Identification and Characterization of EhCaBP2
A SECOND MEMBER OF THE CALCIUM-BINDING PROTEIN FAMILY OF THE PROTOZOA PARASITE ENTAMOEBA HISTOLYTICA

Paramita Chakrabarty‡‡, Dhruv K. Sethi†, Narendra Padhan†, Kanwal J. Kaur*, Dinakar M. Salunke‡, Sudha Bhattacharya†, and Alok Bhattacharya**

From the †School of Environmental Sciences and the ‡School of Life Sciences, Jawaharlal Nehru University, New Delhi, India and the ¶National Institute of Immunology, New Delhi 110067, India

Entamoeba histolytica, an early branching eukaryote, is the etiologic agent of amebiasis. Calcium plays a pivotal role in the pathogenesis of amebiasis by modulating the cytopathic properties of the parasite. However, the mechanistic role of Ca2+ and calcium-binding proteins in the pathogenesis of E. histolytica remains poorly understood. We had previously characterized a novel calcium-binding protein (EhCaBP1) from E. histolytica. Here, we report the identification and partial characterization of an isoform of this protein, EhCaBP2. Both EhCaBP2s have four canonical EF-hand Ca2+ binding domains. The two forms are encoded by genes of the same size (402 bp). Comparison between the two genes showed an overall identity of 79% at the nucleotide sequence level. This identity dropped to 40% in the 75-nucleotide central linker region between the second and third Ca2+ binding domains. Both of these genes are single copy, as revealed by Southern hybridization. Analysis of the available E. histolytica genome sequence data suggested that the two genes are non-allelic. Homology-based structural modeling showed that the major differences between the two EhCaBP2s lie in the central linker region, normally involved in binding target molecules. A number of studies indicated that EhCaBP1 and EhCaBP2 are functionally different. They bind different sets of E. histolytica proteins in a Ca2+-dependent manner. Activation of endogenous kinase was also found to be unique for the two proteins and the Ca2+ concentration required for their optimal functionality was also different. In addition, a 12-mer peptide was identified from a random peptide library that could differentially bind the two proteins. Our data suggest that EhCaBP2 is a new member of a class of E. histolytica calcium-binding proteins involved in a novel calcium signal transduction pathway.

Ca2+ is a ubiquitous second messenger involved in signal transduction processes in eukaryotes (1). Intracellular Ca2+ gradients initiate a number of cellular processes including cell migration, exocytosis, activation of killer lymphocyte cells, cellular secretion, transcellular ion transport, neurotransmitter release, and gap junction regulation (2). These processes are essentially mediated by a variety of Ca2+-binding proteins (CaBPs),1 which are involved in binding Ca2+ and transducing the signal through downstream effectors. Calmodulin (CaM), a four EF-hand, highly conserved CaBP has been found in almost all eukaryotic cells and has been implicated in a large number of cellular processes (for review, see Ref. 3).

Ca2+ signaling also plays a crucial role in pathogenesis of many protozoan parasites (4). In Plasmodium, the expression of CaM has been shown to be stage-specific and is involved in erythrocyte invasion as well as schizont maturation (5). Chelation of Ca2+ can prevent hepatocyte invasion by merozoites (6). Ca2+ is also involved in invasion of host cells by trypanosomatids (7). Treatments that decrease or increase cytoplasmic Ca2+ modify Trypanosoma cruzi infectivity (8). Besides, Ca2+ also regulates various other cellular processes, such as apoptosis, loss of motility, environmental sensing, and excystation (9, 10). In the protozoan parasite Entamoeba histolytica, the causative agent of amebiasis, Ca2+ is reported to be involved in its pathophysiology by initiating the amebic cytolytic activity (for a recent review, see Ref. 11). Amebic cytolytic activity could be blocked by Ca2+ channel blockers, or by treatment with EGTA (12), whereas stimulation of amebic PKC activity with phorbol esters enhanced lysis of target epithelial cells (13). Changes in the Ca2+-profile were also related to the cell cycle and the developmental stages of the parasite i.e. the cyst or the trophozoite stage (14). Furthermore, extracellular Ca2+, amebic intracellular Ca2+ flux, bepridil-sensitive Ca2+ channels and a putative CaM-dependent signal transduction pathway have been implicated in the growth and encystation of Entamoeba histolytica (15). Ca2+ is thus speculated to play a direct role in precipitating the cytopathic effects of E. histolytica.

A number of CaBPs have been identified in E. histolytica (4). Among these are two related EF-hand-containing proteins, grainin 1 and 2, which are localized in intracellular granules (16). They may be involved in phagocytosis, control of endocytic pathways and Ca2+-dependent granular discharge. Another protein, URE3-BP, was shown to have a transcription regulatory function with Ca2+-dependent DNA binding properties (17, 18). CaM-dependent secretion of collagenases from electron dense granules has been demonstrated using Entamoeba lysate but a direct evidence for the presence of CaM is still lacking (19, 20). We had previously characterized a CaBP (EhCaBP1) from E. histolytica (21, 22). EhCaBP1 has 134 amino acid residues with

1 The abbreviations used are: CaBP, calcium-binding protein; CaM, calmodulin; nt, nucleotide; MD, molecular dynamics; ORF, open reading frame; pC112, synthetic peptide C112; pC61, synthetic peptide C61; RT-PCR, reverse transcription-PCR; TMB, 3,3',4,4'-tetramethylbenzidine.

