Molecular Microbiology (1993) **7**(5), 755–763

Structural analysis and demonstration of the 29 kDa antigen of pathogenic *Entamoeba histolytica* as the major accessible free thiol-containing surface protein

Becky M. Flores,[1] Mark A. Batzer,[2] Murry A. Stein,[1] Carolyn Petersen,[3] Dana L. Diedrich[1] and Bruce E. Torian[1]*

[1]Department of Pharmaceutical Sciences, College of Pharmacy, Idaho State University, Pocatello, Indiana 83209-8334, USA.

[2]Human Genome Center, Biomedical Sciences Division, L-452, Lawrence Livermore National Laboratory, P.O. Box 5507, Livermore, California 94551, USA.

[3]Parasitology Laboratory, San Francisco General Hospital and the University of California San Francisco, San Francisco, California 94110, USA.

Summary

The 29 kDa protein of pathogenic *Entamoeba histolytica* is a cysteine-rich surface antigen which we recently characterized by cDNA sequencing and by using monoclonal antibodies which differentiated between pathogenic and non-pathogenic clinical isolates. To determine the structure and biochemical attributes of this protein, a repertoire of immunological techniques using monoclonal antibodies, and radiolabelling were employed. We demonstrated that the 29 kDa protein forms a 60 kDa dimer and a high-molecular-mass oligomer(s) on the surface of the organism through disulphide bonds, and is the major accessible free thiol-containing surface protein of *E. histolytica*. The deduced amino acid sequence encoding the 29 kDa protein was found to share a common amino acid domain with sequences reported for *Helicobacter pylori*, *Salmonella typhimurium*, *MER5* gene expressed in murine erythroleukemia cells, *Clostridium pasteurianum*, and a *Bacillus* spp.

Introduction

The protozoan *Entamoeba histolytica* continues to cause significant worldwide morbidity and mortality infecting nearly 500 million people annually (Walsh, 1988). While a large proportion of the infected individuals are asymptomatic, approximately 50 million people develop colitis which may result in extraintestinal disease or even death. We recently reported the nucleotide sequences of one full-length and four partial cDNA clones isolated from a library constructed from pathogenic *E. histolytica* (strain H-303:NIH) mRNA (Reed *et al.*, 1992; Torian *et al.*, 1990a). The cDNAs encoded a 29 kDa peripheral membrane protein which contained a cysteine-rich region with an unusual motif and the full length cDNA possessed an overall cysteine residue content of 5.1%. Monoclonal antibodies specific for the 29 kDa protein localized the antigen on the surface of the amoebae (Torian *et al.*, 1990a) and differentiated between pathogenic and non-pathogenic clinical *E. histolytica* isolates on the basis of a distinct epitope(s) on the protein (Reed *et al.*, 1992). Northern blot analysis and DNA–DNA hybridization experiments demonstrated that messenger RNA and the gene were apparently present on both pathogenic and non-pathogenic clinical strains. However, we were unable to detect this protein on non-pathogenic isolates using monoclonal antibodies, rabbit polyclonal antiserum, or murine polyclonal antiserum. Recently, the partial nucleotide sequence of a cDNA from pathogenic *E. histolytica* strain HM1:IMSS, which encoded a 30 kDa protein (Tachibana *et al.*, 1991) was shown to have complete amino acid identity with the deduced amino acid sequence of the partial cDNA reported by us. These investigators demonstrated differences in the restriction enzyme patterns of PCR-amplified homologous genomic DNA isolated from pathogenic and non-pathogenic strains. To date, no studies on the structure or function of the 29 kDa protein have been reported.

Because the 29 kDa antigen possessed pathogen-specific epitopes, we analysed the structure of the 29 kDa protein from live *E. histolytica* trophozoites, the protein purified by immunoaffinity chromatography, and the protein from whole trophozoite lysates. The data from the combined experiments show that the native protein was a high-molecular-mass complex on the amoebic surface. Alkylation experiments showed that the antigen was the major surface protein containing accessible free thiol groups. Protein similarity searches of data bases revealed that the antigen shares a striking identity in a 13-amino-acid region with protein sequences reported from several other organisms, suggesting a potential common functional domain.

Received 16 July, 1992; revised 2 November 1992 accepted; 9 November, 1992. *For correspondence. Tel. (208) 236 3088; Fax (208) 236 4482.

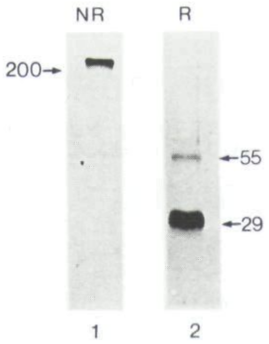

Fig. 1. Immunoaffinity-purified protein (2 µg) was solubilized in Laemmli sample buffer in the absence or presence of a reducing agent, and subjected to 10% SDS–PAGE followed by staining with silver. Numbers to the left and right of the lanes indicate the positions of the molecular-mass standards (in kDa). Lane 1: pure protein electrophoresed in the absence of a reducing agent showing the high-molecular-mass complex (>200 kDa). Lane 2: protein reduced with 10% 2-ME demonstrating a 29 kDa band and a 60 kDa band.

Results

Analysis of the 29 kDa protein in the presence or absence of a reducing agent

In order to demonstrate the purity of immunoaffinity purified 29 kDa protein and to determine if the protein formed an oligomeric structure, we analysed protein under reducing and non-reducing conditions by SDS–PAGE. In the absence of a reducing agent, the 29 kDa protein migrated as a high-molecular-mass complex greater than 200 kDa (Fig. 1). Protein reduced with 10% 2-mercaptoethanol (2-ME) showed a 29 kDa band and a faint band with an apparent molecular mass of 60 kDa. To clarify whether the 60 kDa protein species was present because of incomplete reduction, we titred varying protein concentrations against varying concentrations of 2-ME. In order to ensure adequate sensitivity for detection of the 60 kDa band, all gels were stained with silver and allowed to overdevelop. It appeared that a 1000-fold molar excess of 2-ME to protein was required for complete reduction of the protein (data not shown). However, when this concentration of 2-ME was used to reduce purified ^{35}S-labelled protein, a 60 kDa band was detected on the fluorograph (data not shown) suggesting that the amount of partly reduced protein was below the limit of detection by silver staining.

