

Characterization of a Secretory Type *Theileria parva* Glutaredoxin Homologue Identified by Novel Screening Procedure*

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The schizont stage of the protozoan parasite *Theileria parva* induces features characteristic of tumor cells in infected bovine T-cell lines. Most strikingly *T. parva*-infected cell lines acquire unlimited growth potential *in vitro*. Their proliferative state is entirely dependent on the presence of a viable parasite within the host cell cytoplasm. It has been postulated that parasite proteins either secreted into the host cell or expressed on the parasite surface membrane are involved in the parasite-host cell interaction. We used an *in vitro* transcription-translation-membrane translocation system to identify *T. parva*-derived cDNA clones encoding secretory or membrane proteins. Within 600 clones we found one encoding a 17-kDa protein which is processed by microsomal membranes to a 14-kDa protein (11E), presumably by signal peptidase. The processed form is expressed in the T-cell line TpM803 harboring viable parasites. By immunolocalization we show that the 11E protein mostly resides within the parasite, often in close vicinity to membranous structures, but in addition it appears at the surface membrane. Amino acid sequence comparison suggests that 11E belongs to the glutaredoxin family, but is unique so far in containing a signal sequence for endoplasmic reticulum membrane translocation.

Theileria parva is an obligate intracellular protozoan parasite that infects cattle and causes a usually fatal disease called East Coast Fever. The major pathogenic stage of the life cycle, the schizont, is found in the cytoplasm of infected bovine lymphocytes and replicates synchronously with the infected host cell. Infected lymphocytes undergo blast transformation and multiplication, resulting in a massive lympho-proliferation followed by lymphocytolysis and death of the host (for review, see Ref. 1). Schizont-containing cell lines can be maintained *in vitro* as permanently transformed lymphoblastoid cells exhibiting unlimited growth potential (reviewed in Ref. 2). Proliferation of *T. parva*-infected cell lines is accompanied by the presence of high levels of activated NF- κ B in the nucleus (3), the constitutive expression of high affinity IL-2¹ receptors and

the secretion of IL-2. After killing the parasite by antitheilericidal drug BW720c levels of activated NF- κ B decrease rapidly, expression of high affinity IL-2 receptor and IL-2 ceases, and the lymphocytes stop to proliferate.

This reversibility of the system implies that parasite factors, most likely proteins, induce and maintain host cell transformation. Parasite proteins, which directly interact with the host cell, have to be exported into the host or expressed on the parasite surface membrane. In eucaryotes most secretory and surface proteins use a common intracellular transport pathway. This pathway is entered by translocation of the nascent polypeptide across the ER membrane (4, 5). This entry step can be reconstituted in heterologous cell free translation systems (6, 7). For proteins of different protozoan parasites it has been shown that they can be translocated across microsomal membranes and processed by a signal peptidase (8–10).

We have employed an *in vitro* transcription-translation-translocation assay to identify secretory and membrane proteins of the *T. parva* schizont. 600 individual *T. parva* cDNA clones were expressed in the cell-free system and screened for translation products, which were translocated across microsomal membranes. One cDNA clone (plasmid 11E) was identified encoding a secretory type protein, which is synthesized as a 17-kDa precursor protein and becomes processed by microsomal membranes to the 14-kDa mature form (11E). Based on the amino acid sequence we predict a signal sequence of 24 amino acids (11). In a *T. parva*-infected bovine T-cell line (TpM803) only the 14-kDa form is expressed. By immunofluorescence staining we found 11E protein located within the parasite. Higher resolution analysis by immuno-electron microscopy suggests that 11E is predominantly associated with intraparasitic membrane structures and the surface membrane.

11E shows significant homology on the amino acid level to glutaredoxins including conservation of the active center motif. Glutaredoxins belong to a superfamily of thiol active oxidoreductases and have been defined by their ability to reduce glutathione mixed disulfides, which distinguishes them from the related thioredoxin family (reviewed in Ref. 12). Glutaredoxins are ubiquitously found in diverse organisms as *Escherichia coli* (13–15), yeast (16), plants (17), mammals (18–21), and even encoded by viruses (22–24). Glutaredoxins act *in vivo* as cofactor for ribonucleotide reductase (12, 14). Further physiological functions of glutaredoxins are assumed but less established, like cofactor function for other enzymes (25, 26), regeneration of cytoplasmic ascorbic acid (26), and restoration of oxidatively damaged proteins (27, 28).

Compared with the described glutaredoxin sequences *T. parva* 11E has a cleavable signal peptide for entering the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U48417 (clone 11E).

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¹ The abbreviations used are: IL-2, interleukin-2; ER, endoplasmic

reticulum; PK, protease K; RM, rough microsomes; TPA, phorbol 12-myristate 13 acetate; ConA, concanavalin A; PAGE, polyacrylamide gel electrophoresis.

classical secretory pathway and an additional N-terminal extension of 26 amino acids. So far no secreted glutaredoxin family member has been described. However, a human thioredoxin family member was shown to be secreted and involved in activation and transformation of virally infected lymphocytes (29, 30).

