Surface Localization, Regulation, and Biologic Properties of the 96-kDa Alcohol/Aldehyde Dehydrogenase (EhADH2) of Pathogenic Entamoeba histolytica

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The 96-kDa surface antigen of Entamoeba histolytica was demonstrated through extensive immunologic evaluation with monoclonal and monospecific antibodies to be identical to or an isoform of the amebic alcohol/aldehyde dehydrogenase (EhADH2). EhADH2 was secreted, excreted, or shed into the culture medium in quantities commensurate with amebic growth when studied in a novel culture system. Of importance, using RNase protection assays, specific mRNA coding for the EhADH2 gene product(s) was up-regulated by treatment of viable trophozoites with the enzyme substrate ethanol. These data provide insight into the biology of this enzyme and its regulation by appropriate stressors.

Materials and Methods

Cultivation and radiolabeling of E. histolytica trophozoites. Axenic strains of E. histolytica HM1:IMSS (ATCC 30459) and 303:NIH (ATCC 30887) were cultured in 25-cm² flasks at 35°C in Diamond’s TYI-S-33 (trypticase, yeast extract, iron-serum) medium [5] supplemented with 15% heat-inactivated bovine serum (Bio-Fluids, Rockville, MD) as described [2] or in cell culture plates (Transwell; Costar, Cambridge, MA) incubated in an anaerobic environment as described below. Logarithmic-phase amebae were harvested from flasks and washed twice in ice-cold 0.01 M NaPO₄/0.15 M NaCl, pH 7.6 (PBS) before use.

The Transwell culture system was used only in experiments for analysis of released or secreted amebic proteins and for growth curve determinations. After the upper and lower compartments of each well of each plate were filled with TYI-S-33 medium in volumes recommended by the manufacturer, the upper compartment of each well was seeded with 3 × 10⁴ to 3 × 10⁵ logarithmic-phase trophozoites (depending on the particular experiment) and 0.5 mCi of [³⁵S]methionine/cysteine (specific activity 1050 Ci/mmol; Tran³⁵S-Label; ICN, Irvine, CA). The plates were placed in an anaerobe jar and incubated at 35°C in an anaerobic environment generated by Gas Pak Plus envelopes (BBL Microbiology Systems, Cockeysville, MD). For the generation of the growth curve, time points were taken every 4 h for 60 h. Trophozoites were harvested from the upper compartment for cell count determination, and the medium in the lower chamber was used for isolation of released proteins. The upper and lower compartments of the culture plates are separated by a 0.4-μm pore size collagen-coated microporous membrane, which prevents amebae from transiting the membrane and contamination of the lower compartment by cellular debris. This system is more reliable for the assessment of released or secreted soluble proteins than use of conditioned medium (separation of organisms from the medium by centrifugation) because of the labile nature of the trophozoites.

Trophozoites were surface-labeled with carrier-free ¹²⁵I as previously described [2]. After labeling, unincorporated ¹²⁵I and cellular debris were separated from intact trophozoites on a discontinuous Percoll gradient. Trophozoites were harvested at the 30%/50% gradient interface and washed before use.
**Purification of *E. histolytica* proteins.** The 170-kDa heavy chain of the adherence lectin was purified by immunoprecipitation with monoclonal antibody E-13 and protein A–linked Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) as described [2]. The immunoprecipitated protein was resolved by 7.5% SDS-PAGE [6] and purified as previously described [4].

The 96-kDa protein was purified by immunoaffinity chromatography or by fibronectin-Sepharose affinity chromatography. Briefly, an immunoaffinity column was prepared by linking purified monoclonal antibody (EH335) to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer’s recommendations. Trophozoites were lysed by vortexing for 4 min in 0.01 M PBS (pH 8.0) and 1% TCA containing the following proteinase inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, St. Louis), 1% aprotinin, 1 mM leupeptin, and 100 μM E-64 (trans-epoxy-succinyl-leucylamido(4-guanidino)butane; Boehringer Mannheim, Indianapolis). After centrifugation at 40,000 g for 30 min, the supernatant was applied to the EH335 monoclonal antibody affinity column, and the protein was purified as described [7]. Alternatively, the centrifuged lysate was incubated with a suspension of fibronectin-Sepharose for 3 h. After extensive washes in increasing NaCl concentrations, the protein bound to fibronectin was eluted in 10 mM TRIS-Cl (pH 8.0) and 500 mM NaCl/0.1% Nonidet P-40, further purified by fast protein liquid chromatography (FPLC) as previously described, and tested for alcohol/aldoxide dehydrogenase activity [1].