To characterize further the oligomeric nature of the protein, the antigen from a whole trophozoite lysate was analysed by immunoblotting with monoclonal antibodies and detected by chemiluminescence. Lysates were either boiled (to inactivate proteinases) or not boiled before treating the sample for SDS–PAGE analysis under non-reducing or reducing conditions. When the lysate was boiled to inactivate proteinases before denaturation, the protein detected by specific monoclonal antibody was a high-molecular-mass complex (Fig. 2, lane 1) and even under reducing conditions, some of the high-molecular-mass complex was still present (Fig. 2, lane 3). Without prior inactivation of proteinases, the non-reduced protein appeared to be degraded (Fig. 2, lane 2).

Reconstitution of the high-molecular-mass complex

The ability of the reduced disulphides of the protein to oxidize and reassemble to form the high-molecular-mass complex was examined to support further the assertion of a surface oligomeric structure(s). Affinity-purified ^{35}S-labelled protein was treated with 2-ME to generate reduced protein, then the sample was passed through a Sephadex G-25 column for removal of the reducing agent. The individual samples were incubated for various times (5–60 min) at 4°C, 20°C, or 37°C without the addition of an enzyme or oxidation–reduction buffer system to promote the reforming of disulphide bonds. The sample incubated for 5 min at 4°C showed a faint high-molecular-mass band corresponding to the unreduced, control protein sample on the fluorograph (Fig. 3). There was no apparent visual difference in the amount of high-molecular-mass complex formed in the sample incubated for 10 min compared with the sample incubated for 60 min (Fig. 3). No apparent differences were observed over the different incubation temperatures tested (data not shown). In addition to a range of faint bands, a prominent

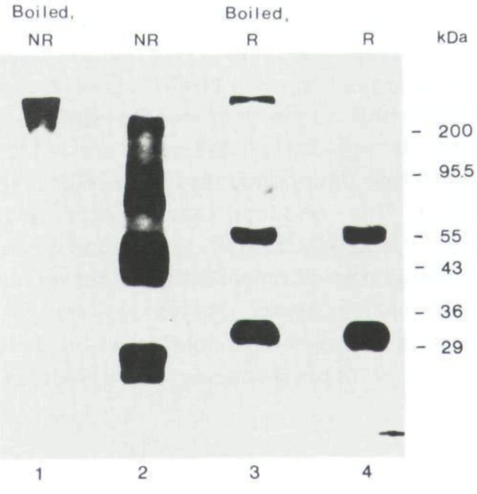

Fig. 2. Analysis of the 29 kDa antigen from whole amoebic lysates detected by immunoblotting enhanced by chemiluminescence. Samples were boiled or not boiled before the addition of non-reducing or reducing sample buffer as described in the *Experimental procedures*. Numbers to the right of the lanes indicate the positions of the molecular-mass standards (in kDa). Lanes 1 and 3: samples were boiled to inactivate proteinases, then denatured with Laemmli sample buffer without reducing agent (lane 1) or with 10% 2-ME (lane 3). Lanes 2 and 4: samples were denatured with Laemmli sample buffer without reducing agent (lane 2) or with 10% 2-ME (lane 4).

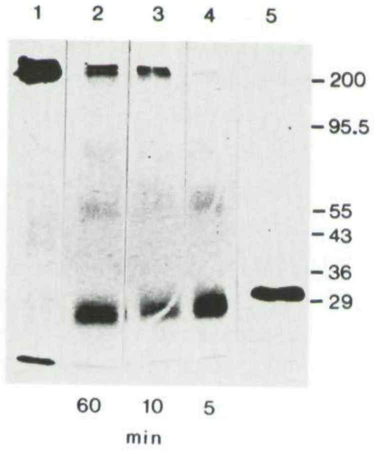

Fig. 3. Fluorograph demonstrating the *in vitro* reassembly of the high-molecular-mass complex from reduced protein. Purified ^{35}S-labelled protein was reduced with excess 2-ME followed by subsequent removal of the reducing agent. Following incubation for the reforming of disulphide bonds, samples were subjected to SDS–PAGE under non-reducing conditions. Numbers at right indicate positions of the molecular mass standards (expressed in kDa) on reducing SDS–PAGE. Lane 1: non-reduced protein, control. Lanes 2–4: reconstitution at 4°C for 60 min (lane 2), 10 min (lane 3), and 5 min (lane 4). Lane 5: reduced protein, control.

broadly diffuse 29 kDa band was seen. The presence of 5 mM EDTA in the buffer failed to prevent reassembly of the reduced protein but when reduced protein was alkylated with 0.1 M iodoacetamide, the reassembly of complexes was not observed (data not shown).

Molecular-mass determination of the oligomeric form(s) of the 29 kDa antigen

Since high-molecular-mass proteins are poorly resolved on SDS–PAGE gels, we fractionated protein on a Sephacryl S-300 column to obtain an estimate of the size of the affinity purified protein. Enzyme-linked immunosorbent assay (ELISA) of the column fractions identified two antigen-containing peaks with the majority of antigen eluted in peak 2 (Fig. 4B). Using a calibration curve determined from molecular-mass standards used, peak 1 was estimated to be 600 kDa and peak 2 was 420 kDa (Fig. 4A).

