Co-localization of Calcium-modulating Cyclophilin Ligand with Intracellular Calcium Pools

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The calcium-modulating cyclophilin ligand (CAML) protein activates Ca²⁺ influx signaling when overexpressed in Jurkat T cells. Although CAML appears to directly participate in Ca²⁺-dependent signaling initiated by the transmembrane activator and CAML interactor cell surface receptor, its mechanism of action is unknown. To address this issue, we have determined its membrane topology, subcellular localization, and ability to mobilize intracellular Ca²⁺ pools. Fractionation of cell extracts on discontinuous sucrose gradients and indirect immunofluorescence indicate that CAML co-localizes with sarcoplasmic/endoplasmic reticulum calcium/ATPase-2 and calreticulin at membrane-bound cytosolic vesicles. Limited trypsin digests indicate that the hydrophilic NH₂-terminal domain of CAML is directed toward the cytoplasm. Functionally, CAML overexpression was shown to deplete thapsigargin-sensitive intracellular Ca²⁺ pools. These data suggest that CAML may initiate Ca²⁺ signaling through activation of a capacitative Ca²⁺ influx pathway.

The T lymphocyte-specific transcription factor NF-AT³ requires a Ca²⁺ influx signal for activation (1). This signal is ordinarily provided following T cell receptor stimulation, and can be mimicked by pharmacologic treatment with thapsigargin, a specific inhibitor of Ca²⁺/ATPase (SERCA) pumps (2). We initially characterized the CAML protein as a potential regulator of Ca²⁺-dependent signaling, because its overexpression in Jurkat T cells constitutively activated an NF-AT reporter in cells co-stimulated with phorbol ester (3). Subsequently, we found that CAML overexpression shifts the dose-response to thapsigargin to lower concentrations, thus suggesting a possible mechanism of CAML action through release of intracellular Ca²⁺ pools, and consequent activation of NF-AT through capacitative Ca²⁺ influx (4). In addition, indirect immunofluorescence localization of epitope-tagged CAML revealed a punctate, reticular pattern suggesting a localization within cytoplasmic vesicles (3).

We have begun to address the question of the role of CAML in Ca²⁺ signaling by assigning functions to subdomains of the protein. Sequence analysis indicates that the carboxyl-terminal third of the protein has three hydrophobic domains that are of sufficient length to span a membrane. Overexpression of the two most carboxyl-terminal of these three predicted membrane spanning regions was sufficient for NF-AT activation in the presence of phorbol 12-myristate 13-acetate (4). The hydrophilic NH₂-terminal domain of CAML was shown to act as a dominant negative regulator of NF-AT activation following cell-surface cross-linking of the newly discovered receptor protein, TACI (5). Therefore, the hydrophilic domain of CAML was proposed to function in the regulation of the signaling properties of its hydrophobic domain.

Although the involvement of CAML in signal transduction seems to be dependent on Ca²⁺ influx, it is still uncertain how the protein acts in relation to known signaling pathways in lymphocytes, involving depletion of intracellular Ca²⁺ pools. To obtain data to help resolve these issues and confirm the effect of CAML on Ca²⁺ influx in a direct manner, we used Ca²⁺ imaging to determine if overexpression of CAML leads to release of intracellular Ca²⁺ pools. We have also determined the topology and subcellular localization of endogenous CAML, and compared it to the known markers of Ca²⁺ storage organelles, calreticulin (6–8) and SERCA2 (9, 10).

MATERIALS AND METHODS
CAML Protein Preparation and Antibodies—Rabbit polyclonal anti-CAML serum was prepared by Rockland (Boyertown, PA) by immunization with bacterially expressed CAML protein produced using the plasmid pGEX-CAML. pGEX-CAML was constructed by inserting the first 600 base pairs of the CAML cDNA, coding for the hydrophilic domain of CAML into pGEX-4T-2 (Amersham Pharmacia Biotech) to produce a glutathione S-transferase-CAML fusion protein. Glutathione S-transferase-CAML was expressed and isolated by glutathione-Sepharose (Amersham Pharmacia Biotech) affinity chromatography (20). The anti-CAML serum was affinity-purified using the glutathione S-transferase-CAML fusion protein conjugated to a glutathione Sepharose 4B matrix (Amersham Pharmacia Biotech) with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) affinity chromatography (20). The anti-CAML serum was affinity-purified using the glutathione S-transferase-CAML fusion protein conjugated to a glutathione Sepharose 4B matrix (Amersham Pharmacia Biotech) with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) affinity chromatography (20). The anti-CAML serum was affinity-purified using the glutathione S-transferase-CAML fusion protein conjugated to a glutathione Sepharose 4B matrix (Amersham Pharmacia Biotech) with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) affinity chromatography (20).