Protein samples were electrophoresed on SDS-polyacrylamide gels, and purity was assessed by Coomassie blue or silver staining of the gels.

**Immunologc assessment.** Purified proteins (1 μg) were electrophoresed on 10% SDS-polyacrylamide gels and electrotblotted to nitrocellulose membranes [8] for testing by immunoblotting with specific antibodies as previously described [4]. The 170-kDa protein was tested with monoclonal antibody, 3F-4, specific for the adherence lectin [9], and confirmed the identity of the purified protein (data not shown). The 96-kDa protein was tested with monoclonal antisera against the 97-kDa EhADH2. Antibody reactivity was visualized with horseradish peroxidase–conjugated Protein A (1:500 dilution; Zymed Laboratories, South San Francisco, CA), 4-chloro-1-naphthol, and H202. Monoclonal antibody 3F-4 was the gift of W. A. Petri, Jr., University of Virginia, Charlottesville.

The EhADH2 protein was tested by an ELISA as previously described [2] using EH335 and five other monoclonal antibodies previously shown to be specific for the 96-kDa protein by immunoprecipitation [2]. Briefly, pure enzymatically active EhADH2 (15 ng) or pure 96-kDa protein (50 ng) was adsorbed to wells of a microtiter plate (Dynatech, Alexandria, VA) at 4°C overnight. The wells were blocked with 5% bovine serum albumin, then incubated with monoclonal antibodies (1:500 dilution) for 1 h at 37°C. After four washes, horseradish peroxidase–conjugated rabbit anti-mouse IgG, IgA, and IgM (1:1500 dilution; Zymed) was added to each well. After incubation for 1 h, the reactions were developed with o-phenylenediamine and H2O2. For controls, matched isotype monoclonal antibodies to *Pneumocystis carinii* (IgG2b) or *Trichomonas vaginalis* (IgM) were used.

**Harvest from culture plates, radioimmunoprecipitation, and analytical densitometry.** For growth curve determinations, trophozoites in the upper compartment were harvested and counted at 4-h intervals after incubation for 24–60 h. Amebae were harvested by removing the upper well and chilling it on ice to permit amebe adherence to the collagen-coated membrane to detach. The suspension was transferred to a tube and ice-cold PBS was added to the well to wash and recover any remaining amebae. Suspensions were pooled, amebae were counted in a hemocytometer, and total organisms were determined. In addition, an aliquot of the trophozoites was examined by Trypan blue exclusion for membrane integrity.

Before harvest of medium, the lower compartments of the culture plate were examined microscopically for bacterial contamination and to ensure that no amebae had passed through the membrane. The harvested medium was mixed with 2X solubilization buffer (0.02 M PBS, pH 8.0, 2% Nonidet P-40, 2% aprotinin, and 2 mM PMSF), vortexed for 4 min, and centrifuged at 40,000 g at 4°C for 30 min. The supernatants were transferred to siliconized tubes, and the radioimmunoprecipitations were done using 200 μg of purified monoclonal antibodies specific for the adherence lectin (E-13, IgG1) or for the 96-kDa antigen (EH335, IgG2b) as described [2]. The antibody concentration used in immunoprecipitations had been titered to assure that an excess antibody-to-antigen ratio was present (for antigen released and detected in the bottom wells) during the growth period containing the highest number of organisms. Subsequent experiments were done using 200 μg of purified monoclonal antibody for each assay. Monoclonal antibodies used for immunoprecipitations were purified by standard protein A affinity chromatography. Immunoprecipitated samples were subjected to SDS-PAGE and fluorography. Controls for immunoprecipitations were matched isotype monoclonal antibodies specific for *P. carinii* (IgG2b) or *T. vaginalis* (IgG1) used at equivalent concentrations. Quantitation of specific radiolabeled bands representing diffusible amebic products visualized by fluorography was obtained by soft-laser analytical densitometry (model SLR-2D/ID; Biomed Instruments, Fullerton, CA).