Estimation of the size of the protein on the surface of live trophozoites

In order to ascertain whether a high-molecular-mass complex was present on the amoebic surface, we performed immunoprecipitations using live *E. histolytica* trophozoites. Monoclonal antibodies were incubated with the live amoebae for 1 h and the reaction mixture was maintained at 4°C to prevent capping and shedding of antigen/antibody complexes (Calderón and Avila, 1986). The membrane integrity of trophozoites during the incubation remained intact as determined by exclusion of Trypan blue dye. The immunoprecipitated proteins were immediately subjected to non-reducing SDS–PAGE (7.5% gel). Two methods were used for added sensitivity in the detection of the immunoprecipitated antigen. In the first, metabolically [^{35}S]-methionine/cysteine-labelled live amoebae were subjected to immunoprecipitation. A single protein band that migrated above the 200 kDa molecular-mass standard was detected on the fluorograph (Fig. 5B). In the second method, unlabelled live trophozoites were subjected to immunoprecipitation and detection of the antigen was accomplished by immunoblotting enhanced by chemiluminescence. A single band >200 kDa as well as a band corresponding to the position of non-reduced monoclonal antibody (used in the

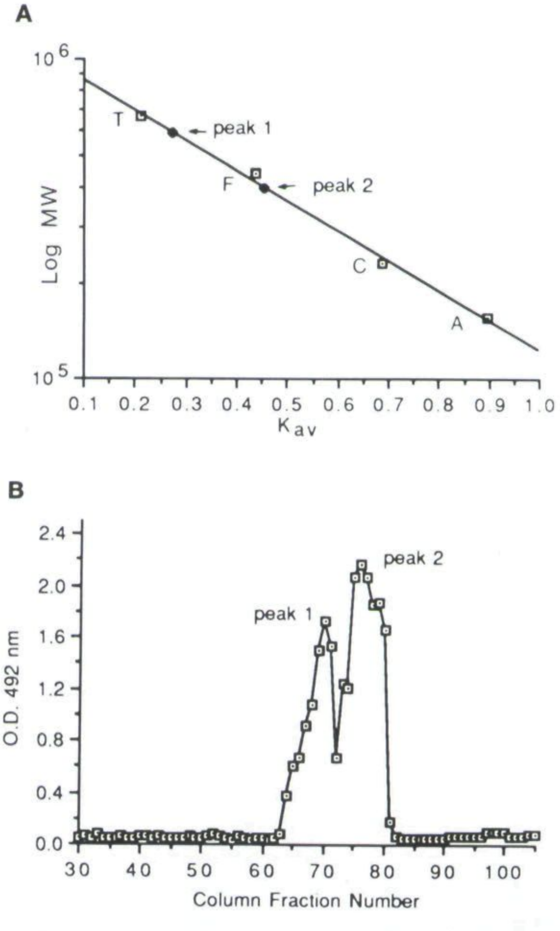

Fig. 4. Gel filtration chromatography. Immunoaffinity-purified protein was fractionated on a Sephacryl S-300 column, and the molecular mass was estimated by using a standard curve determined by the migration of the molecular-mass calibration standards.

A. Calibration curve for molecular-mass determination of the native protein: T, thyroglobulin, 669 kDa; F, ferritin, 440 kDa; C, catalase 232 kDa; and A, aldolase, 158 kDa.

B. OD_{492} measurements of column fractions tested by an ELISA using specific monoclonal antibody demonstrating the presence of two elution peaks.

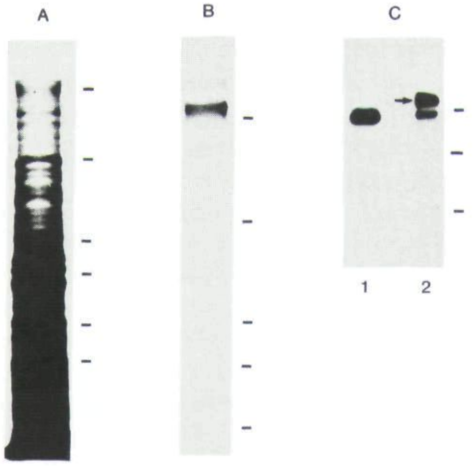

Fig. 5. Immunoprecipitation of the 29 kDa antigen from live *E. histolytica* trophozoites. Protein was immunoprecipitated from ^{35}S-labelled or unlabelled live trophozoites and the protein was separated on 7.5% SDS–polyacrylamide gels under non-reducing conditions. Marks to the right of each panel indicate the positions of the molecular mass standards from top to bottom: 200, 95.5, 55, 43, 36, 29 (expressed in kDa).

A. Fluorograph of whole profile of ^{35}S-labelled *E. histolytica* proteins separated by reducing 10% SDS–PAGE.

B. Fluorograph of immunoprecipitated antigen from ^{35}S-labelled live *E. histolytica* trophozoites analysed under non-reducing conditions demonstrating a high-molecular-mass (>200 kDa) protein(s).

C. Protein immunoprecipitated from unlabelled live trophozoites, analysed under non-reducing conditions and detected by immunoblotting and chemiluminescence. Lane 1: purified monoclonal antibody, FP-21, demonstrating the binding of the Protein A conjugate to non-reduced antibody. Lane 2: immunoprecipitated antigen (indicated by arrow), and the monoclonal antibody FP-21.

immunoprecipitation) were detected by the Protein A conjugate (Fig. 5C, lane 2).

Peptide mapping of the reduced and non-reduced protein

The deduced amino acid sequence of the full-length cDNA which encodes the 29 kDa protein predicted three tryptophan residues (Reed *et al*., 1992). In order to verify this prediction we performed peptide mapping of the purified protein with *N*-chlorosuccinimide (NCS) which selectively cleaves tryptophan bonds in proteins. In addition, we wanted to determine whether the reduced species (29 kDa and 60 kDa) and the high-molecular-mass complex had similar peptide maps to demonstrate identity between these three forms of the protein. NCS cleavage of reduced and non-reduced ^{35}S-labelled protein was carried out in gel slices followed by separation of fragments on 14% SDS–PAGE. The peptide maps of both non-reduced and reduced (60 kDa and 29 kDa) proteins were identical revealing four peptides (Fig. 6, data shown for non-reduced and 29 kDa proteins). Some uncleaved protein was also seen (compare with control, Fig. 6). Although gel slices were excised from the location corresponding to the migration of each form of the protein (29 kDa, 60 kDa, and >200 kDa), reformation of complexes occurred during the treatment of the gel slices containing the reduced protein (Fig. 6, indicated by arrow and bracket).