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‡‡ To whom correspondence should be addressed: School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi-110067, India. Tel.: 91-11-26704516; Fax: 91-11-26165886; E-mail: alok0200@mail.jnu.ac.in.

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four canonical EF-hand Ca\textsuperscript{2+}-binding domains. Though this protein has some structural similarity with CaM, it is functionally quite distinct (23). Inducible expression of EhCaBP2 antisense RNA demonstrated its role in cellular proliferation (24, 25).

Structural studies on EhCaBP1 have revealed an organization similar to CaM, having two independent globular domains connected by a flexible linker. It consists of four canonical EF-hands, a pair each in the N- and C-terminal domains. The Ca\textsuperscript{2+} binding loops in the protein consist of 12 contiguous residues from two helices, which are, oriented nearly perpendicular to each other, a feature shared by other members of the family of EF-hand proteins (26). EhCaBP1 is structurally related to CaM and Troponin C (TnC) despite low sequence homology with these proteins. The major differences in the structure of EhCaBP1 with respect to CaM and TnC are in the Ca\textsuperscript{2+} binding loops, interhelical angles and exposed hydrophobic surface. These differences have led to a more open conformation for the Ca\textsuperscript{2+} binding loops in the C-terminal domain of EhCaBP1 compared with the ones in the N-terminal domain and higher solvent exposure of the hydrophobic residues (27, 28). In EhCaBP1, the linker region exhibits a large structural flexibility, leading to an ill-defined orientation of the two domains (N- and C-terminal) with respect to each other. These structural features make EhCaBP1 functionally distinct from other CaM-like Ca\textsuperscript{2+}-binding proteins. In this study, we report the identification and partial characterization of EhCaBP2, a paralog of EhCaBP1, and show that these two proteins are functionally distinct.

EXPERIMENTAL PROCEDURES

Strains and Cell Growth—E. histolytica strain HM1:IMSS was maintained in TYI-S-33 medium with appropriate antibiotics. Southern and Northern Hybridization—Total DNA was purified from late-log phase grown cells, as described earlier (30). Genomic DNA, digested with appropriate restriction enzymes and separated on an agarose gel, was transferred to nylon membranes. Southern blots were hybridized in a solution containing 1% SDS, 1 μM NaCl, and 3 × 10\textsuperscript{5} cpm ml\textsuperscript{-1} of DNA probe at 65 °C for 16 h. Blots were washed according to the manufacturer’s instructions and exposed for autoradiography. Radiolabeled DNA probes were prepared using the NEBlot random priming kit (NEB).

Total RNA was purified using TriPure reagent (Roche Applied Science) according to the manufacturers’ instructions. RNA samples (30 μg) were denatured by incubating with 2 μl deionized glyoxal, 50% (v/v) Me2SO and 1× gel running buffer (12 μl Tris-HCl, 6 mM sodium acetate, and 0.3 mM EDTA, pH 7.0) at 50 °C and electrophoresed in a 1.2% (w/v) Tris-acetate-EDTA (TAE) agarose gel. After electrophoresis, the gel was blotted on to a GeneScreen plus (GS+) nylon membrane (NEN), and hybridization was carried out at 42 °C in formamide-containing hybridization buffer according to the manufacturer’s instructions.

Polymerase Chain Reaction—Primers to amplify the EhCaBP2 ORF were designed based on the Entamoeba GSS sequence data base. The primers used (Primer 1: 5′-GGGAAACATAGTGGATCACAAGGATTGAT-T3′ and Primer 2: 5′-GGGGAGAGGTCATTGAAAGGGTAGTGC-3′) were obtained from Microsynth, Switzerland. PCR was performed with 400 μM of each primer and 200 μM of nucleotide triphosphates. The conditions were: 92 °C, 5 min; 36 °C, 1 min; 70 °C, 1 min (5 cycles); 92 °C, 1 min; 56 °C, 1 min; 70 °C, 1 min (25 cycles). The PCR products were cloned in pGEMTeasy vector (Promega) and subsequently in pET30a vector (Novagen) for expression in E. coli BL21 (DE3) cells.

The primers set 3–4 was used to amplify reverse-transcribed products obtained with total E. histolytica genomic RNA (primer 3: 5′-GTTTCAAGTATATCATGATTACAAGGATTGATATTGATTACACAAGGATTGTGTC-3′ and Primer 4: 5′-GTTTCTTTAACGACGCCGTCGA-3′). The conditions used were 92 °C, 1 min, 56 °C, 1 min, 70 °C, 1 min (30 cycles).