Western Blotting—SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose sheets was performed as described in Sambrook et al. (11).

Triton X-114 Protein Extraction—× 10⁶ Jurkat cells were extracted with Triton X-114 by the method of Bordier (12) as modified by Brusca et al. (13). The cells were suspended in 0.1 ml of cold phosphate-buffered saline and brought to 2% precondensed Triton X-114 (Boehringer Mannheim). The suspension was gently rotated at 4 °C for 2 h, and centrifuged at 13,000 × g at 4 °C for 10 min to remove insoluble material. The supernatant was incubated at 37 °C for 10 min and...
antibody. 3T3 cells were transfected with the pFLAG mammalian expression vector so that the Flag epitope is expressed at the amino terminus of the protein. 

blot for detection with anti-CAML antibody. 3T3 cell lysates were run on a 12% SDS-PAGE gel, and the gel used for transfer to nitrocellulose for Western blotting as described under “Materials and Methods.” The presence of a single band of the correct size demonstrates the high degree of specificity of this antibody preparation.

centrifuged 10 min at 13,000 x g at room temperature. The aqueous phase was again brought to 2% Triton X-114, incubated at 4 °C for 10 min, and separated as above. The second detergent phase was discarded. The second aqueous phase was centrifuged and the Triton X-114 phase was acetone-precipitated prior to electrophoresis. Protein concentrations were determined by the method of Bradford (14) using the Bio-Rad protein assay reagent.

Cell Fractionation—Jurkat cells were homogenized by nitrogen cavitation and fractionated over a discontinuous sucrose gradient as described by Bourguignon et al. (15, 16). Briefly, 100 ml of culture grown to 10^6 cells/ml were resuspended in 10 ml of cold phosphate-buffered saline, incubated at 37 °C, and centrifuged as above. The second aqueous phase was discarded. The Triton X-114 phase was acetone-precipitated prior to electrophoresis. Protein concentrations were determined by the method of Bradford (14) using the Bio-Rad protein assay reagent.

Enzyme Assays—Galactosyltransferase activity was measured as described in Goldberg and KornelI (17) and Schientek et al. (18). NADPH-dependent cytochrome c oxidase was assayed by the method of Fleischer and Fleischer (19). Glucosaminidase was assayed as described in Bourguignon et al. (20). Mannosyltransferase was assayed as described in Wolfe et al. (21).

Limited Trypsin Digest—2 x 10^7 Jurkat cells were lysed as described in the cell fractionation protocol above, with the exception that incubation with high pressure nitrogen was done at 60 psi. Protein concentration was measured by the Bradford (14) method, and 225 μg of protein were digested with the concentrations of trypsin (Sigma type IX, 17,600 units/mg of protein), indicated in Fig. 2, for 30 min at room temperature. The digestion was stopped with the addition of 100 μg of trypsin inhibitor (Sigma) and 200 μg of tRNA. Protein was trichloroacetic acid-precipitated, separated by SDS-polyacrylamide gel electrophoresis, and Western blotted for detection with anti-CAML antibody.

Indirect Immunofluorescence—CAML cDNA was inserted into the pFLAG mammalian expression vector so that the Flag epitope is expressed at the amino terminus of the protein. 3T3 cells were transfected by use of LipofectAMINE reagent (Life Technologies, Inc.) following the manufacturer’s protocol. NIH 3T3 cell fixation and incubation with primary and secondary antibodies was carried out as described by Bourguignon et al. (22). Flag epitope-tagged CAML-transfected cells were incubated with 1.7 μg/ml anti-flag M2 monoclonal antibody (Eastman Kodak Co.). Rabbit antisera against calreticulin (Affinity Bioreagents) was made into a 50% glycerol stock for storage at −20 °C and used at a 1:100 dilution according to the manufacturer’s suggested protocol. Incubations with affinity-purified rabbit antisera against CAML were carried out at a concentration of 600 ng/ml. Rabbit anti-mouse lissamine rhodamine (Jackson Immunoresearch Laboratories), biotinylated goat anti-rabbit IgG, and avidin fluorescein isothiocyanate (Vector Laboratories) were used at 1:1000 dilution according to the manufacturer’s suggested protocol. Fixed and stained cells were mounted with p-phenylenediamine in 50% glycerol as described by Johnson and Nogueira Araujo (23).