Detection of free thiol groups on surface antigens of live trophozoites

Because free thiol groups on proteins can be integral to protein function, we reacted live *E. histolytica* trophozoites with [^{14}C]-iodoacetamide which alkylates accessible thiol groups. After a 10 min incubation at 4°C followed by removal of unreacted alkylating agent, the radiolabelled organisms (which were carefully monitored by Trypan blue exclusion for membrane integrity) were solubilized, and subjected to immunoprecipitation. The whole profile of proteins containing alkylated surface thiols analysed under reducing conditions revealed eight faintly labelled bands and a prominent 29 kDa band (Fig. 7, lane 1). After the alkylation of proteins on live trophozoites, immunological identity was established by immunoprecipitation of the antigen (Fig. 7, lane 2). To compare the profile of alkylated thiols on surface proteins with that of alkylated thiol groups on proteins of lysed trophozoites, a freeze-thawed lysate was reacted with [^{14}C]-iodoacetamide. In contrast to the experiment using live trophozoites, this experiment was performed with a lysate containing four-fifths fewer amoebae and reacted with four-fifths less alkylating reagent. The alkylation of thiols on proteins of lysed amoebae demonstrated a marked increase in the number of alkylated proteins (Fig. 7, lane 3).

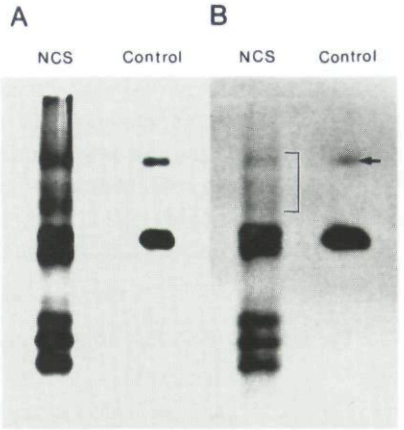

Fig. 6. *N*-chlorosuccinimide (NCS) peptide maps of the non-reduced and reduced proteins. Purified ^{35}S-labelled protein in gel slices was treated with NCS as described in the *Experimental procedures*.

A. Non-reduced protein cleaved with NCS and control.

B. Reduced 29 kDa protein cleaved with NCS and control. Arrow and bracket indicate complexes formed while in the gel slice during treatment of the protein.

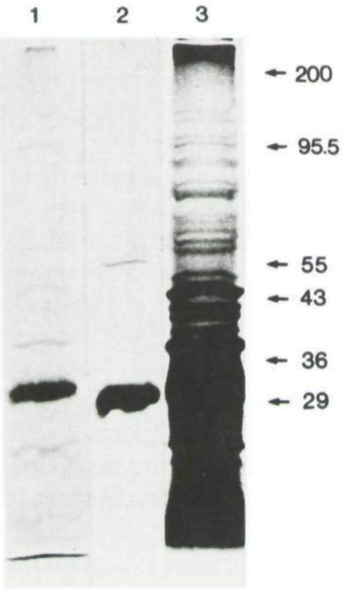

Fig. 7. Fluorograph of [^{14}C]-iodoacetamide alkylation of free thiol groups. Live *E. histolytica* trophozoites were incubated with [^{14}C]-iodoacetamide for 10 min. After removal of unbound radioisotope by washing, the labelled antigen was immunoprecipitated with a pool of monoclonal antibodies specific for the 29 kDa antigen and subjected to reducing 10% SDS–PAGE. The numbers at the right indicate positions of molecular mass standards (expressed in kDa). Lane 1, whole profile of alkylated surface proteins containing free thiols from live *E. histolytica*. Lane 2, immunoprecipitation of alkylated antigen from live *E. histolytica*. Lane 3, profile of alkylated proteins from a freeze–thaw lysate from *E. histolytica*.

Amino acid identity

A search of Swiss Protein (release 21) and PIR (release 32) data bases was conducted using the IFIND program (Wilbur and Lipman, 1983) from IntelliGenetics Suite in order to obtain any previously reported proteins with sequences similar to that of the 29 kDa protein. The 29 kDa protein shared a 13-amino acid region of identity with five other proteins (Fig. 8) as well as other smaller interspersed regions of amino acid identity. These proteins include the *MER5* gene product from murine erythroleukemia cells (Yamamoto *et al.*, 1989), a 20 kDa protein from the anaerobic nitrogen-fixing *C. pasteurianum* (GenBank accession number M60116), *aphC* gene product (C22 subunit of the alkyl hydroperoxide reductase) of *S. typhimurium* (Tartaglia *et al.*, 1990), an open reading frame upstream from the NADH dehydrogenase gene from an alkalophilic *Bacillus* spp. strain YN-1 previously suggested to be part of the *ndh* operon (Xu *et al.*, 1991), and a species-specific 26 kDa protein from *H. pylori* (O'Toole *et al.*, 1991) (Fig. 8). This domain may represent a common functional or physical feature shared by all six proteins, but does not appear to be a commonly reported structural motif.