Expression and Purification of Recombinant EhCaBP2 in E. coli—The purification of recombinant EhCaBP2 was carried out essentially as described earlier (22). Induced bacterial cells were resuspended in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA and passed through EhCaBP-Sepharose 4B-column (4–5 mg protein per ml of bed volume). Unbound proteins were washed with 10 bed volumes of Buffer C. The bound proteins were eluted with Buffer D (same as Buffer C except that CaCl\textsubscript{2} is replaced with 2 mM EGTA) as 1-ml fractions. The chromatographic steps were carried out at 4 °C. The eluates were dialyzed against water and then concentrated by centrifugal evaporation.

Laser Ionization Time of Flight Mass Spectrometry (MALDI-TOF)—MALDI-TOF spectrometry was used to analyze EhCaBP-binding proteins. The matrix was prepared using sinapinic acid in 30% (v/v) acetonitrile, and 0.33% trichloroacetic acid, and the samples were analyzed by a MALDI-TOF spectrometer (Kratos PCKOMPACT SEQ model). The mass calibration was carried out using the MS-CAL1 mass calibration kit for MALDI-MS (Sigma).

In Vitro Kinase Assay—Total Entamoeba cell extract was prepared and the activity of Ca\textsuperscript{2+}-dependent kinases was estimated according to Chandok and Sopory (30). The reaction mixture contained 30 mM HEPES, pH 7.5, 5 mM MgCl\textsubscript{2}, 40 μg of histone (Type I/II-Sigma), 30 μg of Entamoeba cell extract, 1 mM calmodulin inhibitory peptide (Microbiotech), 10 μg of biotinylated, and 100 pmol of peptide where indicated. The reaction volume was adjusted to 50 μl. The reaction was initiated by adding 100 μM [γ-32P]rATP (specific activity 5000 Ci/mmol, Amersham Biosciences) and allowed to proceed at 30 °C for 10 min. The reaction was terminated by adding 10% trichloroacetic acid, which precipitated total protein in the reaction mixture. The pellet was washed with diethyl ether/ethanol (1:1) mixture and after drying the pellet, and spotting the rehydrated pellet on G/F/C paper, the amount of radioactivity was determined in a scintillation counter. For visualization of phosphorylated products, the reaction mixture after termination was separated in a 10% SDS-PAGE. The fluorographed gels were dried and exposed to an x-ray film.

Fluorescence experiments involving peptides, reactions were performed under identical conditions using E. histolytica total cell lystate preincubated with indicated amounts of peptide(s).

Ca\textsuperscript{2+}-dependent Phage ELISA—Phages were prepared according to standard procedures and phage ELISA assays were performed essentially as described earlier (32). However, certain changes were introduced to ensure that the phage binding was Ca\textsuperscript{2+}-dependent. The phages were allowed to bind to the EhCaBPs in the presence of 2 mM CaCl\textsubscript{2}. The plates were washed in TBST containing either 2 mM CaCl\textsubscript{2} or 4 mM EGTA, and the bound phages were detected using horseradish peroxidase-labeled anti-M1 monoclonal antibody (Amersham Biosciences; 1:3000 dilution) with TMB substrate (Amersham Biosciences) at 400 nm. Plasmid Rearrangement Analysis—Binding kinetics of phages to protein was determined using IAsys Auto+ (Affinity sensor). Amplified phages (10\textsuperscript{11} pfu/ml) from a 20-ml culture were precipitated and dissolved in 50 μl of phosphate-buffered saline, pH 7.4. Phages were biotinylated using established protocols (33). Biotinylated phages were immobilized onto streptavidin bound on a biotin-coated surface. For determination of association rate constants, EhCaBPs (25–400 μM) in the binding buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM CaCl\textsubscript{2}) were used. Following analyte binding, dissociation rate constants were measured by replacing the sample with binding buffer. The surface was regenerated with the regeneration buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 4 mM EGTA). Kinetics of the interaction of EhCaBPs with phages was analyzed by non-linear regression using FASTFIT software package supplied with IAsys instrument.

Sequence Analysis—Preliminary sequence data for E. histolytica is deposited regularly into the GSS division of GenBank\textsuperscript{TM} and was used for this analysis. The sequencing effort is part of the International Entamoeba Genome Sequencing Project and is supported by award number 5P50GM58533-04 of the National Institute of General Medical Sciences, National Institutes of Health.

The Entamoeba genome sequence data base from the Sanger Centre (www.sanger.ac.uk) supported by the Welcome trust was also used. BLAST searches (NCBI: www.ncbi.nlm.nih.gov/BLAST) were used for finding homologues of EhCaBPs. ClustalW (EMBL: www.ebi.ac.uk) was used to align sequences of multiple Entamoeba species.

Molecular Modeling—Molecular modeling was carried out using the Modeler (34) interface of INSIGHTII (Molecular Simulations Inc.) The model was built on the basis of the NMR solution structure (1JFK) of the Ca\textsuperscript{2+}-bound form of EhCaBP1 (28). The constructed model was

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minimized initially with full backbone constraints, which were gradually relaxed as the minimization proceeded. The final model was validated by PROCHECK (35).