Staining with one or the other primary antibody separately revealed that there was no interspecies cross-reactivity with the secondary antibodies. Incubation of the anti-mouse lissamine rhodamine-conjugated antibody after incubation with the rabbit polyclonal anti-CAML antibody, for instance, produces only background staining comparable to cells incubated with the secondary antibody alone.

Measurement of [Ca^{2+}], in transiently transfected TAg-Jurkat Cells—Transient overexpression of CAML in TAg-Jurkat cells was performed by electroporation of 3 μg of CAML expression plasmid CLX12, as described previously. Co-transfection of the pHOK2 plasmid (2 μg, Invitrogen) was used to facilitate isolation of CAML-overexpressing cells, as follows. After electroporation, cells were incubated at 37 °C in 5% CO_{2} for 4 h to allow expression of CAML and the HOOK protein, which is a hemagglutinin epitope-tagged cell surface protein lacking a cytoplasmic domain. Cells were collected and incubated with 12CA5 (anti-HA) monoclonal antibody (2 μg/ml, Boehringer Mannheim) and with fura-2AM (3 μM, Molecular Probes) for 30 min at room temperature in the dark, in Hepes-buffered RPMI 1640 with 10% fetal bovine serum. After washing, cells were allowed to bind to glass-bottom dishes (Matek) that had been precoated with goat anti-mouse IgG antibodies (10 μg/ml, Sigma) for 15–30 min in the dark, after which untransfected cells were removed by gentle washing with Ringer’s solution containing 2 mM CaCl_{2}. Control experiments verified that mock-transfected cells do not adhere under these conditions, while HOOK-expressing cells attach firmly.

Cytosolic free Ca^{2+} was measured in fura-2-loaded cells using an Attofluor Ratiovision system with a Zeiss Axiovert 135TV inverted microscope (Carl Zeiss, Rockville, MD). Cells at room temperature were excited with alternating 340 and 380 nm light, and emitted wavelengths at 520 nm were detected using an ICCD camera. Excitation light was attenuated with the use of neutral-density filters to prevent damage to cells and photo-bleaching of fura-2, and to maintain the detected levels of fluorescence within the linear response range of the camera. Free [Ca^{2+}], was calculated from the 393/580 fluorescence ratios following the method of Grynkiewicz et al. (24). For each experiment, approximately 45 individual cells were monitored at 5-s intervals for a total of 1000 s. After determining base-line [Ca^{2+}], in Ringer’s solution containing 2 mM CaCl_{2}, the bathing solution was replaced with Ringer’s solution without Ca^{2+} for 1–2 min, followed by addition of thapsigargin to 1 mM final concentration, as indicated. Once the release of intracellular Ca^{2+} stores was complete, extracellular Ca^{2+} was restored (2 mM) as indicated.
RESULTS

CAML Is an Integral Membrane Protein—To test our hypothesis that CAML is an integral membrane protein, we asked whether the endogenous protein partitions into TX-114 detergent micelles. Phase separation of proteins based on their affinity for such hydrophobic detergent micelles has been used as an indicator of the ability of a protein to insert into a lipid bilayer (13). Protein lysates were prepared from Jurkat cells and separated into membrane-soluble and aqueous fractions. The majority of endogenous CAML protein was found to be incorporated into the detergent fraction (Fig. 2), confirming that it is capable of anchoring within a membrane, as was suggested by the primary sequence and cell fractionation studies (see below) (3, 25).