MATERIALS AND METHODS

Cell Culture—The characteristics and maintenance of the bovine lymphocyte cell line TpM803 persistently infected with *T. parva* Muguga have been described previously (31). TpM803 cells were cured of the parasite by culturing for 7 days in the presence of the theilericidal drug BW720c (Coopers Animal Health Ltd., Beckhamsted, United Kingdom) at a concentration of 50 ng/ml. For cultivation of cured TpM803 for more than 8 days, 10 units/ml of recombinant human IL-2 (Amersham Corp.) and 50 ng/ml TPA were added to the medium. Concanavalin A-stimulated bovine lymphocytes were cultured as described (32).

cDNA Library Construction—*T. parva* schizont poly(A)⁺ RNA was prepared from cell line TpM803 as described previously (32). cDNA was synthesized (Amersham synthesis kit), ligated to *Eco*RI linkers and inserted into plasmid vector pGem3 (Promega).

cDNA Screening by *in Vitro* Transcription-Translation-Translocation—The *E. coli* strain DH5 α was transformed by the *T. parva*-pGem3 cDNA library. Plasmid DNA of 600 randomly picked clones was digested with *Eco*RI, and the insert size was analyzed on 1% agarose gels. Clones containing inserts larger than 0.2 kilobase pair were analyzed further.

For *in vitro* transcription *T. parva*-pGem3 plasmids were linearized by *Sca*I and transcription was carried out in a 20- μ l volume containing 1 μ g of linearized DNA and 5 units of T7 or SP6 RNA polymerase at 37 °C for 1 h (33).

2 μ l of the transcription reaction was translated in wheat germ lysate in the presence of L-[³⁵S]methionine as described (14). For membrane translocation of synthesized polypeptides, rough microsomes derived from dog pancreas were included in the translation (7).

To test for membrane translocation of *in vitro* synthesized proteins across, or insertion into, the microsomal membrane accessibility to proteinase K (PK) was used. A 10- μ l aliquot of the translation mixture containing rough microsomes was incubated for 15 min at 25 °C with either 0.3 mg/ml of PK or 0.3 mg/ml of PK and 0.5% Nonidet P-40. Further proteolysis was stopped by addition of phenylmethylsulfonyl fluoride to 0.1 mg/ml. Samples were analyzed by SDS-PAGE and fluorography (34).

To remove secretory and peripheral membrane proteins, rough microsomes were subjected to carbonate extraction with 0.1 M Na₂CO₃, pH 11 (35).

Southern and Northern Blots—Genomic DNA from concanavalin A-stimulated lymphocytes, TpM803 cells, *T. parva* piroplasm was prepared according to standard procedures as was poly(A)⁺ RNA from infected and drug-treated TpM803 cells (32, 36). Genomic Southern and Northern blot analyses were performed in a standard fashion (37). Blots were washed for 30 min each in 2 \times SSC, 0.1% SDS at room temperature, 1 \times SSC, 0.1% SDS at 65 °C, 2 \times with 0.1 \times SSC at 65 °C.

DNA Sequencing and Analysis—The cDNA insert 11E was subcloned as a *Hind*III-*Pvu*II fragment into plasmids pUC18/19 (Life Technologies, Inc.) and sequenced. Nucleotide and amino acid sequence analysis and comparison were carried out using the GCG program and the GenBank™/EMBL data base (38).

Preparation of 11E Fusion Protein—A *Hind*III-*Pvu*II fragment of plasmid 11E-pGem3 encoding the C-terminal portion of protein 11E, starting with residue 56 was cloned into the *Sma*I site of plasmid pEX3. The bacterial β -galactosidase-11E fusion protein was prepared and gel-purified as described (39).

Antiserum Preparation—A female Chinchilla rabbit was injected intradermally with 200 μ g of the purified β -galactosidase-11E fusion protein, emulsified in complete Freund's adjuvant, on days 1, 9, 15, 22, and 43. Preimmune serum was collected on day 1 prior to immunization, and the immune serum used in the experiments was prepared on day 50.