Discussion

Free thiols on intracellular proteins or surface proteins possess critical roles in many biological systems. Cell surface thiols present on cloned murine natural killer lymphocytes were required for binding and lysis of tumour target cells (Ristow *et al.*, 1985). The cytotoxicity of diphtheria toxin in Chinese Hamster ovary cells is also surface sulphydryl dependent (Ryser *et al.*, 1991). Alkylation of thiol groups has been demonstrated to completely or partially inhibit the activity of some enzymes (Avila *et al.*, 1985; Dailey, 1984; Keene *et al.*, 1986; McLaughlin and Faubert, 1977). For parasitic protozoans, particularly *E. histolytica*, little information is available regarding free thiol groups on individual proteins. The proteolytic activity of a purified major cysteine proteinase (Keene *et al.*, 1986), and other proteinases of *E. histolytica* (Avila *et al.*, 1985; McLaughlin and Faubert, 1977) were completely inhibited by thiol reagents. It has been demonstrated that surface thiols on *Giardia lamblia* and *E. histolytica*, and the reducing agents cysteine and ascorbic acid in culture medium, were required for attachment of organisms to plastic for growth and cell survival (Gillin and Diamond, 1981a,b; Gillin *et al.*, 1984). The presence of cysteine in culture medium also protected the amoebae from the lethal effects of 4% oxygen tension (Gillin and Diamond, 1981b).

In this report, alkylation of proteins containing free thiols on live *E. histolytica* trophozoites with [^{14}C]-iodoacetamide has demonstrated that the 29 kDa protein is the major accessible free thiol-containing protein on the amoebic surface. Although iodoacetamide may penetrate cell membranes, trophozoites were incubated with the reagent for only 10 min and the cell membrane of the amoebae remained intact (i.e., excluded Trypan blue dye). If penetration of the membrane by the reagent had occurred, a much greater range, as well as more intense radiolabelling of free thiol-containing proteins would

E. histolytica	118	F	Y	P	L	D	W	T	F	V	C	P	T	E	130
MER5	100	F	Y	P	L	D	F	T	F	V	C	P	T	E	112
C. pasteurianum	41	F	Y	P	L	D	F	T	F	V	C	P	T	E	53
S. typhimurium	38	F	Y	P	A	D	F	T	F	V	C	P	T	E	50
Bacillus spp.	19	F	Y	P	A	D	F	T	F	V	C	P	T	E	31
H. pylori	40	F	W	P	K	D	F	T	F	V	C	P	T	E	52

Fig. 8. Amino acid sequence identity between the 29 kDa protein of *E. histolytica*, and sequences reported for the following proteins: *MER5* gene product from murine erythroleukaemia cells (Yamamoto *et al.*, 1989), 20 kDa protein from *C. pasteurianum* (GenBank accession number M60116), *ahpC* gene product (C22 subunit of the alkyl hydroperoxide reductase) of *S. typhimurium* (Tartaglia *et al.*, 1990), an open reading frame upstream from the *ndh* gene from *Bacillus* spp. (Xu *et al.*, 1991) and a species-specific 26 kDa protein from *H. pylori* (O'Toole *et al.*, 1991). The numbers indicate the positions of the amino acid residues from each sequence.

be expected to occur (compare lanes 1 and 3, Fig. 4). Incubation of live amoebae with a membrane-impermeant thiol reagent, monobromotrimethylammoniobimane (Kosower *et al.*, 1981), yielded similar results (unpublished data).

The molecular mass of the purified protein was estimated by gel filtration chromatography. The approximate molecular mass of most of the protein fractionated was determined to be 420 kDa. Although SDS–PAGE does not adequately resolve very high-molecular-mass proteins, a single high-molecular-mass species (>200 kDa) was seen when the antigen was subjected to non-reducing 7.5% SDS–PAGE immediately after purification or immunoprecipitations. Use of gradient gels (4–12%) also failed to resolve more than one high-molecular-mass band.

While other proteins, such as the insulin receptor, may require the addition of an oxidation–reduction buffer to promote the reforming of disulphide bonds after reduction (Massagué and Czech, 1982), the apparent partial reassembly of the high-molecular-mass complex from reduced protein *in vitro* occurred in the absence of such a reagent. Although the reassembly of reduced protein may have been caused by spontaneous air oxidation, the rapidity at which this occurred suggests that the folded, disulphide-linked species may be the most thermodynamically favoured native form of the protein. Extensive kinetic experiments would be required to prove this. The predominant forms detected were >200 kDa and 29 kDa. Faint, intermediate-molecular-mass bands may represent randomly assembled complexes or intermediate forms of the high-molecular-mass complex. Alternatively, they may represent inappropriately formed disulphide-linked species. Whether the reassembled complexes have the disulphide bond formation identical to that of the intact protein in the native form is currently unknown, although the reassembled high-molecular-mass complex was immunologically reactive.

Although the 60 kDa protein was not always seen by silver staining, detection of the 60 kDa species was enhanced when ^{35}S-labelled protein was analysed; this may be due to the high specific activity of radiolabelling achieved by the substantial number of cysteines (14) predicted by analysis of the cDNA sequence that encodes the protein (Reed *et al.*, 1992). NCS cleavage of the 29 kDa protein, the 60 kDa protein, and high-molecular-mass complex showed the same peptide map (four cleavage products). This result corroborates the number of tryptophan residues in the protein predicted by the deduced amino acid sequence. Analysis of the purified protein under reducing and non-reducing conditions, use of reconstitution experiments, and NCS peptide mapping suggest that the basic unit is a 29 kDa monomer linked by interchain disulphide bonds to form the multimeric mature complex on the cell surface, likely through a 60 kDa dimeric intermediate. Experiments on the assembly of the protein on the amoebae are currently being performed.

For many proteins, the stability or resistance to unfavourable conditions (such as proteolysis) has been attributed to the folding of the protein and three-dimensional structure mediated by disulphide bonds (Chang, 1991; Matsumura *et al.*, 1989; Pace *et al.*, 1988). Since this surface antigen is present in an environment rich in cysteine proteinase (Keene et *al.*, 1986), as well as other proteinases (Avila *et al.*, 1985; McLaughlin and Faubert, 1977), on or near the *E. histolytica* cell surface, its structural characteristic may protect this protein from proteolysis, at least from the neutral cysteine proteinase. Intact purified protein has been demonstrated to be resistant to proteolysis by purified neutral cysteine proteinase of pathogenic *E. histolytica,* while denatured or reduced proteins were rapidly degraded (unpublished data).

