkat cells were performed with the 10 mutant CAML constructs together with the control and full-length vectors. Fig. 2A shows the portions of CAML-coding sequence left in each construct, while Fig. 2B indicates the effectiveness of each construct at activating NFAT as a percentage of maximal activation due to CAML plus thapsigargin. Deletions removing a portion of or all of the N-terminal half of the protein had no important effect in the assay (compare residues 189–296 with the full-length 1–296) nor did removal of the last seven amino acids from the short hydrophilic C terminus (1–289). By contrast, removal of 46 residues from the C-terminal end (1–250) effectively abolished NFAT activity.

To further localize the activating activity within the hydrophobic domain, we next tested plasmids encoding subregions of the three putative transmembrane domains. One that encoded a surprisingly small part of the protein, the predicted second and third membrane-spanning regions (residues 240–289), was active in this assay. The loop region (residues 220–240) between the putative membrane domains 1 and 2 was not necessary, as it could be deleted without any apparent effect (compare 220–289 with 240–289). Domains 1 and 2 (195–265) or either domain 2 or 3 alone (220–265 and 268–289) lacked significant activity in the NFAT assay. We conclude from these experiments that domains 2 and 3 are necessary and sufficient to form the minimum active fragment of CAML. This interpretation is supported by independent comparison of human and chicken CAML genes showing that the greatest area of sequence similarity is within the C terminus (19). Indeed, of the 50 residues within the 240–289 domain, 46 were identical between the two species and two others were highly conserved.

Because overexpression of CAML shifts the dose-response curve of thapsigargin to lower drug levels, we inferred that CAML acts positively in this signaling pathway to activate NFAT. To examine the possibility of a dominant negative effect in any of the mutant CAML constructs we similarly investigated dose-response curves for a shift to higher thapsigargin levels. The 240–289 active fragment, containing the putative second and third membrane-spanning domains, displays elevated NFAT activity and increased sensitivity to thapsigargin, as indicated by the left shift of the titration curve (Fig. 3, triangles). The assay results with this plasmid resemble those of transfections with the full-length CAML plasmid, although the values are often higher with the short fragment, suggesting the possibility of an inhibitory or modulatory region within the N-terminal half of the protein. Each mutant plasmid with NFAT-stimulating activity (1–289, 44–296, 189–296, 220–289, and 240–289) also showed a similar shift of thapsigargin response to lower levels. By contrast, each of the inactive mutant plasmids (1–250, 1–201, 195–265, 220–265, 268–289), while directing appropriate protein expression as measured by Western blot analysis, did not shift the response of cells to thapsigargin to higher levels by comparison with control transfections (Fig. 3, squares and data not shown). Thus, we can find no evidence for a dominant negative effect of any of our constructs. Similar results were obtained using ionomycin titration instead of thapsigargin.

To further localize the effects of overexpression of mutant CAML constructs, we asked whether calcineurin was an obligate intermediate in the activation of NFAT. Cells transfected with the 240–289 expression plasmid were stimulated with PMA in the presence or absence of cyclosporin A at 100 ng/ml. Addition of this immunosuppressive level of cyclosporin A completely abrogated NFAT expression (data not shown), consistent with our model in which this CAML fragment triggers NFAT through activation of calcineurin.

To verify that the activation of calcineurin was due to elevation of Ca\(^{2+}\), we measured the effect of the residue 240–289 expression plasmid on cytoplasmic Ca\(^{2+}\) levels. Cells transfected with full-length CAML and those expressing the 240–289 deletion mutant were loaded with indo-1 and identified by staining for a cell surface co-transfected marker. Control transfections had normal resting Ca\(^{2+}\) fluorescence levels (Fig. 4A), while cells overexpressing either CAML or the 240–289 fragment displayed a significant number showing markedly elevated Ca\(^{2+}\) to drop down to baseline within 4–5 s (data not shown). Thus, overexpression of this relatively small peptide is sufficient to elevate [Ca\(^{2+}\)], to levels similar to that of the full-length protein, an effect likely produced by increased influx of Ca\(^{2+}\) from outside the cell.

The structure-function data presented here demonstrate that residues 240–289 of CAML are necessary and sufficient to activate Ca\(^{2+}\) influx when overexpressed in J urkat T cells. These residues overlap the previously noted transmembrane domains 2 and 3. Because the N-terminal half of the protein does not activate Ca\(^{2+}\) influx and is not necessary for the activity of the 240–289 fragment, we propose that it may regulate CAML function under normal circumstances. However, we cannot exclude a regulatory role for other factors (e.g. altered interference by interacting proteins or altered subcellular
localization). Studies to address this issue are under way.

The finding that the modest 50-residue fragment of CAML can activate Ca\(^{2+}\) influx argues against an enzymatic role for CAML in this context, since it seems unlikely that such a small peptide could catalyze a reaction producing a small second messenger. We favor instead a model in which this domain of CAML forms a direct protein interaction site by which it contacts and activates the next signaling protein in the pathway. The downstream target of CAML is currently unknown but could include such well characterized molecules as the inositol trisphosphate receptor or the endoplasmic reticulum Ca\(^{2+}\) pump or the hypothesized intracellular Ca\(^{2+}\) leak channel (20). To date, we have not been able to ascertain the filling state of intracellular stores in CAML-overexpressing cells and hence cannot yet place the putative target upstream or downstream of the site where the capacitative Ca\(^{2+}\) influx signal is generated. Indeed, because of the extreme hydrophobic nature of residues 240–289, CAML may itself be a component of an internal Ca\(^{2+}\) channel.

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