Blot Adsorption—Antiserum 27 was depleted of antibodies toward β -galactosidase by adsorption to bacterial proteins blotted onto nitrocellulose membranes. pEX3-transformed *E. coli* cells were spread on LB agar plates, grown overnight at 30 °C to confluent layers, transferred to nitrocellulose membranes, then induced at 42 °C for 2 h and lysed. Bacterial proteins were blotted onto the nitrocellulose, DNase-treated, washed, and blocked for 1.5 h as for Western blots (39). Antiserum 27

was diluted 1:20 in 0.2% gelatin, phosphate-buffered saline, 0.1% Tween 20, 20 μ g/ml phenylmethylsulfonyl fluoride, 0.05% NaN₃ and incubated with the filters for 18 h at 4 °C. The solution was collected and lack of reactivity toward β -galactosidase was confirmed by Western blotting. Next, the lysate of TpM803 cells was separated on preparative 18% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The localization of the antigens specifically recognized by anti-serum 27 was determined by Western blotting with test strips using the β -galactosidase-depleted antibody fraction, the preimmune and immune serum 27 serving as controls. The region containing the 14-kDa antigen was excised, and the remainder of the membrane was incubated with the β -galactosidase-depleted antiserum as above. The adsorption to TpM803 cell blots, from which the 14-kDa 11E antigen had been removed, was repeated twice. For these experiments cell lysates were used in which the 10-kDa antigen was not detectable. The reactivity of the depleted serum was tested toward proteins of the TpM803 cell lysate by Western blotting, as above. The resultant antibody preparation reacted exclusively with the 14- and 10-kDa antigens in Western blots (see Fig. 5). The specificity of depleted antiserum 27 was controlled on Western blots by recognition of a glutathione *S*-transferase fusion protein containing the identical 11E C-terminal segment as in the β -galactosidase fusion protein (data not shown).

Metabolic Cell Labeling and Immunoprecipitation—TpM803 cells were sedimented, resuspended at 5 \times 10⁵ cells/ml in methionine/cysteine-free minimum essential medium supplemented with 10 mM HEPES, pH 7.4, 2 mM L-glutamine, 0.5% fetal calf serum, 3.75 MBq/ml each of L-[³⁵S]methionine and L-[³⁵S]cysteine and cultured for 18 h. Cells were washed with phosphate-buffered saline and lysed on ice with precooled buffer containing 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 20 μ g/ml phenylmethylsulfonyl fluoride, 1 μ M leupeptin, 0.3 μ M aprotinin, and 1 μ M pepstatin. Debris was pelleted by a 5-min spin in a microcentrifuge, and the resultant supernatant was clarified by ultracentrifugation for 1 h at 4 °C, 100,000 \times g. After posttranslational *in vitro* assays, antigens in a 50- μ l aliquot were solubilized by adding 2 volumes of cell lysis buffer. Samples were incubated with 5 μ l of preimmune or antiserum 27 for 2 h at 4 °C. Immunocomplexes were collected on 40 μ l of 50% (w/v) protein A-Sepharose slurry, washed, and prepared for SDS-PAGE as described (34).

Western Blots—Protein lysates of bacteria and lymphocytes were prepared, separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and probed with rabbit sera as described (34). Detection of the primary antibody was with peroxidase-coupled donkey anti-rabbit Ig (Amersham diluted 1:5000) and diaminobenzidine substrate reaction (40).

Immunofluorescence—Indirect staining and Hoechst 33258 staining of formaldehyde-fixed, permeabilized TpM803 cells with depleted antiserum 27 or preimmune serum was performed essentially as described (34). Briefly cells were washed with phosphate-buffered saline, centrifuged onto coverslips (Shandon cytofuge, 1000 rpm, 5 min), fixed, and incubated with depleted rabbit serum 27 diluted 1:10. Detection was with rhodamine-conjugated donkey F(ab')₂ anti-rabbit IgG (Dianova, Hamburg, diluted 1:400). For double staining with the DNA dye Hoechst 33258, 1 μ g/ml was included in the secondary reagent solution. Photographs were taken on a Zeiss Axioskop microscope using Fuji Neopan 1600 professional film.

Immuno-electron Microscopy—Cell suspensions were fixed for 1 h at room temperature in 4% glutaraldehyde, 0.5 mM calcium chloride, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The cells were centrifuged at 500 \times g for 5 min and processed according to (41), except that cells were dehydrated in ethanol and embedded in Lowicryl HM 20. Ultrathin sections were cut and immunolabeled with depleted immune serum 27 as described (42). Control sections were incubated in preimmune serum. Detection was with 5-nm colloidal gold particles conjugated with goat anti-rabbit IgG (Amersham) followed by silver enhancement (Amersham kit).