CAML Projects into the Cytoplasm—The CAML amino acid sequence does not contain an NH₂-terminal signal sequence that could help in predicting the protein’s orientation in a membrane. The “positive inside” rule of von Heijne, however (26), predicts that the majority of the protein lies on the cytoplasmic face of a membrane. To test this prediction, a microsome fraction from Jurkat T cells was prepared and briefly treated with low concentrations of trypsin to determine the relative accessibility of CAML to the protease. Calreticulin was chosen as the control since it resides inside purified vesicles and should be relatively protected from limited exposure (27). Western blotting analysis (Fig. 3) using both CAML- and calreticulin-specific antibodies demonstrate that CAML is not protected from protease digestion, while the lipid bilayer does protect the luminal protein calreticulin from exposure to the enzyme. We conclude that the hydrophilic domain of CAML is on the outer surface of membrane-bound structures in vivo.

CAML Co-localizes with Ca²⁺ Pools—Jurkat T cells were finely homogenized by nitrogen cavitation, and membranes were separated in a sucrose step gradient. Fractions were probed for the presence of CAML and several known Ca²⁺-regulating proteins by Western blotting. Membranes containing CAML comigrated in the sucrose gradient with those containing both calreticulin and SERCA2 (Fig. 4). The CAML-containing membranes are present in fractions containing enzymatic markers for smooth and rough endoplasmic reticulum, lysosome, and Golgi membranes, although the bulk of CAML appears in the endoplasmic reticulum-containing fractions. Inositol 1,4,5-trisphosphate receptor-containing membranes sediment more slowly (as has been shown previously by others) (15) and are clearly distinct from membranes containing CAML.

To further identify the physical location of CAML, we observed the native protein in cells using indirect immunofluo-
rescence. The human glioblastoma cell line U87 was found to have an easily detectable level of endogenous CAML, as well as a more convenient physical architecture than Jurkat T cells. Cells fixed and stained with affinity-purified rabbit polyclonal antibody to CAML (whose purity is demonstrated in Fig. 1) showed a cytoplasmic, punctate pattern of staining, consistent with previous reports of the pattern of expression of epitope-tagged CAML (Fig. 5A). Indirect immunofluorescence using a mouse monoclonal antibody to the SERCA2 protein also revealed a punctate, reticular pattern of distribution similar to that already reported for SERCA2 in several cell types (27, 30).

To compare co-localization of CAML with a second marker for intracellular Ca\(^{2+}\) pools, we also examined the distribution of calreticulin using a mouse calreticulin-specific antibody. For this purpose, we used NIH3T3 cells, which were transiently transfected with a plasmid encoding a FLAG-epitope-tagged derivative of CAML. In this experiment, the CAML stain overlapped with a portion of the calreticulin stain, indicating that CAML is expressed in close proximity to a subset of calreticulin containing membrane structures (Fig. 5E-G). We conclude from these and the subcellular fractionation experiments that CAML is expressed in the SERCA2-containing subset of the intracellular Ca\(^{2+}\) pool.

Overexpression of CAML Depletes Ca\(^{2+}\) Pools—We found previously that transient overexpression of CAML in Jurkat T cells led to a constitutive increase in cytoplasmic free Ca\(^{2+}\), in a subset of transfected cells (3). The results described above indicate that the endogenous protein co-localizes with SERCA2, raising the possibility that the mechanism of CAML action might involve depletion of intracellular Ca\(^{2+}\) stores. This would serve to activate the capacitative Ca\(^{2+}\) influx mechanism. Alternatively, CAML could act “downstream” of intracellular Ca\(^{2+}\) stores, instead acting by generating a third-messenger signal (such as calcium influx factor) (28, 29) able to open plasma membrane Ca\(^{2+}\) channels. To distinguish between these two possibilities, we examined the filling state of thapsigargin-sensitive Ca\(^{2+}\) stores in CAML-overexpressing Jurkat cells.

As it has been impossible to establish cell lines that stably overexpress CAML (data not shown), we performed these experiments following transient transfection of expression plasmids into TAg-positive Jurkat cells. To identify transfected cells, we included a plasmid (pHook2) that encodes a cell surface marker. Transfected cells were physically separated from nontransfected cells by panning with a monoclonal antibody able to recognize a specific epitope on the Hook protein. Because the Hook protein does not have an intracellular domain, it serves as a useful cell marker, without having an effect on normal Ca\(^{2+}\) signaling.