Molecular dynamics simulation was carried out to explore the conformational variation existing in the flexible linker region between the two domains in EhCaBP1 and EhCaBP2. 10 ps of equilibration at 300 K were followed by 100 ps of molecular dynamics (MD) simulation. The MD simulation was carried out for the 13 residues of the linker region with maximum variability, while the rest of the structure was constrained. RMSD for 10 representative MD-derived structures of the linker region (residues 55–67) was found to be 0.32 Å suggesting no significant variation from the average structure.

Circular Dichroism Spectroscopy—CD measurements were performed using a Jasco-810 spectropolarimeter. Each spectrum was measured in the far-UV region (200–270 nm) and was an average of five scans. Scans were done at a protein concentration of 1 μM using a cuvette of pathlength 1 cm. Percentage helical content was calculated using the method described by Barrow et al. (36).

RESULTS
A Second E. histolytica Calcium-binding Protein (EhCaBP2)

Our laboratory had previously characterized a calcium-binding protein, EhCaBP, from E. histolytica (21). A search for the
The presence of EhCaBP homologs in the *E. histolytica* genome sequence data base at NCBI revealed (in addition to an entry with complete match with EhCaBP) a sequence that showed a very high score and low p value (score = 163; p = 4e⁻³⁸) using BLAST. The identified sequence was then used to find a contig that carried the entire coding region, from the genome data base at the Sanger Centre, UK (contig 4494). The second gene was designated as EhCaBP2 whereas the original gene was renamed EhCaBP1.

The overall identity at the nucleotide level between EhCaBP2 and EhCaBP1 was 79% (Fig. 1A). Of the 80 nucleotides that differed in the two genes, 28 were in the third codon position, leaving the encoded amino acids unchanged. The conceptual translation product of EhCaBP2 was compared with EhCaBP1 and the alignment of the two sequences is shown in Fig. 1B. The amino acid sequences of the two proteins showed an identity of 78%. Both the proteins have four typical EF-hand Ca²⁺ binding domains. The level of similarity between the two proteins varied along the length of the sequence. The maximum identity was observed in the Ca²⁺ binding domains with a total of 6 amino acid substitutions (Fig. 1B). The minimum identity was seen in the 27 amino acid central linker region between Ca²⁺ binding domains 2 and 3, which had 10 substitutions. The sequence comparison shows that the overall structure of both the proteins is similar, but the two may perform different functions due to variations in the central linker.

The nucleotide sequences upstream and downstream of the ORF of EhCaBP1 and EhCaBP2 showed no significant sequence identity (Fig. 1A). In addition, genes present in the vicinity of each EhCaBP were different. The putative ORF located upstream of EhCaBP1 was the human homolog of AMP-deaminase (Score = 72, p = 2e⁻¹²) whereas the ORF upstream of EhCaBP2 had similarity to myosin heavy chain (Score = 56, p = 3e⁻⁷). Therefore, these two EhCaBP genes are unlikely to be allelic copies.

Currently, genomic sequences from different species of *Entamoeba* are being deposited in the GSS databases maintained at the Sanger Centre. The putative coding regions of several EhCaBP-like sequences were identified from this data base using standard bioinformatics tools. A CaM-like protein displaying 50–78% identity to other calmodulins was also identified in this data base (contig 5693). A phylogenetic analysis of the EhCaBPs and related orthologs and paralogs and different calmodulins revealed that all *Entamoeba* CaBPs are distinctly different from CaMs including that from *E. histolytica* (Fig. 2). A close examination of the tree revealed minor differences among the CaBPs from different *Entamoeba* species. Among the *Entamoeba* CaBP orthologs, *E. invadens* displayed the maximum divergence. None of the EhCaBP-like *Entamoeba* CaBPs is close to CaM as the two segregate out in different clusters. The groupings were well supported by high bootstrap values. Interestingly, none of the sequences were found more than once in the data base, which may imply all of these isoforms may not be duplicated pseudogenes. From this analysis, it appears that EhCaBP1 and EhCaBP2 are paralogs of each other as they branched out separately.
To determine the copy number of EhCaBP2, E. histolytica genomic DNA was digested with the restriction enzymes BglII and EcoRI. From the nucleotide sequence of EhCaBP2, it was determined that BglII does not have a site in the coding region whereas EcoRI has a single site. Genomic DNA digested with these enzymes was blotted and hybridized with EhCaBP2 ORF as described under “Experimental Procedures.” Panel C, Southern hybridization of cloned EhCaBPs with the radiolabeled EhCaBP2 probe was carried out as described for panel B. The plasmid DNAs were first separated in 0.8% agarose gel before transfer and hybridization. Lane 1, EhCaBP1; lane 2, EhCaBP2.