E. histolytica lacks mitochondria, grows anaerobically or in the presence of low oxygen tension when cysteine is used in culture medium (Gillin and Diamond, 1981b) and has been reported to lack glutathione metabolism (Fahey *et al.*, 1984). This cysteine-rich protein containing accessible thiol groups may potentially play a role in providing the organism with some protection against oxygen toxicity, especially as an oxygen-rich micro-environment exists at the site of colonic lesions. The thiol data in this study suggests a potential role for this protein as a critical moiety for cell survival and these data will facilitate further detailed study into the function, regulation of expression, and potential role in the pathogenicity of the organism. Interestingly, the 29 kDa protein of *E. histolytica* shares a common domain of 13 amino acids with proteins from five unrelated organisms. The C22 subunit of the alkyl hydroperoxide reductase of *S. typhimurium* possesses a critical free thiol for function (Storz *et al.*, 1990), and like the 29 kDa protein, forms oligomeric structures. The *MER5* housekeeping-type gene expressed in murine erythroleukaemia cells may initially appear unrelated to any protection from host oxidative defense mechanisms until consideration is given to the need for leukaemia cells to escape detection and destruction from host surveillance cells. Although the gene products of the sequences reported for *H. pylori*, *C. pasterianum*, and *Bacillus spp.* have not been well characterized and their functions are currently unknown, it is interesting that a common domain of amino acids exists among such diverse gastrointestinal organisms. In addition, *Cryptosporidium parvum*, a gastrointestinal protozoan pathogen like *E. histolytica*, has recently been shown to express a 25 kDa protein which contains this 13-amino acid domain (unpublished observation). This domain may have a biologically functional role or may simply be an amino acid domain critical for proper protein folding. Although some amino acid identity between these organisms is present on numerous other segments of the proteins, the identities are small with respect to length and therefore speculation on a common function, although intriguing, is premature.

Experimental procedures

Cultivation, harvesting, and radiolabelling of E. histolytica

Axenic *E. histolytica* strain H-303:NIH was cultivated at 35°C in Diamond's TYI-S-33 medium (Diamond *et al.*, 1978) supplemented with 10–15% (v/v) heat-inactivated bovine serum (Biofluids). Early to mid-logarithmic-phase trophozoites were harvested and washed in 0.01 M PBS, pH 7.6 as previously described (Torian *et al.*, 1987). Early-logarithmic phase *E. histolytica* trophozoites were incubated with [^{35}S]-methionine/cysteine (EXPRE^{35}S^{35}S; 83 μCi ml^{-1}; specific activity, 1077.5 Ci mmol^{-1}; Dupont, NEN Research Products) for 24 h.

Monoclonal antibodies, immunoaffinity chromatography, and immunoprecipitation

Production of monoclonal antibodies specific for the 29 kDa *E. histolytica* antigen has been previously described (Torian *et al.*, 1990a). Demonstration of the specificity of the antibodies for the antigen on axenic pathogenic strains of *E. histolytica* and on pathogenic clinical isolates has also been reported (Reed *et al.*, 1992). Monoclonal antibodies were purified from ascites using Protein A affinity chromatography. The monoclonal antibodies used in this study included: FP 4, FP 10, FP 14, FP 21, and FP 37 (all IgG$_{2a}$ isotypes). Monoclonal antibody to *Pneumocystis carinii* was used as a matched isotype, unrelated antibody control for immunoprecipitation experiments and did not react with any *E. histolytica* antigens.

For immunoaffinity chromatography, four monoclonal antibodies used to immunoprecipitate the 29 kDa antigen (FP 4, FP 10, FP 21, and FP 37) were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Inc.) according to recommendations of the manufacturer. *E. histolytica* trophozoites were solubilized by continuous vortexing for 4 min in ice-cold PBS containing 1% (v/v) Triton X-100 followed by centrifugation at 35 000 × *g* for 45 min to remove insoluble material. Solubilized protein was applied to the column, and unbound material was removed by washing the column with 20 bed-volumes of 0.1 M NaHCO$_3$, pH 8.3. The antigen was eluted with 0.05 M glycine, pH 2.5 containing 0.15 M NaCl and 0.1% (v/v) Triton X-100, and the pH of the eluates were immediately neutralized with 0.5 M Tris-Cl, pH 9.0 as the fractions eluted from the column. Protein concentrations were estimated using the BCA assay (Pierce) with bovine serum albumin (Fraction V; Sigma) as a standard. Antigen purity was assessed by silver-staining the gels (Wray *et al.*, 1981).

Immunoprecipitations were performed as previously described (Torian *et al.*, 1987). Trophozoite antigens were solubilized in solubilization buffer (0.01 M PBS, pH 8.0, 1% Nonidet P-40, 1% Aprotinin, 0.001 M PMSF) and immunoprecipitated with a pool of monoclonal antibodies (FP 4, FP 10, FP 21, and FP 37). Immunoprecipitations using live trophozoites were performed by incubating the amoebae with the pool of monoclonal antibodies for 1 h at 4°C to prevent antigen/antibody capping and shedding (Calderón and Avila, 1986). Trophozoites with bound monoclonal antibody were washed three times with 15 ml ice-cold PBS; then solubilization and immunoprecipitations were carried out as previously described (Torian *et al.*, 1987). During the antibody incubation procedure and subsequent washing steps, the membrane integrity of the trophozoites was closely monitored by Trypan blue exclusion.