**Treatment of *E. histolytica* with ethanol.** Logarithmic phase *E. histolytica* trophozoites were harvested, resuspended in fresh TYI-S-33 medium, and counted on a hemocytometer. Trophozoites (3.2–3.5 × 106) were seeded into borosilicate glass tubes (13 × 100 mm) containing 8 mL of fresh prewarmed medium and then incubated at 37°C for 18 h. Immediately before treatment, the tubes were centrifuged at 250 g for 5 min to pellet organisms not attached to the glass to prevent aspiration of trophozoites during the procedure. The medium was aspirated and replaced with the treatment reagent (2, 6, 12, 18, or 24 mM ethanol) in prewarmed TYI medium (without serum, vitamins, or antibiotics) for 2 h at 37°C. Alternatively, the trophozoites were treated with 12 mM ethanol for 15 min, 30 min, 1 h, 2 h, and 4 h at 37°C. At the end of treatment, the tubes were placed in ice for 10 min to allow for detachment of organisms from the glass. After centrifugation, the medium was aspirated, and amebae were suspended immediately in lysis solution (Direct Protect—lysaté ribonuclease protection assay [RPA] kit; Ambion, Austin, TX). For each assay, controls included organisms prepared and treated identically, except that no ethanol was added. Cell counts of control and treated organisms after manipulation ranged from 8.0 to 8.4 × 10⁶ amebae.

**RNA transcripts and RPA.** A 400-bp HindIII fragment from the cDNA encoding EhADH2 was subcloned into the vector pGEM4 (Promega, Madison, WI) and linearized with PvuII. The 266-nucleotide (nt) RNA transcript was synthesized using T7 RNA polymerase and an in vitro transcription kit (Maxiscript; Ambion) according to the manufacturer’s protocol, except that digoxigenin-UTP (Boehringer Mannheim) was used in place of radiolabeled
UTP. After labeling, the probe was purified from unincorporated nt by use of a spin column (Sephadex G-50; Pharmacia). An E. histolytica actin template for RNA transcription was generated by subcloning the DraI-EcoRI region of a genomic actin DNA clone (provided by G. Bailey, Morehouse School of Medicine, Atlanta) into the Smal-EcoRI cloning site of the vector pGEM4. The construct, which contained a 445-bp fragment, was linearized with AccI, and a 307-nt antisense RNA transcript was synthesized using SP6 RNA polymerase.

The RPA was done using a commercial kit (Ambion) in accordance with the manufacturer’s protocol. Reaction conditions were optimized with respect to the number of amebae and amount of probe used for each assay and conditions for RNase digestion of unprotected RNA. In brief, the organisms were harvested and suspended in lysis solution at $10^9$ organisms/mL. An aliquot of the lysate equivalent to $2.5 \times 10^7$ organisms was hybridized at $37^\circ C$ overnight to 300 pg of the digoxigenin-labeled RNA probe. An antisense RNA probe to E. histolytica actin was used in the assay and served as an internal standard for quantification of mRNA levels [10]. Because the actin- and EhADH2-protected mRNAs were not significantly different in size (297 and 251 nt, respectively), a single assay could not be used to quantitate the respective mRNA levels. Thus, samples containing identical cell numbers were hybridized to each probe in separate assays. After treatment of unprotected RNA with 200 U of RNase T1, protected mRNA fragments were denatured and electrophoresed on a 5% acrylamide-7 M urea gel. The resolved RNAs were transferred to a nylon membrane by electroblotting. Protected RNA fragments were detected using anti-digoxigenin antibodies and a chemiluminescence substrate (Lumi-Phos 530; Boehringer Mannheim) according to the manufacturer’s recommendations. The chemiluminescent signals were recorded on x-ray film.

For quantitation of mRNA levels, fluorographs were analyzed by a scanning analytical densitometer (ImageMaster DTS; Pharmacia) and software package (Protein + DNA ImageWare Systems; PDL, Huntington Station, NY). Actin mRNA levels of trophozoites were used as an internal standard for normalization of potential minor variability in amounts of target mRNA between samples. Fold increases were determined after densitometric scanning, and optical densities were multiplied by the area of the band (in square millimeters) and normalized with respect to actin by comparing the ratios of the density of EhADH2 mRNA to the density of actin mRNA.