RESULTS

Identification of cDNA Clone 11E—We were interested in determining proteins which are directly involved in the interaction of *T. parva* and its host the bovine T-lymphocyte. Since the schizont stage of *T. parva* is obligate intracellular and cannot be cultured in a free form, we developed a general method to identify proteins which enter the secretory pathway of eucaryotic cells. Our screening system is based on cell-free expression of cDNA in an *in vitro* transcription-translation-

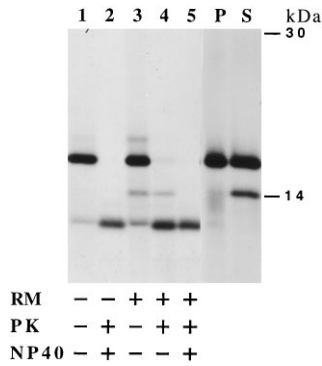


FIG. 1. Cell-free expression of cDNA clone 11E. 11E protein was synthesized in a wheat germ cell-free translation system in the absence (lanes 1 and 2) or presence (lanes 3–5) of dog pancreas microsomes (RM). Following translation, proteinase K (PK) was added to an aliquot of the reaction mixtures, either in the absence (lane 4) or in the presence (lanes 2 and 5) of nonionic detergent Nonidet P-40 (NP40). An aliquot of the reaction mixture shown in lane 3 was subjected to carbonate extraction. The pellet (P) and supernatant (S) were processed separately. Samples were reduced and separated by SDS-PAGE (15%). Molecular mass standards are indicated.

membrane translocation system. Proteins which fulfil the criterion of being translocated across ER-derived membranes are prime candidates to be exported or transported to the cell surface via the secretory transport route.

Screening about 600 clones of a schizont cDNA library we identified clone 11E, which upon cell-free expression encodes a protein product with the characteristics of a secretory protein (Fig. 1). The 11E transcript was translated into a polypeptide with apparent molecular mass of 17 kDa (lane 1). When translation was carried out in the presence of rough microsomes (RM), an additional form of 14 kDa size was observed (lane 3). Following addition of PK, the 17-kDa form was digested, whereas the 14-kDa form was protected from protease (lane 4). After solubilization of the microsomal membranes and PK treatment, the 14-kDa form was digested to a 10-kDa PK-resistant fragment (lane 5). Similarly bacterial chloramphenicol acetyltransferase has been shown to be partially resistant to PK digestion (43). We conclude that the primary translation product of 17 kDa was translocated across the microsomal membrane and processed to a 14-kDa form presumably by signal peptide cleavage.

11E transcript was translated in the presence of RM and membrane integration of the 14-kDa form was tested by carbonate extraction of salt-washed microsomes at pH 11 (35). The pellet fraction (P) and the supernatant fraction (S) were analyzed by SDS-PAGE (Fig. 1). The 14-kDa form was exclusively found in the S fraction, which indicates that this form is not integrated into the membrane and behaves like secretory type proteins. The 17-kDa form is found in both fractions. Its presence in the supernatant can be explained by polypeptide chains not translocated but loosely associated with the microsomes. Its presence in the pellet fraction is due to inefficient processing by signal peptidase as has been observed for other proteins (44).

cDNA Clone 11E Is Derived from the T. parva Genome and Expressed in T. parva-infected Lymphocytes—After having identified the cDNA clone 11E we wanted to show that it is *T. parva*-specific and not derived from the host cell. We performed Southern blot analysis with genomic DNA from concanavalin A-stimulated lymphocytes, a *T. parva*-infected T-cell line (TpM803), and *T. parva* piroplasms. As shown in Fig. 2, a single specific band was detected for TpM803 cells and piroplasms upon hybridization with the 11E cDNA probe. No signal was obtained with DNA from concanavalin A-stimulated bo-

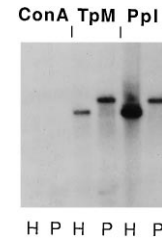


FIG. 2. cDNA clone 11E is derived from *T. parva*. 12.5 μ g of genomic DNA from ConA-stimulated bovine lymphocytes (ConA), *T. parva*-infected TpM803 cells (TpM) and *T. parva* piroplasms (Ppl) was digested with *Hind*III (H) or *Pst*I (P). Southern analysis was performed using radiolabeled 11E cDNA as a hybridization probe.

vine lymph node cells. DNA from isolated *T. parva* piroplasms was used to confirm that 11E gene was of parasite origin. Integrity of bovine genomic DNAs used in this experiment was controlled by hybridization with bovine actin cDNA (data not shown).

To show expression of the 11E gene we performed Northern analysis using 11E cDNA as hybridization probe (Fig. 3). Non-infected concanavalin A-stimulated bovine lymph node cells (ConA) were negative. A single transcript of 0.6 kilobase pair was detected in TpM803 cells (TpM). In TpM803 cells that had been cured from the parasite by treatment with the theilericidal drug BW720c (cured) as well as in cured TpM803 cells stimulated to proliferate with recombinant IL-2 and TPA (cured + IL2/TPA), no signal was obtained. Rehybridization of the stripped filter with bovine actin cDNA as a probe resulted in specific signals for all samples (data not shown). These results demonstrate that the 11E cDNA clone is of parasite origin and that the 11E gene is expressed in the schizont.