In control transfections with pHook2 alone, (Fig. 6A) release of intracellular Ca\(^{2+}\) stores with thapsigargin in the absence of external Ca\(^{2+}\), caused a transient rise of free Ca\(^{2+}\) of approximately 120 nM above baseline. Examination of CAML + pHook2-transfected cells revealed that 33% (15 out of 45, in a
typical experiment) of cells displayed a resting Ca\(^{2+}\) higher than 220 nm (Fig. 6B, solid line), which was the highest value seen in control cells. The other 67% of cells most likely did not take up sufficient CAML plasmid DNA to activate constitutive Ca\(^{2+}\) influx, and were separately analyzed (Fig. 6B, dashed line). Following removal of extracellular Ca\(^{2+}\), intracellular Ca\(^{2+}\) in the high base-line transfected cells declined to control levels, indicating its apparent requirement for Ca\(^{2+}\) influx. Subsequent treatment with thapsigargin caused an insignificant increase in [Ca\(^{2+}\)]\(_{i}\), when compared with low base-line transfected cells (Fig. 6B). Thus, we conclude that overexpression of CAML induces a high cytoplasmic Ca\(^{2+}\) at the expense of intracellular Ca\(^{2+}\) stores, most consistent with a model in which it acts through activation of a capacitative influx mechanism.

**DISCUSSION**

The data presented here demonstrate that CAML is an integral membrane protein and that a portion of the protein is available for binding to other proteins in the cytoplasm. The membrane anchor of the protein is provided by the carboxy-terminal third of the protein, which contains the three regions predicted to be membrane-spanning domains (3). That the hydrophilic domain of the molecule projects into the cytoplasm is consistent with previous work demonstrating a physical interaction of this portion of the protein with the cytoplasmic tail of the plasma membrane receptor, TACI (5).

Co-localization with calreticulin via immunofluorescence and sucrose gradient fractionation places CAML within membranes that store Ca\(^{2+}\) inside the cell. However, it is apparent that CAML does not completely co-localize with calreticulin, which is located in many types of membrane-bound compartments, including Golgi, lysosomes, endoplasmic reticulum, and the nucleus (27, 30). CAML appears to be targeted to the subset of these membrane structures that also contain the Ca\(^{2+}/\)ATPase SERCA2.

Such localization of CAML to SERCA2-containing vesicles raised the possibility that CAML might function by causing depletion of Ca\(^{2+}\) from internal stores. This had been previously suggested by observation of a synergistic effect between CAML and the SERCA2 inhibitor thapsigargin on activation of NF-AT (4). In this current study, therefore, we measured the effects of CAML overexpression on internal Ca\(^{2+}\) stores, and demonstrated a marked depletion due to the protein. Thus, it appears that CAML may act by directly releasing intracellular Ca\(^{2+}\), a process that would be expected to activate cell surface Ca\(^{2+}\) channels responsive to the filling state of intracellular stores (31, 32).

We previously found that the COOH-terminal 50 amino acid residues of CAML were necessary and sufficient to cause a constitutive activation of Ca\(^{2+}\) influx, when overexpressed in Jurkat T cells (4). This observation argued against an enzymatic function for CAML (such as generation of inositol 1,4,5-trisphosphosphate, or other known second messenger.) A simple model that could explain both sets of data is that CAML could itself form an internal Ca\(^{2+}\) “leak” channel. In that scenario, regulation of the channel function might be controlled by the cytoplasmic NH\(_2\) terminus of the protein, which we have more recently shown is responsible for TACI-mediated activation of Ca\(^{2+}\) dependent signaling. An alternative hypothesis would be that CAML might physically contact and modulate the function of a known Ca\(^{2+}\) channel or transporter, such as SERCA2 itself. This latter hypothesis is somewhat attractive, given that CAML has no obvious sequence homology with known channels.

Before meaningful studies on any potential channel properties of CAML can be performed, it will be necessary to more fully understand how its activity is regulated. Currently, we do not know if its activity is normally gated by diffusible small molecules, by post-translational modification (such as phosphorylation) or by direct physical interactions with receptors at the cell surface. Given the recent isolation of the tumor necrosis factor receptor family member TACI by virtue of its ability to bind to CAML, and work from other laboratories demonstrating the importance of the cytoskeleton in transducing signals leading to Ca\(^{2+}\) influx (33), the latter explanation may ultimately prove to be correct.

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