Copy Number of EhCaBP2

To determine the copy number of EhCaBP2, E. histolytica genomic DNA was digested with the restriction enzymes BglII and EcoRI. From the nucleotide sequence of EhCaBP2, it was determined that BglII does not have a site in the coding region whereas EcoRI has a single site. Genomic DNA digested with these enzymes was blotted and hybridized with EhCaBP2 probe. The EcoRI-digested DNA hybridized to two bands of sizes 6.0 kb and 4.36 kb while BglII-digested DNA hybridized with a single band of 4.36 kb (Fig 3B). Under the conditions of hybridization, the EhCaBP2 coding region probe specifically hybridized to EhCaBP2 DNA and not EhCaBP1 DNA (Fig. 3C). The sizes of bands obtained by Southern hybridization matched very well with the expected sizes from nucleotide sequence of the 18-kb TIGR contig 318109, which contains the EhCaBP2 ORF (Fig. 3A). The Southern data show that EhCaBP2 is a single copy gene.

Expression of EhCaBP2 in E. histolytica HM-1:IMSS

Expression of EhCaBP2 was checked in order to rule out the possibility that it could be a pseudogene. A search of the data base revealed an EST (accession no. AB002775) with 100% identity with EhCaBP2 at the nucleotide level, suggesting that this gene may be expressed in E. histolytica trophozoites (40). The presence of EhCaBP2 transcript was confirmed using both RT-PCR and Northern hybridization. EhCaBP2-specific primers were designed from the regions that showed maximum variability with respect to EhCaBP1 (marked in Fig 4A, top panel). The specificity of the primers was checked by a PCR reaction using cloned EhCaBP1 and EhCaBP2 genes as templates (Fig. 4A, lanes 1 and 2). A band of 225 bp was detected after RT-PCR of RNA isolated from axenic trophozoites (Fig. 4A, lane 3). In the absence of reverse transcriptase (Fig. 4A, lane 4), no such amplification was detected, showing the absence of any contaminating DNA.

In addition, Northern hybridization of total HM1:IMSS RNA, carried out at high stringency as described under “Experimental Procedures,” revealed a single species of RNA transcript of 0.57 kb (Fig. 4B). Under the conditions used, the EhCaBP2 probe did not hybridize with EhCaBP1 (Fig. 4B, inset a). The data indicate that EhCaBP2 is expressed in E. histolytica.

Ca$^{2+}$ Binding Properties of Recombinant EhCaBP2

The EhCaBP2 ORF (402 bp) from strain HM-1:IMSS was amplified using primers 1–2 designed from the GSS entry AZ542766. The PCR-amplified product was cloned into the expression vector pET30a as described under “Experimental Procedures.” The recombinant protein was purified from the induced bacterial lysate (data not shown) using a method developed for purification of recombinant EhCaBP1 (22). The Ca$^{2+}$-free form of the protein was used for all experiments involving Ca$^{2+}$ binding which was evaluated by CD spectroscopy. CD studies were carried out on both holo and apo forms of EhCaBP2. The CD profiles of the two protein forms showed
significant differences, which could be directly attributed to conformational differences. Thus, a distinct conformational change, evident in terms of the increase in the helical content, was observed in the holo-EhCaBP2 as compared with the apoprotein (Fig. 5). The helical content in the case of holo-EhCaBP2 was about 56% and that in the case of the apoprotein was found to be 35%. A similar trend was also reported earlier with regard to the helical contents of the apo- and the holo-EhCaBP1 (31).

Structural Model of EhCaBP2

A model for EhCaBP2 was built using methods described under “Experimental Procedures.” (34). A comparative study of the two proteins was done at the primary as well as the tertiary level. The high sequence homology of the two proteins, EhCaBP1 and EhCaBP2 was reflected in similar structural topologies for the two proteins namely, the basic tertiary structure of two globular domains with the four well-conserved EF-hands (Fig. 6). The Ca$^{2+}$ binding domains show high degree of structural similarity between the two EhCaBPs. Residues responsible for Ca$^{2+}$ binding in EhCaBP1 were mapped using CONTACT (CCP4 Suite); only one residue, Ala$^{16}$, in the Ca$^{2+}$ binding site of EhCaBP1 was found to be mutated to Ser$^{16}$ in EhCaBP2. However, significant differences were seen in the flexible linker region. A reduction in helicity, leading to a more compact structure in the EhCaBP2 linker region was evident, with its helix being four residues shorter than that of EhCaBP1. The resultant structural compaction might be due to the absence of several bulky side chains in the linker region of EhCaBP2. Charge differences also exist between EhCaBP1 and EhCaBP2 (Fig. 6, b–c); the surface of EhCaBP1 is more positively charged compared with that of EhCaBP2. Due to the compact packing within the EhCaBP2 linker region (Fig. 6a) K74, which is a major component of a positively charged patch in EhCaBP1 gets buried in EhCaBP2 by E67 (Gly$^{67}$ in EhCaBP1). Lys$^{69}$ and Arg$^{70}$, which are located on the surface of EhCaBP2.