11E Is a Secretory Type Protein with Homology to Glutaredoxin—The 11E cDNA has a length of 574 nucleotides, including a poly(A) tail of 49 nucleotides. The *in vitro* translation product of 17 kDa can be assigned to an open reading frame encoding a polypeptide of 151 residues starting at nucleotide 14. Other open reading frames starting with an ATG codon are too short to encode a 17-kDa polypeptide. The deduced primary amino acid sequence, shown in Fig. 4, contains an amino-terminal signal peptide for ER translocation with a predicted cleavage site at cysteine 24 (11). The calculated molecular masses of the primary product of 17,528 and 14,853 Da of the processed form agree well with the apparent molecular masses of the cell-free translation products (see Fig. 1). Furthermore, when the cDNA is expressed in *E. coli*, two forms corresponding to the precursor product and the processed product are observed (data not shown).

The sequence of the processed 11E polypeptide can be divided into two parts. First, the carboxyl-terminal segment spanning residues 51–151 shows significant homology to glutaredoxins, a family of thiol-active oxidoreductases (reviewed in Ref. 12). The highest homology was found to glutaredoxin from calf thymus (*bovGlx*) (18). There was 37% identity over amino acids 51–151 of 11E. Nikkola and co-workers (44) have identified 29 highly conserved residues of glutaredoxins by sequence comparison. In the 11E sequence 15 of those are identical and 8 can be regarded as conservative changes (Fig. 4). Importantly, the active center motif Cys-Pro-Tyr-Cys is conserved in 11E. The two cysteines, assumed to form an intramolecular disulfide bridge in several mammalian family members, are absent in the 11E sequence like in the yeast glutaredoxin (16). Second, the amino-terminal region of processed 11E (residues 25–50) has no homology to described sequences and does not contain known sequence motifs.

Intracellular Processing and Expression of the 11E Protein in TpM803 Cells—In the cell-free expression system the 11E pro-

tein is processed by a signal peptidase to a 14-kDa form. If the same processing occurs *in vivo* the cellular protein and the cell-free expressed RM-processed form of 11E would have identical molecular weights. However, further modifications of 11E might occur *in vivo*. To test for signal peptide cleavage of the 11E protein in the schizont, its electrophoretic mobility in SDS-PAGE was compared with its *in vitro* synthesized, processed form (Fig. 5). 11E was translated *in vitro* both in the absence (not shown) and in the presence of RM. In the presence of microsomes the 17-kDa precursor was partially processed to the 14-kDa form (lane 1). An aliquot of the translation assay with RM, shown in lane 1, was treated with PK. The 17-kDa precursor form was digested, whereas the 14-kDa form was protected by microsomes (lane 2). To an aliquot of the translation assay with RM, which had been posttranslationally treated with PK (lane 2), detergent buffer was added to solubilize the microsomes. The detergent lysate was split and used for immunoprecipitation. The 14-kDa form could be specifically immunoprecipitated with antiserum 27, but not with the pre-

immune serum (lanes 3 and 4). Likewise, 11E antigen was immunoprecipitated with antiserum 27 from detergent lysates of metabolically labeled TpM803 cells (lane 6). The *in vitro* synthesized RM-processed 14-kDa form and the 11E antigen from schizont-infected cells had identical electrophoretic mobility (compare lanes 4 and 6). These results demonstrate that the 11E protein is proteolytically processed, most likely by a signal peptidase.

To corroborate the specificity of antiserum 27 we performed immunoblots using lysates of infected and cured TpM803 cells (Fig. 6). Three antigens with apparent molecular masses of 28, 14, and 10 kDa were specifically recognized by the immune serum (strip 2). The 14-kDa antigen corresponds well to the processed form synthesized *in vitro* and the antigen found in infected cells. The 10-kDa antigen is likely to be a degradation product rather than that generated by specific proteolytic processing, because it was observed in some experiments only. To determine whether the 28-kDa antigen represents a modified form of the 11E gene product, we depleted antiserum 27 of binding activities to antigens other than the 14-kDa antigen by sequential immunoabsorption (see "Materials and Methods"). The depleted antiserum fraction no longer recognized the 28-kDa antigen, but still reacted with the 14- and the 10-kDa antigens (strip 3), further supporting the assumption that the 10-kDa form is derived from the 14-kDa form. The loss of binding to the 28-kDa antigen indicates that it is not an unreduced 14-kDa homodimer and that recognition is due to cross-reactivity to another *T. parva* protein. Samples cured of the parasite showed that the expression of 28-, 14-, and 10-kDa antigens is specific for TpM803 cells (strips 4 and 5).