Results

Identity of the 96-kDa protein with EhADH2. To determine whether the 96-kDa surface protein that we had previously characterized was identical to or an isoform of the recently reported EhADH2, we tested both antigens for immunologic cross-reactivity. Immunofinity-purified 96-kDa antigen or enzymatically active EhADH2 purified by fibronectin-Sepharose chromatography and FPLC [1] were cross-tested with respective monospecific or monoclonal antibodies. Immunoblotting of the purified 96-kDa antigen with monospecific antibody to EhADH2 demonstrated a single highly reactive band (figure 1B, lane 1), while horseradish peroxidase–conjugated goat anti-rabbit antibodies used as a control failed to show reactivity (figure 1B, lane 2). The purity of immunofinity-purified 96-kDa antigen was assessed by a silver-stained gel (figure 1A) and was 90% pure by analytical densitometry. Furthermore, purified enzymatically active EhADH2 was recognized by monoclonal antibody EH335 and five other monoclonal antibodies specific for the 96-kDa protein by ELISA, thus demonstrating immunologic cross-reactivity ($A_{492}>1.0$ for all six monoclonal antibodies; $A_{492}=0.059$ for controls). In addition, EhADH2 was isolated by fibronectin chromatography from $^{125}$I-labeled trophozoites and had alcohol/aldehyde dehydrogenase activity (figure 2), further corroborating the surface localization and identity of the protein. Hereafter, we will refer to the proteins as EhADH2, as designated by Yang et al. [1].

Detection of released antigens. Because EhADH2 was previously reported to be an integral membrane protein and 14% of the protein was membrane associated [3], we were prompted to investigate whether this antigen might be released. In an analysis of conditioned medium, Petri et al. [11] demonstrated that the adherence lectin was released and was active in blocking adherence of trophozoites to target tissue in competition assays. Thus, during assays for released EhADH2, concurrent isolation of the adherence lectin from the medium in the culture plate system (Transwell) served as a positive control.

Since the culture plate system was a novel technique for culturing E. histolytica, we generated a growth curve for the amebae and determined that midlogarithmic-phase growth occurred between 24 and 48 h (figure 3). Concomitantly, we isolated the newly synthesized, radiolabeled, released 170-kDa heavy chain of the adherence lectin and EhADH2 antigen by radioimmunoprecipitation of the medium in the lower compartment. Immunoprecipitated antigens were resolved by SDS-PAGE and detected by fluorography (figure 4, lanes 1 and 2 [data for one time point]). Analytical densitometric analysis of released proteins on fluorographs showed an increase in the amount of newly synthesized released antigens commensurate with the length of incubation and increase in the number of organisms (figure 3, data for EhADH2 antigen). A decrease in the amount of EhADH2 protein after 60 h of incubation may be attributed to lower levels of protein expression by the amebae during late log to early stationary phase together with degradation by released E. histolytica proteinases or serum...
enzymes. The lower bands (55 kDa) in each lane represent bovine immunoglobulins present in the serum used in the growth medium that were chemically labeled with the $[^{35}S]$methionine/cysteine. Labeled bovine immunoglobulins were not observed when ultra-pure grade $[^{35}S]$methionine was used (data not shown). However, the use of pure $[^{35}S]$methionine for the entire project was cost-prohibitive and unnecessary after the initial determination that the 170-kDa adherence lectin and EhADH2 antigens were metabolically and not chemically labeled. Matched isotype monoclonal antibodies specific for *P. carinii* or *T. vaginalis* failed to immunoprecipitate *E. histolytica* proteins (figure 4, lanes 3 and 4).

To determine whether the antigens were released in soluble form, the medium from bottom wells of the culture system were subjected to ultracentrifugation at 100,000 g for 2 h at 4°C followed by radioimmunoprecipitation of supernatants and pellets. The antigens were isolated from the supernatants but not from the pellets (data not shown), suggesting that the proteins were likely released in soluble form, although the possibility of a small organelle releasing the antigen due to centrifugal force exists.

**RPA.** Trophozoites were subjected to various concentrations of ethanol for 2 h, then EhADH2 mRNA was assayed by RPA. We detected increases in the EhADH2 mRNA levels of ~2.5-fold in response to 12 and 18 mM ethanol when compared to the untreated control (data not shown). Since treatment of amebae with 12 and 18 mM ethanol yielded similar mRNA levels, a time-course assay was done using the lower concentration of ethanol. Trophozoites were treated with 12 mM ethanol for 15 min, 30 min, 1 h, 2 h, and 4 h. The RPA revealed a 3-fold increase in mRNA levels in amebae treated for 1 and 2 h compared with untreated organisms (figure 5). After incubation for 4 h in the presence of ethanol, the mRNA level was 1.8-fold more than the untreated control. A distinct 200-nt mRNA, in addition to EhADH2 mRNA (251 nt), was always protected, suggesting mRNA from a gene family of *E. histolytica* dehydrogenases.