FIG. 4. Expression of EhCaBP2 RNA in E. histolytica. Panel A, amplification of EhCaBP2 RNA by RT-PCR. The location of the primers is indicated with arrows at the top of the figure. The positions of the calcium binding domains are marked I–IV in this scheme. The specificity of the primers for EhCaBP2 was checked by PCR amplification of EhCaBP1 and EhCaBP2-containing plasmid templates. The PCR products were electrophoresed in 1.2% agarose gel at 4 V/cm. Lane 1, EhCaBP2 plasmid DNA; lane 2, EhCaBP1 plasmid DNA; lane 3, total HM1:IMSS RNA and lane 4, same as lane 3 except no reverse transcriptase was added. Panel B, Northern blot analysis of EhCaBP2. 30 μg of purified total RNA was separated in a 1.2% denaturing formaldehyde agarose gel and transferred onto a nylon membrane. The blot was hybridized with a radiolabeled EhCaBP2 coding region probe as described under “Experimental Procedures.” Inset a, the probe was also hybridized with plasmid DNA (5 ng) containing cloned insert under the same condition. 1, EhCaBP2 and 2, EhCaBP1.

FIG. 5. CD Spectrum of EhCaBP2. The CD profile of EhCaBP2 was obtained in aqueous buffer. The thick continuous line represents the profile for the holo and dotted discontinuous line is that of the calcium-free apo protein.

EhCaBP1, contributing to a significant positively charged patch, are changed to Ser$^{69}$ and Pro$^{68}$ in EhCaBP2 leading to significant reduction in the positive charge on the surface. The CD spectra also showed that the Ca$^{2+}$-bound form of EhCaBP2 exists in a largely helical conformation (Fig. 5).

Functional Analysis of EhCaBP2

Analysis of Binding Proteins—The purified EhCaBP1- and EhCaBP2-binding proteins were analyzed by MALDI/TOF
mass spectrometer. The spectrum differed considerably for the two sets of binding proteins (Fig. 7). Whereas there was essentially a single major species of 95 kDa among the Eh-CaBP2-binding proteins, a number of polypeptides were observed among EhCaBP1-binding proteins. No common peak was observed in both the spectra suggesting that the two proteins bind different targets. Mass spectrometric pattern of binding proteins was found to be somewhat different from that obtained by SDS-PAGE analysis of [35S]methionine-labeled binding proteins prepared in the same way (data not shown here). It may be due to uneven labeling of proteins with [35S]methionine independent of size or relative amount or lack of effective separation at high molecular weight range in SDS-PAGE.

Activation of Endogenous Kinase—EhCaBP1 was shown to activate endogenous kinase(s) that may be an important component of the Ca$^{2+}$-signal transduction pathway mediated by this protein (23). The ability of EhCaBP2 to activate an endogenous kinase was similarly tested. The in vitro system for assaying kinase activity included E. histolytica cell lysate as the source of kinase, and histone as the substrate. The reaction was carried out in the presence of 10 and 100 μM Ca$^{2+}$ with varying concentrations of EhCaBP2 (Fig. 8B). The two Ca$^{2+}$ concentrations were chosen on the basis of our previous work where two Ca$^{2+}$-dependent kinase activities were found in the amebic lysate, with optimum activation at 10 and 100 μM added Ca$^{2+}$ respectively (23). The data showed that, indeed, EhCaBP2 could also activate an endogenous kinase in the presence of 100 μM added Ca$^{2+}$ and at 2 nM protein concentration. In contrast, EhCaBP1-dependent kinase activity was optimal at 10 μM added Ca$^{2+}$ as reported earlier (Fig. 8A). Therefore, these two proteins differ in the Ca$^{2+}$ concentrations required for optimal activity.

In order to identify the endogenous substrate of Ca$^{2+}$/EhCaBP2-dependent kinase, a phosphorylation assay was performed in the absence of any exogenous acceptor, and the products were separated by SDS-PAGE. The result was compared with that obtained with EhCaBP1 to see if the two
proteins share the same substrate. Both these proteins were found to stimulate phosphorylation of a 47 kDa protein present in the cell-free lysate of *E. histolytica* (Fig. 8C). However, the extent of phosphorylation by EhCaBP2 was at least 20 times more than that by EhCaBP1 under identical experimental conditions (Fig. 8C). The relative ability of the two EhCaBP-dependent kinases to phosphorylate histone or endogenous polypeptide as substrates differed significantly and may be due to their relative affinities for the different substrates. The phosphorylated band in the case of EhCaBP2 was diffuse suggesting that the level of phosphorylation and or glycosylation may also be different giving rise to a heterogeneous band.

In order to infer whether the two EhCaBPs bind to different sets of target proteins the *E. histolytica* lysate was first depleted of EhCaBP2-binding proteins by incubating with EhCaBP2-Sepharose. This was used to assay for both EhCaBP-dependent kinases. After depletion there was very low endogenous EhCaBP-dependent kinase activity. On addition of EhCaBP1 to depleted lysate the level of activation was found to be nearly at the same level as that obtained with normal lysate (Fig. 9, lanes 3 and 5). However, the level of activation of EhCaBP2-dependent kinase activity in depleted lysate was about 50% compared with that of 300% when normal lysate was used as a source of kinase (Fig. 9, lanes 6 and 7). The results suggest EhCaBP2-Sepharose could deplete specifically the kinase activated by it but not that activated by EhCaBP1.