Subcellular Localization of the 11E Protein—To localize the secretory type protein 11E within the parasite-host cell system, we performed indirect immunofluorescence staining on formaldehyde-fixed, permeabilized TpM803 cells. As the primary antibody a 14-kDa antigen-specific preparation of antiserum 27 was used (compare with Western analysis, Fig. 6). This preparation reacted specifically with distinct subcellular regions of TpM803 cells (Fig. 7A). Double labeling with the DNA binding dye Hoechst 33258 revealed that the immunolabeled clusters always lay in the region of infected cells where the nuclei of the multinucleate schizonts localized (Fig. 7C). When cured TpM803 cells were analyzed we observed no region-specific immunostaining (Fig. 7B). Double labeling with Hoechst 33258 was used to confirm the loss of the parasite (Fig. 7D). Preimmune serum was used as a control and resulted in nonspecific background staining in infected and cured cells (data not shown).



FIG. 3. The 11E gene yields a single transcript in TpM803 cells. 2.5 μ g of poly(A)⁺ RNA from ConA-stimulated bovine lymphocytes (ConA), *T. parva*-infected TpM803 cells (TpM), and BW720c-treated TpM803 cells (cured), cultured either with or without IL-2 and TPA, was transferred onto nylon membrane (GeneScreen Plus, DuPont) and hybridized with radiolabeled 11E cDNA. The mobility of RNA size standards is indicated.

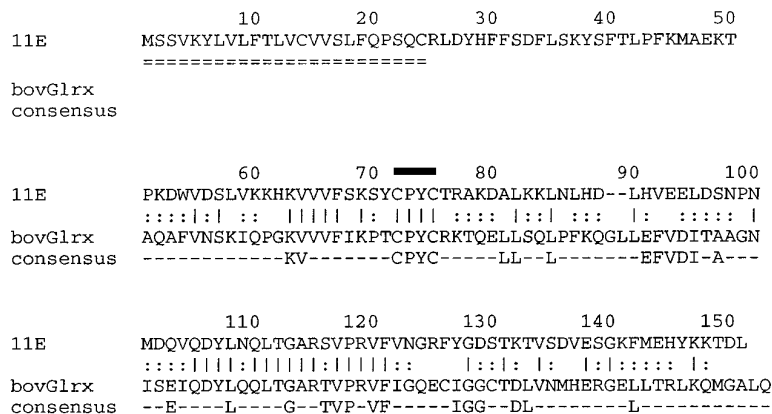


FIG. 4. Amino acid sequence of cDNA clone 11E. The primary sequence of the 151-residue polypeptide 11E encoded by the open reading frame from nucleotides 14 to 466 is aligned to the sequence of glutaredoxin from calf thymus (*bovGlxr*; Ref. 18). Vertical bars indicate amino acid identity, and colons indicate conservative amino acid changes. To obtain maximal alignment, a gap marked by dashes was introduced. The signal peptide is indicated by a broken double underline, and the active center motif of glutaredoxins is overlined. A glutaredoxin consensus sequence is shown in capital letters below (consensus; Ref. 44).

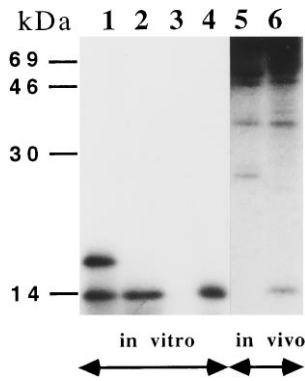


FIG. 5. **Immunoprecipitation of *in vitro* and TpM803 expressed 11E protein.** cDNA clone 11E was transcribed *in vitro* and translated in the presence of rough microsomes (lane 1). An aliquot of the reaction was treated with proteinase K (PK) (lane 2). PK-treated microsomes were solubilized and used for immunoprecipitation with either preimmune serum (lane 3) or immune serum (lane 4). Detergent lysates of metabolically labeled TpM803 cells were subjected to immunoprecipitation with either preimmune serum (lane 5) or immune serum (lane 6). Samples were analyzed by SDS-PAGE (18%) and fluorography. Molecular mass standards are indicated.

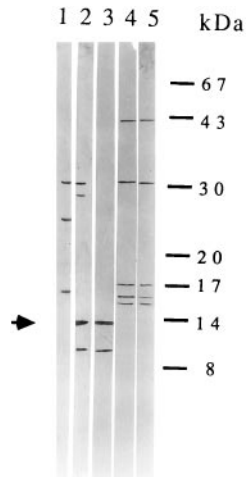


FIG. 6. **Cured TpM803 cells do not express 11E protein.** Lysates of 10^6 cells/lane were electrophoretically separated on an 18% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After staining with Ponceau S (Sigma), the lanes were cut off and probed with antisera as follows: TpM803 cells (lane 1) and BW720c-treated TpM803 cells (lane 4) with preimmune serum, diluted 1:200; TpM803 cells (lane 2) and BW720c-treated TpM803 cells (lane 5) with immune serum 27, diluted 1:200; and TpM803 cells (lane 3) with depleted immune serum 27. Detection of bound antibody was with horseradish peroxidase-conjugated secondary antibody and diaminobenzidine reaction. The arrow marks the mobility of the specifically recognized 14-kDa 11E antigen. Molecular mass standards are indicated.