Gel filtration chromatography

Purified protein (300 μg) was applied to a Sephacryl S-300 (Sigma) column equilibrated with 0.02 M Tris-Cl, pH 7.5 and 0.15 M NaCl. Each of the column fractions was tested by ELISA as previously described (Torian *et al.*, 1987) using FP 21 (1:2000 dilution) and antigen was detected with horseradish-peroxidase conjugated Protein A (1:2000 dilution; Zymed Laboratories). The molecular mass of the protein was estimated from a standard curve determined by using a calibration kit (Pharmacia) which consisted of thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa).

Reconstitution of the high-molecular-mass complex

Samples of immunoaffinity purified ^{35}S-labelled protein (3 × 10^3 c.p.m.; specific activity, 4 × 10^3 c.p.m. μg^{-1}) were reduced with 10% 2-ME. After heating at 100°C for 5 min, all samples were placed on ice and maintained at 4°C in the presence of excess reducing agent. Immediately prior to reconstitution of the high-molecular-mass complex, the reducing agent was removed by passing the sample through a Sephadex G-25 column (Pharmacia). After removal of 2-ME, the samples were incubated at 37°C for 60 min, 25°C for 60 min, or at 4°C for 60 min, 50 min, 40 min, 30 min, 20 min, 10 min, or 5 min. A control sample was reduced, then alkylated with 0.1 M iodoacetamide for 1 h at 4°C before reconstitution, as described above.

Electrophoresis and immunoblotting

Purified protein (2 μg) or a lysate of freshly harvested whole trophozoites (1 × 10^5 per lane) was electrophoresed on 7.5% or 10% SDS–PAGE gels in the presence or absence of a reducing agent in a discontinuous buffer system (Laemmli, 1970). Samples were denatured in Laemmli sample buffer and reduced with 2-ME, and then heated at 100°C for 5 min. Non-reduced samples were prepared similarly except in the absence of a reducing agent, and were electrophoresed on a separate gel to eliminate any possibility of the reducing agent diffusing into neighbouring lanes. After electrophoresis, gels containing unlabelled purified protein were silver stained and gels containing ^{35}S- or ^{14}C-labelled proteins were treated with En^{3}Hance® (Dupont) according to the manufacturer's recommendation, dried *in vacuo*, then subjected to fluorography. For detection of the protein from whole amoebic lysate, electrophoretically separated proteins were transferred to nitrocellulose membranes (Towbin *et al.*, 1979) and immunoblotted with purified monoclonal antibodies specific for the 29 kDa antigen (FP 14; 1:2000 dilution) and horseradish-peroxidase conjugated Protein A (1:3000). Blots were developed using enhanced chemiluminescence (ECL reagent) according to the recommendations of the manufacturer.

N-chlorosuccinimide peptide mapping

Purified ^{35}S-labelled protein samples (2.4×10^3 c.p.m. for control or 1.2×10^4 c.p.m. for NCS cleavage; specific activity, 4×10^3 c.p.m. μg^{-1}) were electrophoresed on SDS–polyacrylamide gels in the presence or absence of a reducing agent. After electrophoresis, gel slices corresponding to the positions of the reduced proteins or the non-reduced protein were excised and subjected to NCS cleavage in a urea-acetic acid system (Lischwe and Ochs, 1982). Samples treated in the urea–acetic acid buffer without NCS served as uncleaved controls. The gel slices were equilibrated in 1× Laemmli sample buffer containing 10% 2-ME , then subjected to SDS–PAGE (14% gel). The cleaved protein fragments were detected by fluorography.

Detection of cell surface accessible thiol groups

[^{14}C]-iodoacetamide (50 μCi; 5 μCi ml^{-1}; specific activity 21.1 mCi mmol^{-1}; Dupont) was added to a washed (1× in PBS) suspension of 10^7 live trophozoites and incubated at 4°C for 10 min with gentle rocking. Unbound radioisotope was removed by six 10 ml washes in ice-cold PBS. The final pellet was solubilized in buffer and the antigen was immunoprecipitated as described above. The membrane integrity of trophozoites was assessed by Trypan blue exclusion during each step of the procedure. Greater than 99% of the amoebae excluded the dye after the final wash. Alternatively, a sample containing 2×10^6 trophozoites was lysed by three cycles of freezing and thawing, then reacted with 10 μCi of [^{14}C]-iodoacetamide. The samples were electrophoresed on a 10% SDS–PAGE gel under reducing conditions, and subjected to fluorography as described above.

Acknowledgements

We gratefully acknowledge James C. K. Lai for critical review of this manuscript. This work was supported by Public Health Service Grants AI28188 (B.E.T.) and GM 34838 (D.L.D.) from the National Institutes of Health. Work by M.A.B. was performed at the Lawrence Livermore National Laboratory under the auspices of the US Department of Energy, contract W-7405-ENG-48.

References

Avila, E.E., Sanchez-Garza, M., and Calderón, J. (1985) *Entamoeba histolytica* and *E. invadens*: sulfhydryl-dependent proteolytic activity. *J Protozool* **32:** 163–166.

Calderón, J., and Avila, E.E. (1986) Antibody-induced caps in *Entamoeba histolytica*: isolation and electrophoretic analysis. *J Infect Dis* **153:** 927–932.

Chang, J.-Y. (1991) Stability of hirudin, a thrombin-specific inhibitor. *J Biol Chem* **266:** 10839–10843.

Dailey, H.A. (1984) Effect of sulfhydryl group modification on the activity of bovine ferrochelatase. *J Biol Chem* **259:** 2711–2715.

Diamond, L.S., Harlow, D.R., and Cunnick, C.C. (1978) A new medium for the axemic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans R Soc Trop Med Hyg* **72:** 431–432.

Fahey, R.C., Newton, G.L., Arrick, B., Overdank-Bogart, T., and Aley, S.B. (1984) *Entamoeba histolytica*: a eukaryote without glutathione metabolism. *Science* **224:** 70–72.

Gillin, F.D., and Diamond, L.S. (1981a) *Entamoeba histolytica* and *Giardia lamblia*: growth responses to reducing agents. *Exp Parasitol* **51:** 382–391.