**Fig. 7.** MALDI-TOF mass spectrometric analysis of EhCaBP-binding proteins. The binding proteins were purified using affinity chromatography over respective EhCaBP-Sepharose matrix as described in the text. The proteins eluted in the presence of EGTA were dialyzed against water and then concentrated before mass spectrometric analysis. MALDI spectrum of EhCaBP1-binding proteins (A) and EhCaBP2-binding proteins (B).
EhCaBP2 of E. histolytica

**FIG. 8. Stimulation of E. histolytica protein kinase by EhCaBP.**

E. histolytica cell-free lysate (30 μg) was used as the source of the kinase and histone type III (40 μg) as the substrate. The amount of EhCaBP is 2 nM, unless otherwise indicated. The concentration of Ca^{2+} used in the experiments is indicated. EGTA was used at 500 μM. The assay was performed as described under “Experimental Procedures” using [γ-32P]ATP as the phosphate donor. The amount of radioactivity incorporated into histone was determined either by PhosphorImager (Fuji BAS 800) analysis of 10% SDS-PAGE-separated phosphorylated products (panel A) or by scintillation counting of total trichloroacetic acid-precipitable proteins (panel B). Endogenous substrate phosphorylation by EhCaBP-dependent protein kinase, was performed with cell-free E. histolytica lysate (30 μg) and 2 nM each of EhCaBP1 (lanes 2–5) or EhCaBP2 (lanes 6–9) in the presence of 100 μM added Ca^{2+} (lanes 2 and 3 and lanes 6 and 7) or 500 μM EGTA (lanes 4 and 5 and lanes 8 and 9) as described earlier, except that histone was not added in the reaction mixture. lane 1 did not have Entamoeba cell lysate but contained Ca^{2+} (panel C). The phosphorylated reaction mixes were visualized after separation on a 10% SDS-PAGE followed by autoradiography.

Therefore it appears that the two kinases may be distinctly different from each other.

**Peptide C112 Binds EhCaBP2 Specifically**—The two EhCaBPs were further distinguished on the basis of their binding to a dodecamer peptide, C112 (SMNLQTPGYKDG). This peptide was identified from a random dodecamer peptide phage display library by affinity purification using an EhCaBP-Sepharose column. Phage-displayed peptides obtained from the above screen were tested for their differential binding to the two EhCaBPs. Binding was assayed using Ca^{2+}-dependent phage ELISA as described under “Experimental Procedures.” The phage-displayed peptide C112 showed at least 5-fold higher Ca^{2+}-dependent binding to EhCaBP2 compared with EhCaBP1 (Fig. 10).

The extent of phage 112 binding to EhCaBP1 was negligible as the value was close to that obtained in the presence of EGTA. The parental M13 phage showed no significant binding. To check for nonspecific binding, bovine serum albumin was used for coating the ELISA plates instead of EhCaBP. The level of nonspecific binding was low (about 5% of specific binding). The binding of phage C112 to both the EhCaBPs was also checked by a real-time assay employing surface plasmon resonance. Using binding conditions as described under “Experimental Procedures,” the affinity constant was found to be 14 μM for EhCaBP2 (Fig. 11). No binding was observed with EhCaBP1 under identical binding conditions, demonstrating the specificity of phage C112 for EhCaBP2.

**FIG. 9. Depletion of EhCaBP2-dependent kinase activity by EhCaBP2-Sepharose.** E. histolytica cell-free lysate was incubated with EhCaBP2-Sepharose in the presence of 100 μM Ca^{2+} at 4°C for 1 h, and the supernatant is referred to as a depleted source of protein kinase. Equal amounts (30 μg) of depleted lysate and normal undepleted lysate was used for checking the efficacy of either EhCaBP2- or EhCaBP1-dependent protein kinase activity. The respective EhCaBPs were used at 2 nM concentration. The amount of radioactivity incorporated into histone was determined by PhosphorImager analysis (Fuji BAS 800) of 10% SDS-PAGE-separated phosphorylated products. Representative bands obtained by autoradiographic exposure are shown.

**FIG. 10. Binding of C112 phage to EhCaBP2 and EhCaBP1.** Indicated amounts of phage C112 were allowed to bind to 4 μg each of EhCaBP2 or EhCaBP1 immobilized on an ELISA plate. Binding assay was carried out in the presence of Ca^{2+} (2 mM) or EGTA (4 mM) as described under “Experimental Procedures.” Bound phages were detected immunologically by incubating each well with HRPO-tagged anti-M13 antibody. The plate-bound HRPO was detected with TMB substrate and absorbance recorded at 410 nm. -o-, EhCaBP2/Ca^{2+}; -o-, EhCaBP2/EGTA; -o-, EhCaBP1/Ca^{2+}; -x-, EhCaBP1/EGTA.

