INTRODUCTION

Calreticulin (CR) is a widely distributed protein in eukaryotic cells except for yeast and erythrocytes. Filogenetically, it is a highly conserved protein in both its amino acid sequence and molecular organization. CR is a resident endoplasmic reticulum (ER) membrane protein that belongs to the family of KDEL proteins. It contains an N-terminal signal sequence and a C-terminal KDEL-ER retrieval sequence. CR binds Ca^{2+} with high capacity and plays an important role in control of intracellular Ca^{2+} homeostasis. CR also modulates steroid-sensitive gene expression and cell adhesion. This protein possesses chaperone activity, binding specifically to partially processed monoglucosylated N-linked oligosaccharides; therefore, it is not surprising that the most frequently reported location of this interesting protein is within the ER.

In the present work, we report partial sequencing of a 51 kDa protein of Entamoeba histolytica that is highly immunogenic in humans. Partial sequencing of the N-terminal end showed that 18 of the first 20 amino acid residues of the protein were identified uniquely, indicating that the final product was a homogeneous protein preparation. The N-terminal sequence that was found was: KVYFEETFENGWXWSKW. Comparing the 19-amino acid sequence of the protein in automated databases shows significant similarity with amino acid sequences of the calreticulin-like protein of spinach leaves (77%) and of the calreticulin precursor of Dictyostelium discoideum (60%).

MATERIALS AND METHODS

Electrophoresis and Western blot of 51 kDa protein. Membrane antigen fraction of E. histolytica HM1:IMSS was prepared as described previously. Electrophoresis of antigen was performed in 10% polyacrylamide-SDS gels under non-reducing conditions and run at 100 volts for 60 min at room temperature. Gels were stained with Coomassie blue for detection of 51 kDa band. Electroelution of the 51 kDa band was carried out using preparative 10% SDS-polyacrylamide gels stained with Coomassie blue. The 51 kDa band was cut with a scalpel blade and submitted to electroelution in an electroeluter chamber (model 422, Bio-Rad Laboratories, Hercules, CA) using 0.025 M Tris-HCl buffer added to 0.19 M glycerine and 1% SDS for 6 h, 10 mA at 4°C. The electroeluted protein was recovered and then dialyzed against Tris-HCl 0.01 M pH 7.5; finally, the protein was concentrated by ultrafiltration using Centricon 10 Units (Amicon Millipore Co., Bedford, MA) and a filter of 30,000-molecular-weight-cutoff (XM-30) (DIAFLO, Amicon). The 51 kDa band obtained in electroelution was analyzed in a two-dimension electrophoresis gel with a pH gradient of 3–10. The gel was stained with Coomassie blue to identify the homogeneity of the protein and its isoelectric point.

On the other hand, 51 kDa electroeluted protein was run in SDS-PAGE transferred to nitrocellulose and tested for reactivity with goat anti-human CR antibody (sc-6467) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat anti-rabbit skeletal muscle CR antibody (designated CRT283), and antiamebic antibodies obtained from a pool of five serum samples from amebic liver abscess (ALA) patients in Western blot assay. Sites of nitrocellulose membrane strips uncovered with protein were blocked with 50% skim-milk solution in 0.02 mM pH 7.4, and the nitrocellulose membrane strips were washed with PBS added to 0.5% Tween 20 (PBS-Tw) (Sigma Chemical Co., St. Louis). For detection of KDEL-ER retrieval sequence in E. histolytica, 51 kDa protein, goat anti-rabbit skeletal muscle CR (CRT283) was used (donated by Dr. M. Michelak, Department of Biochemistry, University of Alberta, Manitoba, Canada). This antibody was raised against synthetic peptide QAKDEL, which encodes C-terminal amino acids 386-401 of rabbit CR. The CRT283 antibody was affinity-purified using BSA-coupled QAKDEL synthetic peptide affinity column.

Nitrocellulose strips were incubated (1 h at room temperature) with CRT283 antibody (1:300 dilution), goat anti-human CR antibody (1:1000 dilution), or serum antibodies from patients with ALA (1:1000 dilution). After incubation, strips were washed as before and incubated with goat anti-rabbit IgG, rabbit anti-goat IgG, or goat anti-human IgG peroxidase-conjugated antibody (Zymed Laboratories, San Francisco) (1:1,000 dilution) in accordance with the respective origin of the first antibody used. Immunoactive bands were detected with diaminobenzidine tetrahydrochloride solution (50 mg/100 mL in PBS). For analysis of reactivity of 51 kDa protein submitted to two-dimension electrophoresis and Western blot assay, the procedure was the same as described previously.

Reactivity analysis of 51 kDa E. histolytica protein in ELISA system. Enzyme-linked immunosorbent assay (ELISA) was performed in microtiter well strips coupled with electroeluted 51 kDa E. histolytica protein (1 μg/well) Strips
were blocked with 3% solution of bovine serum albumin (BSA) in 0.01 M phosphate buffer at 4°C overnight. Thereafter, strips were washed twice with both 0.01 M phosphate buffer added with 0.5% BSA and 0.5% Tween 20 (Tw). A total of 50 μL/well of proper dilutions of CRT283, anti-human CR antibodies, or serum anti-amebic antibodies from patients with ALA were reacted for 2 h at room temperature and then washed once with PBS-BSA-Tw and twice with PBS-Tw (10 min). Peroxidase-conjugated antibodies used to detect antigen-antibody reaction in this ELISA were the same as described previously in the Western blot assays (50 μL of equivalent dilutions/well). Strips were incubated overnight at 4°C. Substrate consisted of 50 μL/well of 10 mL of 0.1M citrate buffer, pH 4.5 containing 10 mg o-phenylenediamine and 4 μL H₂O₂ (30%). The reaction was stopped after 3 min with 1 M of H₂SO₄ (200 μL/well) and then read in a micro-ELISA reader (Bio-Tek Instruments Co., Winoosky, VT) at 490 nM.