Gillin, F.D., and Diamond, L.S. (1981b) *Entamoeba histolytica* and *Giardia lamblia*: effects of cysteine and oxygen tension on trophozoite attachment to glass and survival in culture media. *Exp Parasitol* **52:** 9–17.

Gillin, F.D., Reiner, D.S., Levy, R.B., and Henhart, P.A. (1984) Thiol groups on the surface of anaerobic parasitic protozoa. *Mol Biochem Parasitol* **13:** 1–12.

Keene, W.E., Petitt, M.G., Allen, S., and McKerrow, J.H. (1986) The major neutral proteinase of *Entamoeba histolytica*. *J Exp Med* **163:** 536–549.

Kosower, N.S., Kosower, E.M. Zipser, Y., Faltin, Z., and Shomrat, R. (1981) Dynamic changes of red cell membrane thiol groups followed by bimane fluorescent labeling. *Biochim Biophys Acta* **640:** 748–759.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lischwe, M.A., and Ochs, D. (1982) A new method for partial peptide mapping using *N*-chlorosuccinimide/urea and peptide silver staining in sodium dodecyl sulfate-polyacrylamide gels. *Anal Biochem* **127:** 453–457.

Massagué, J., and Czech, M.P. (1982) Role of disulfides in the subunit structure of the insulin receptor. *J Biol Chem* **257:** 6729–6738.

Matsumura, M., Signor, G., and Matthews, B.W. (1989) Substantial increase of protein stability by multiple disulphide bonds. *Nature* **342:** 291–293.

McLaughlin, J., and Faubert, G. (1977) Partial purification and some properties of a neutral sulfhydryl and an acid proteinase from *Entamoeba histolytica*. *Can J Microbiol* **23:** 420–425.

O'Toole, P.W., Logan, S.M., Kostryzynska, M., Wadström, T., and Trust, T.J. (1991) Isolation and biochemical and molecular analyses of a species-specific protein antigen from the gastric pathogen *Helicobacter pylori*. *J Bacteriol* **173:** 505–513.

Pace, C.N., Grimsley, G.R., Thompson, J.A., and Barrett, B.J. (1988) Conformational stability and activity of ribonuclease T_1 with zero, one, and two intact disulfide bonds. *J Biol Chem* **263:** 11820–11825.

Reed, S.L., Flores, B.M., Batzer, M.A., Stein, M.A., Stroeher, V.L., Carlton, J.E., Diedrich, D.L., and Torian, B.E. (1992) Molecular and cellular characterization of the 29-kilodalton peripheral membrane protein of *Entamoeba histolytica*: differentiation between pathogenic and nonpathogenic isolates. *Infect Immun* **60:** 542–549.

Ristow, S.S., Starkey, J.R., Stanford, D.R., Davis, W.C., and Brooks, C.G. (1985) Cell surface thiols, but not intracellular glutathione, are essential for cytolysis by a cloned murine natural killer cell line. *Immunol Invest* **14:** 401–414.

Ryser, H.J.-P., Mandel, R., and Ghani, F. (1991) Cell surface sulfhydryls are required for the cytotoxicity of diphtheria toxin but not of ricin in chinese hamster ovary cells. *J Biol Chem* **266:** 18439–18442.

Storz, G., Tartaglia, L.A., and Ames, B.N. (1990) Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248:** 189–194.

Tachibana, H., Ihara, S., Kobayashi, S., Kaneda, Y., Takeuchi, T., and Watanabe, Y. (1991) Differences in genomic DNA sequences between pathogenic and nonpathogenic isolates of *Entamoeba histolytica* identified by polymerase chain reaction. *J Clin Microbiol* **29:** 2234–2239.

Tartaglia, L.A., Storz, G., Brodsky, M.H., Lai, A., and Ames, B.N. (1990) Alkyl hydroperoxide reductase from *Salmonella typhimurium. J Biol Chem* **265:** 10535–10540.

Torian, B.E., Lukehart, S.A., and Stamm, W.E. (1987) Use of monoclonal antibodies to identify, characterize, and purify a 96,000-dalton surface antigen of pathogenic *Entamoeba histolytica. J Infect Dis* **156:** 334–343.

Torian, B.E., Flores, B.M., Stroeher, V.L., Hagen, F.S., and Stamm, W.E. (1990a) cDNA sequence analysis of a 29-kDa cysteine-rich surface antigen of pathogenic *Entamoeba histolytica. Proc Natl Acad Sci USA* **87:** 6358–6362.

Torian, B.E., Reed, S.L., Flores, B.W., Creely, C.M., Coward, J.E., Vial, K., and Stamm, W.E. (1990b) The 96-kilodalton antigen as an integral membrane protein in pathogenic *Entamoeba histolytica*: potential differences in pathogenic and nonpathogenic isolates. *Infect Immun* **58:** 753–760.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4354.

Walsh, J.A. (1988) Prevalence of *Entamoeba histolytica* infection. In *Amebiasis*. Ravdin, J.I. (ed.) New York: John Wiley and Sons, pp. 93–105.

Wilbur, W.J., and Lipman, D.J. (1983) Rapid similarity searches of nucleic acid and protein data banks. *Proc Natl Acad Sci USA* **80:** 726–730.

Wray, W., Boulikas, T., Wray, P., and Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels. *Anal Biochem* **118:** 197–203.

Xu, X., Koyama, N., Cui, M., Yamagishi, A., Nosoh, Y., and Oshima, T. (1991) Nucleotide sequence of the gene encoding NADH dehydrogenase from an alkalophile, *Bacillus* sp. strain YN-1. *J Biochem* **109:** 678–683.

Yamamoto, T., Matsui, Y., Natori, S., and Obinata, M. (1989) Cloning of a housekeeping-type gene (*MER5*) preferentially expressed in murine erythroleukemia cells. *Gene* **80:** 337–343.