The binding of the synthetic peptide C112 to EhCaBP2 also blocked activation of endogenous kinase by EhCaBP2. In the presence of 100 pmoles pC112, the inhibition was found to be about 85% of the EhCaBP2-dependent kinase activity (Fig. 12). No such inhibition was obtained when other 12-mer peptides, p12 and p61, were used in the kinase activation assay. This further demonstrates that the two EhCaBPs are functionally and structurally distinct. The sequence of C112 was used to search the Entamoeba genome data base at both Sanger Center and TIGR. None of the hit sequences obtained had a perfect match. The best match was 7 of 12 amino acids (data not shown).

**DISCUSSION**

EhCaBP1 is the first CaM-like calcium-binding protein that has been identified and characterized from E. histolytica. There

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appears to be a CaM gene encoded in the genome of *E. histolytica* as inferred by sequence similarity and phylogenetic relationship (Fig. 2). The expression of this gene and functional analysis of the gene product has not been studied so far though work done several years ago indicated presence of CaM-like activity in this organism (19, 20, 23). EhCaBP was originally thought to be a form of primitive CaM. However, phylogenetic analysis and structure modeling studies described in this report and some of the experimental observations reported earlier, clearly show that the EhCaBPs are distinctly different from CaM (23). Both the EhCaBPs are phylogenetically close to each other and may have been derived by a duplication event.

Many CaBPs are known to be present in multiple copies (isoforms) in different organisms. Among these, CaM and CaM-like proteins have been studied quite extensively (41, 42, 43). One such CaM isoform, the epithelial cell protein CLP, has the same size and displays 85% sequence identity with CaM (41). Both CaM and CLP can activate CaM kinase II. However, CLP cannot activate four other CaM-dependent enzymes. In plants, soybean has four different CaM variants. Of these, SCaM4 shows the highest divergence at the level of amino acid sequence (44). Unlike SCaM1, SCaM4 did not activate NAD kinase, indicating subtle variations at the functional level that may be due to variations at the primary sequence level. Support for this comes from a study where a number of different single mutations in CaM (e.g. phenylalanine to alanine) altered its properties and the different mutants displayed distinctive functional defects (45). In addition, a number of single amino acid mutations in *Paramecium* CaM that did not alter growth properties, did however, display behavioral aberrations (46).

Thus, changes in the protein interaction domain, without changing the rest of the protein can, in principle, alter the specificities of proteins. The human genome also contains a family of non-allelic CaM genes that are under selective pressure to encode identical proteins, while maintaining divergent nucleotide sequences of non-coding regions (43). Preserving multiple CaM genes with divergent non-coding sequences may be necessary in complex organisms to ensure that the many CaM-dependent processes occur with appropriate spatial and temporal control (43). Two or more non-allelic CaM or CaM-like genes have also been reported in *Xenopus* (47) and trypanosomes (48). Therefore it is not surprising that there are multiple forms of EhCaBP. The length and composition of the central linker region has been shown to be important for maintaining the functionality of CaM. Apart from binding to target peptide stretches, the linker has also been proposed to modulate metal ion binding and interdomain cooperativity (49). The major sequence divergence between the EhCaBPs, interestingly, spans this region, which suggests distinct functional roles for the two proteins. These molecules interacting with specific sets of target proteins may initiate different downstream pathways. This is evident from some of the results presented here that show different sets of binding proteins as revealed by affinity purification and subsequent analysis using MALDI-TOF mass spectrometry and the ability of the EhCaBP2 affinity matrix to deplete only EhCaBP2-dependent kinase activity but not EhCaBP1-dependent activity. Differences in the phosphorylation...
patterns of the endogenous substrates by EhCaBP1 and EhCaBP2-dependent protein kinase(s) also indicate distinct functional pathways for these two closely related proteins.

The data presented here show that EhCaBP1 and EhCaBP2 are non-allelic variants with identical sizes and a high degree of identity at both nucleotide and amino acid sequence level. Since the upstream and downstream sequences flanking the coding regions are totally different, it is likely that the two EhCaBPs are located in different chromosomal regions. It is unlikely that there are other members of this family, since a search of the genome data base available as of now did not reveal any other isoforms.

Commensurate with the observed biochemical properties of the two proteins, the surface features in the two proteins show differences based on modeling studies. In general no difference was found in the regions that bind Ca2+ in these two proteins. The central linker region, connecting the two globular domains in CaM-like proteins has been a region of special interest for biochemists and structural biologists alike. Several site-directed mutageneses have been carried out in these proteins to understand the effect of length and composition of this linker on their individual functions (50, 51, 52). The flexible linker region between the two domains may contribute to the different juxtapositions of the Ca2+ binding domains. The presence of Gly48 and Gly67 in the linker region of EhCaBP1 was shown to be important for the function of EhCaBP1 as it provided flexibility to the linker region. Circular dichroism and mass spectrometric techniques were also used to reveal that the mutant protein was weakened in its ability to bind Ca2+ and its target peptides (53), implying a possible role of these glycines in specific interactions with target peptides. In EhCaBP2, Gly53 and Gly67 have been changed to Ala63 and Glu57, respectively. These changes along with charge differences are likely to affect the specificity of binding of the target proteins leading to altered functional properties. The identification of a peptide C112 that can bind EhCaBP2 selectively suggested distinctive specificity of binding domains. The presence of

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