**Discussion**

Alcohol/aldehyde dehydrogenases play a pivotal role in cellular metabolism and have been linked to microbial pathogenesis. Our study shows that a key enzyme in *E. histolytica* metabolism, the EhADH2 molecule, can be localized to the cell surface of amebic trophozoites and is secreted or shed extracellularly. The immunologic identity of the previously studied 96-kDa surface protein and the EhADH2 protein was demonstrated by monospecific antibody and, of importance, the same identity was established with monoclonal antibodies that are absolutely conserved with respect to reactivity with the 96-kDa protein [2]. These data clearly establish that the 96-kDa antigen and the EhADH2 molecule are identical or are isoforms.

The finding of a surface location for the EhADH2 is consistent with study results for a number of organisms. Pancholi and Fischetti [12] showed that the glyceraldehyde-3-phosphate
P. pylori produces acetaldehyde, a toxic metabolite that may play a role in damaging the gastric mucosa. In addition, a surface-associated glutamine dehydrogenase was identified in the periodontal pathogen *Porphyromonas gingivalis* [16].

We previously analyzed the 96-kDa protein by immunofluorescence and immunogold labeling of live *E. histolytica* trophozoites followed by immunoprecipitation, and agglutination of trophozoites with IgM antibodies to the 96-kDa protein to establish its surface location [2, 3]. Here, we confirm the surface iodination studies and show that the EhADH2 antigen can be surface-labeled with $^{125}$I and can retain its fibronectin-binding activity. Computer analysis of the deduced amino acid sequence of EhADH2 showed that the antigen does not possess a classical eukaryotic signal sequence, but it has two potential transmembrane domains at amino acid positions 550–568 and 713–728. Other researchers have identified proteins that lack classical signal sequences and have evidence of novel secretory pathways [17–20]. It is of interest that the surface-associated *P. gingivalis* glutamine dehydrogenase lacks a defined signal sequence [18].

In previous studies, we used Triton X-114 partitioning to demonstrate that 14% of the 96-kDa protein is associated with the surface membrane, while the rest can be localized to the cytosol [3]. This suggests that EhADH2 may exist in different isoforms, and structural differences or posttranslational modifications may influence the localization of the enzyme to the surface membrane or cytosol. The presence of the EhADH2 isoforms is consistent with data from Southern blotting analysis that suggest that the EhADH2 gene is part of a gene family with at least three members [1]. One possible difference between isoforms may be in their ability to polymerize. The *Escherichia coli* homologue to EhADH2, the adhE protein, requires the formation of multimers called spiroosomes for optimal function [21–23], and there is evidence for EhADH2 multimers [24]. One can speculate that isoforms of the enzyme that do not polymerize might have reduced enzymatic activity but could be localized to the surface and serve alternative functions. A further structural analysis of the genes encoding EhADH2 and of enzyme localized to the cytosol and membrane may be revealing.

A possible function for the surface location or release into the external milieu for an alcohol/aldehyde dehydrogenase, such as EhADH2, may be that a functional EhADH2 is released to protect amebae from toxic products released by gut flora. It is of particular interest that when we administered ethanol (a substrate for EhADH2) to *E. histolytica* trophozoites, mRNA levels for the enzyme were increased. Alternatively, surface membrane–bound EhADH2 might be an adhesin and mediate binding to extracellular matrix proteins or some other target [1] and could be shed after binding the appropriate ligand. Shedding or secretion of the EhADH2 molecule is also consistent with the recent finding that this molecule is a major component of the uroid, a posterior membrane organelle associated with motility, which can form in response to ligand binding (such as antibody) (Guillen N, personal communication).

In summary, our studies document that the EhADH2 enzyme, a pivotal molecule in the *E. histolytica* fermentation pathway, is immunologically homologous to the previously described 96-kDa amebic antigen and can be localized to the surface of *E. histolytica* trophozoites. The antigen is shed or secreted into the extracellular milieu, and mRNA is specifically

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**Figure 5.** Ribonuclease protection assay (RPA). Time course of EhADH2 protein mRNA levels in response to 12 mM ethanol detected by RPA. Protected RNA fragments were resolved on 5% acrylamide/7 M urea gel, transferred to a nylon membrane, and detected with anti-digoxigenin antibodies enhanced by chemiluminescence. A, Fluorograph shows protected EhADH2 and actin mRNAs. B, Bands visualized on fluorographs were analyzed by densitometric scanning and densities (OD x mm$^2$) were normalized with respect to actin (density EhADH2 mRNA/density actin mRNA). Data are means of 2 experiments. C = untreated trophozoites.
up-regulated upon stimulation with the substrate ethanol. The EhADH2 enzyme joins a growing list of dehydrogenase molecules that may serve multiple roles in pathogenic organisms.

References