It was not possible to deduce a clear subcellular localization from the immunofluorescence data. Therefore we analyzed the 11E antigen distribution by immuno-electron microscopy. Ultra-thin sections of TpM803 cells were fixed with glutaraldehyde/picric acid, embedded in Lowicryl HM 20, and labeled with depleted antiserum 27. Detection was with 5-nm gold particle-labeled secondary antibody and silver enhancement. Fig. 8A shows an overview of an infected cell in which the boundary of the schizont (*S*) can be clearly seen. Fig. 8B is taken from the same specimen and shows part of the schizont (*upper right* in A) at higher magnification. Specific labeling was observed only within the schizont, frequently at membranous structures, including the parasite surface. These membranous structures could not be ascribed to defined subcellular compartments, because the schizont lacks clear ultrastructural organi-

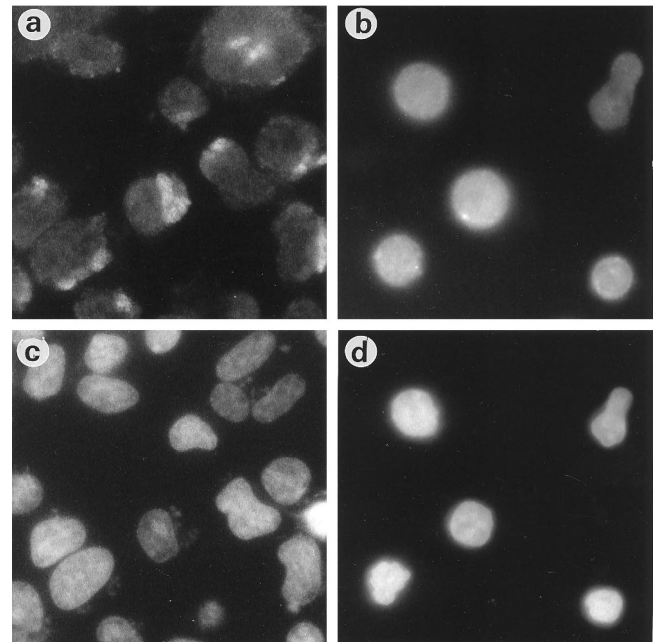


FIG. 7. **The 11E antigen is localized in the schizont.** Formaldehyde-fixed, detergent permeabilized, TpM 803 (A) or cured TpM803 cells treated with BW720c (B) were labeled by indirect fluorescence staining using depleted immune serum 27. C and D show double labeling of specimen A and B, respectively, with Hoechst dye 33258. Specimens were photographed using an automatic device (Wild MPS 45). Exposure times: 20 s (A), 90 s (B), 5 s (C), and 10 s (D).

zation recognizable by electron microscopy (45).

Taken together these data show that the 11E protein is localized in the schizont and is partially associated with intracellular and surface membranes.

DISCUSSION

Since the intracellular schizont stage of *T. parva* induces and maintains the proliferative phenotype of infected lymphocytes, and since it can only be prepared in a form suitable for mRNA isolation but not for analysis of exported proteins, we developed a novel approach to identify secretory and surface proteins. The screening strategy is based on the fact that the entry step of eucaryotic proteins into the secretory transport route, namely the translocation across the membrane of the ER, can be reconstituted and assayed in heterologous, cell-free, coupled translation-translocation systems. This procedure is not limited to our experimental system and may also be useful to identify secretory and surface proteins of other obligate intracellular pathogens or other systems where cells of interest cannot be propagated. Inherent in the technique is the selectivity for full-length cDNA clones since, except for some classes of transmembrane proteins, the signal sequence for ER translocation is in nearly all cases at the extreme amino terminus. However, there is the possibility that incomplete cDNA clones start with an internal transmembrane region that can mimic a functional signal sequence (46). There is a potential for false positives due to incomplete cDNA clones carrying cryptic signal sequences (47). This implies that for any protein identified by this screening procedure, its subcellular localization remains to be determined. So far we have analyzed 600 cDNA clones and obtained two candidates, which unequivocally fulfil the criterion of being translocated across microsomal membranes.