**Sequencing.** Protein sample was prepared and sequenced after SDS-PAGE and electrophrased to polyvinylidenedifluoride membrane (Immobilon, Bio-Rad) by the Towbin method. N-terminal sequence was determined by automated Edman degradation on a gas-phase protein sequencer (LF 3000, Beckman Instruments, Irvine, CA) equipped with an online Beckman System Gold high-performance liquid chromatography system. HPLC equipment included models 126 pump and 168 diode array detector settings at 268 and 293 nm for signal and reference, respectively. The HPLC column used was a Beckman Spherogel Micro PTH (2 X 150 mm) column, while standard Beckman sequencing reagents were used for analysis.

**RESULTS**

Electroeluted 51 kDa *E. histolytica* protein analyzed in two-dimension electrophoresis gel showed a unique spot located in the range of pH 6.7 (Figure 1), demonstrating the presence of only one homogeneous protein in a molecular weight of 51 kDa of *E. histolytica* antigen. Western blot of two-dimension electrophoresis gels reacted with anti-KDEL-ER retrieval sequence, anti-human CR antibodies, or the anti-amebic serum antibodies of patients with ALA showed no reactivity. However, in one-dimension electrophoresis and Western blot of previously electroeluted 51 kDa protein of *E. histolytica*, reactivity of 51 kDa protein with anti-KDEL antibody (CRT283) was positive (Figure 2), demonstrating the presence of KDEL sequence in the studied protein.

To test whether the absence of reactivity with other antibodies tested (anti-human CR, and serum antibodies of patients with ALA) was related to sensitivity of the Western blot assay, we tested electroeluted 51 kDa protein in an ELISA system, obtaining clear and strong reactivity of all antibodies tested (anti-KDEL-ER, anti-human CR, and ALA serum anti-amebic antibodies (Table 1). These results suggest that KDEL-ER retrieval sequence is present in 51 kDa *E. histolytica* protein; the protein also has antigenic similarity with human CR and is highly recognized by anti-amebic antibodies of patients with ALA.

Sequencing of 51 kDa protein showed that 18 of the protein’s first 20 amino acid residues were uniquely identified, indicating that the final product was a homogeneous protein preparation. The N-terminal sequence found was: KVVY-

<table>
<thead>
<tr>
<th>Antibody</th>
<th>51 kDa <em>E. histolytica</em> protein</th>
<th>Value of optical density at 490 nm ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune serum</td>
<td>0.386 ± (0.054)</td>
<td></td>
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<tr>
<td>Serum of patients with amebic liver abscess</td>
<td>0.772 ± (0.083)</td>
<td></td>
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<tr>
<td>Anti-KDEL antibody (CRT283)</td>
<td>0.531 ± (0.065)</td>
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<tr>
<td>Anti-human calreticulin antibody (sc-6467)</td>
<td>0.522 ± (0.051)</td>
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<tr>
<td>CRT283 anti-KDEL-ER retrieval sequence antibody, anti-human calreticulin antibodies (sc-6467), and serum anti-amebic antibodies from a pool of five patients with amebic liver abscess.</td>
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CR-like protein of Entamoeba histolytica. The previous points open an inconcerning an equivalent system to ER and Golgi without several morphologically recognizable organelles. This molecule in diagnostic and epidemiologic importance of the CR-like molecule in other eukaryotic cells (Table 2). The major homology was obtained with the amino acid sequence of CR-like protein of spinach leaves (77%) (Swissprot: accession P30806) and CR precursor of Dictyostelium discoideum (60%) (Swissprot: accession Q23858).

**DISCUSSION**

CR has been detected in Onchocerca volvulus, Schistosoma japonicus, S. mansoni, and Leishmania major. In Leishmania and Schistosoma species, the Ca²⁺ binding property of CR is conserved in vitro. There also is evidence concerning immunogenicity of CR from parasites in humans; anti-CR antibodies have been detected in serum from patients with onchocercosis, and tripanosomiasis. Our results suggest the presence of a CR-like molecule in *E. histolytica* that appears highly immunogenic in patients with invasive amebiasis. This protein also possesses the KDEL sequence characteristic of RE resident proteins observed in other eukaryotic cells (Figure 2, Table 2). In addition to the diagnostic and epidemiologic importance of the CR-like molecule, the presence of this molecule in *E. histolytica*, a parasite without several morphologically recognizable organelles such as mitochondria, Golgi apparatus, RE, centrioles, or microtubules, supplies additional evidence for recent observations concerning an equivalent system to ER and Golgi complex in this protozoan. The previous points open an interesting field of research related to the biologic function of this *E. histolytica* protein. Clonation of the putative gene for CR-like protein of *E. histolytica* is ongoing.

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**REFERENCES**