We have shown here that the 11E signal sequence is functional and cleaved off in a heterologous cell free system. Second, we have also demonstrated that the schizont expresses a processed 11E protein, which together with the subcellular localization data led us to conclude that the signal sequence

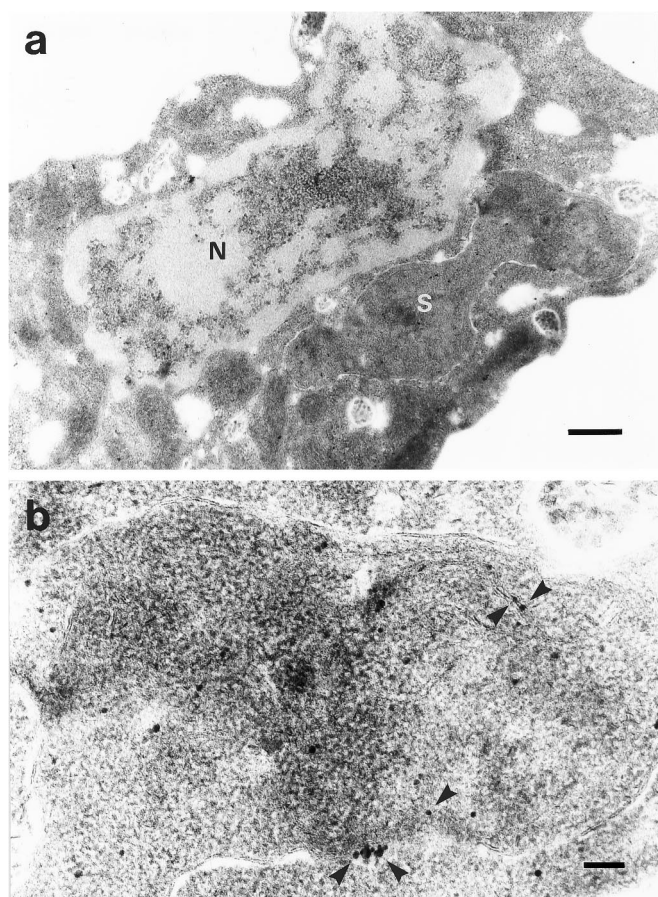


FIG. 8. Subcellular distribution of 11E antigen. TpM803 cells were fixed with glutaraldehyde/picric acid and embedded in Lowicryl HM 20. Ultra-thin sections were immunolabeled with depleted immune serum 27. Detection was with 5-nm colloidal gold-goat anti-rabbit IgG and silver enhancement. A shows a TpM803 cell; the host cell nucleus (N) and the schizont (S) are marked. B shows part of the schizont from the same specimen in higher magnification. Gold particularly localizes to membrane structures within the parasite and at the parasite cell surface (arrowheads). The scale bars represent 0.5 (A) or 0.1 (B) μm , respectively.

operates as topogenic signal in the schizont.

This study on the 11E protein provides direct evidence for the first time that in *T. parva* the classical secretory pathway is operating, as has been shown for other intracellular protozoans (*Trypanosoma brucei* (8); *Plasmodium lophurae* (9); *Plasmodium falciparum* (10); for review, *Toxoplasma gondii* (48) and references therein). Our biochemical evidence for a functional ER is important, since with microscopical techniques an ER-like structure is not detectable in the schizont stage, except when it differentiates at the beginning of merogony (45).

To date the reported glutaredoxin family members lack intracellular transport signals and reside in the cytoplasm. 11E is the first glutaredoxin-like protein described to enter the secretory transport pathway. Interestingly, the *T. parva* homologue of the catalytic subunit of casein kinase II appears to represent a similar case. In cells of other organisms the enzyme is usually found in the cytoplasm and nucleus. The cloned *T. parva* casein kinase II homologue is also preceded by a consensus signal peptide for ER translocation and an intervening sequence without homology to described proteins (49). It appears to be an attractive hypothesis that the schizont uses its secretory pathway to position parasite proteins in the host cell, which then can modulate the host cell physiology.

Various, partially overlapping, cellular functions have been ascribed to glutaredoxins and other members of the oxidoreduc-

tase superfamily they belong to (12, 25–28). It has to be shown whether the 11E protein has any of the described glutaredoxin or other oxidoreductase activities.

Other members belonging to the thiol active oxidoreductase superfamily exist, which are exported. In Gram-negative bacteria, some family members are either integral membrane proteins or bound to the periplasmic face of the inner membrane as part of an oligomeric protein complex (for review, see Ref. 50). Based on the localization of 11E protein, in close vicinity to membranous structures, it is conceivable that 11E protein participates in a membrane protein complex of the schizont. One would expect 11E to be exposed at the extracellular side of the schizont plasma membrane exerting its enzymatic activity toward the host cell cytoplasm.

Adult T-cell leukaemia-derived factor is a protein with more than 90% homology to human thioredoxin. It shows oxidoreductase activity and is secreted by a yet unknown mechanism. It has been shown to induce IL-2 receptor/tac antigen expression and to be involved in lymphocyte immortalization by human T-cell lymphotropic virus, type I and Epstein-Barr virus (29, 30, 51). Increased tac antigen expression is essential for continuous proliferation of *T. parva*-infected bovine lymphocytes (52). It is tempting to speculate that 11E might play a crucial role in the induction of IL-2 receptor expression in TpM803 cells.

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