Cloning and Expression of Mitochondrial Heat Shock Protein 70 of Trypanosoma congolense and Potential Use as a Diagnostic Antigen

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Received 27 December 2002/Returned for modification 17 April 2003/Accepted 12 June 2003

The ability to use mitochondrial heat shock protein 70 (MTP) of Trypanosoma congolense as a diagnostic antigen was examined. One cDNA clone was obtained by immunoscreening of a T. congolense procyclic form (PCF) cDNA library with monoclonal antibody (MAb) 10F9. The cDNA clone contained an open reading frame of 1,977 bp encoding a polypeptide consisting of 659 amino acids. Southern blotting analysis indicated that there were at least three copies of the MTP gene in the haploid genome. Interference of the MTP RNA resulted in complete inhibition, which indicated that MTP is essential at the PCF stage. Northern and Western blotting analyses revealed that MTP is expressed both in the bloodstream form (BSF) and in PCF. The B-cell epitope recognized by MAb 10F9 was located within 206 amino acids from the C terminus. Depending on the conditions of protein extraction, MTP was cleaved into smaller polypeptides by endogenous proteases. However, the C-terminal epitope of MTP was preserved with a high degree of antigenicity, even after cleavage. Antibody detection by enzyme-linked immunosorbent assay with the truncated recombinant MTP revealed that anti-MTP antibodies exist in experimentally infected mouse sera. Thus, MTP may be useful as an antigen for the serodiagnosis of primary T. congolense infection.

Heat shock protein 70 (HSP70) is one of the most highly conserved proteins in eukaryotic and prokaryotic cells. It has been reported that parasite-derived HSP70 plays an important role in the host-parasite interaction (15). An HSP70 of Toxoplasma gondii was reported to be highly antigenic and to induce autoantibodies against a host HSP70 during infection. Several studies indicated that this autoimmunity was related to the pathogenicity of T. gondii (2, 3). HSP70 of Trypanosoma cruzi, the causative agent of Chagas’ disease, was reported to be a major target of humoral immunity in human infections. Despite its high degree of evolutionary conservation, antibodies are highly specific for the parasite HSP70 and do not have cross-reactivity to the human HSP70 (6). In general, HSP70 possesses both a highly conserved N-terminal domain and a parasite-specific C-terminal domain. Thus, T. cruzi HSP70-specific antibodies are considered to recognize unique amino acid sequences in a C-terminal region. In general, HSP70 is expressed at most of all developmental stages of protozoan parasites and possesses parasite-specific antigenicity. Therefore, HSP70 might be a good candidate as a diagnostic antigen. The diagnosis of trypanosomiasis in mammalian hosts essentially relies on visualization of the parasites in blood. However, parasites are occasionally undetectable because of very low levels of parasitemia. Therefore, detection of antibodies against parasite antigens is required for accurate diagnosis. Most existing antibody detection tests are based on the use of trypanosome extracts as antigens (8), which precludes standardization and specific diagnosis. Recently, a serological method for the detection of Trypanosoma congolense with a truncated recombinant HSP70 (Bip homologue) was reported (1). However, since this method showed limited sensitivity for the detection of the organism in cattle with primary infections, more sensitive reagents are required for the accurate detection of primary infections.

African trypanosomes switch their metabolism in response to drastic environmental changes encountered during their life cycle. It is known that the mitochondrion of African trypanosomes in the long slender bloodstream form (BSF) lacks detectable cytochrome activity and that it is missing several key Krebs cycle enzymes. In this developmental stage, the parasite relies almost entirely on glycolysis for energy production. After uptake by the tsetse fly, the procyclic forms (PCFs) in the insect mid gut possess a fully developed mitochondrion and produce ATP by the Krebs cycle and oxidative phosphorylation in the mitochondrion. Thus, the proteins related to the Krebs cycle and oxidative phosphorylation are developmentally regulated in terms of their enzymatic activities and expression levels (4, 18). A mitochondrial HSP70 (MTP), whose amino acid sequence is distinguishable from those of cytosol HSP70 and Bip, is located in the matrix of a mitochondrion and is required for the translocation and refolding of nucleus-encoded mitochondrial matrix proteins. Because the MTP gene of African trypanosomes has not been cloned, the usefulness of recombinant MTP as a diagnostic antigen for African trypanosomiasis has not been clarified. Recently, we reported that monoclonal antibody (MAb) 10F9 recognizes a 76-kDa mitochondrial antigen in Trypanosoma brucei, T. congolense, and Trypanosoma evansi (11). In the present study, we cloned the MTP gene of T. congolense and clarified that a specific antigenic epitope is located in its C-terminal region. Moreover, we revealed that the C-terminal region of MTP is recognized by sera from mice with primary infection.
MATERIALS AND METHODS

Cloning and sequencing of mtp gene. MAbs 10F9, which recognizes a mitochondria-antigen of 76 kDa, was used for immunoscreening (11). A Uni-ZAP cDNA expression library constructed from T. congolense PCF mRNA was screened with MAbs 10F9 by using the picoBlue immunoscreening kit (Stratagene, La Jolla, Calif.). To clone the 5’ region of the MTP gene, PCRs with oligonucleotide primers specific for the consensus sequence of the spliced leader RNA (5’-ACGAGGTTCCTGACTATAT-3’) and the partial cDNA sequence of MTP were performed. The nucleic acid sequence was determined with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.).

Trypanosomes. T. congolense IL-3000 PCFs were grown in TVM-I medium as described by Hirumi and Hirumi (10). PCFs were obtained from the culture supernatant by centrifugation at 1,500 × g for 10 min at 4°C and washed three times with phosphate-buffered saline. BSFs were grown in male BALB/c mice (age, 8 to 10 weeks; CLEA Japan Inc., Tokyo, Japan). The mice were infected by intraperitoneal injection of BSFs (10 ⁷ parasites/ml, 0.1 ml/mouse). When the mice showed levels of parasitemia greater than 10 ⁸ parasites/ml, the infected blood was collected by cardiac puncture. The trypanosomes were purified by anion-exchange column chromatography (14). Then, the parasites were centrifuged and washed as described above.

Southern blotting analysis. Five micrograms of PCF genomic DNA was digested with restriction enzymes that cut at a single site within the probe-specific sequence (EcoRV and PstI) and with restriction enzymes that did not cut within the probe-specific sequence (XhoI and EcoRI). The DNA was electrophoresed on a 0.8% TAE (Tris-acetate-EDTA)-agarose gel and transferred to a nylon membrane (Hybond-N+; Amersham Biosciences, Little Chalfont, United Kingdom). The blot was probed with a 601-bp fragment (nucleotides 515 to 1,116) of the T. congolense MTP gene (nucleotides 736 to 1,329) under high-stringency conditions. The blot was also probed with a 18S rRNA-specific oligonucleotide (nucleotides 736 to 1,329) under high-stringency conditions. The probe was labeled by using AlkPhos direct labeling reagents (Amersham Biosciences) and unquenched. The membrane was washed several times. The probe-specific hybridization signal was visualized by using CDP-star (Amersham Biosciences) for 2 h at 37°C. The membrane was washed four times with with the washing solution (TBS with 0.05% Tween 20) and incubated at room temperature for 2 h. The plates were washed four times with the washing solution, and 100 μl of diluted serum (diluted 1:100 with TBS) was added to each well. After incubation at room temperature for 2 h, the plates were washed four times with the washing solution, and 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:2,000 with TBS) was added to each well. After incubation at room temperature for 30 min, 25 μl of stop solution (2 M NaOH) was added. Then, the absorbance at 415 nm was read.

Nucleotide sequence accession number. The sequence of the MTP gene of T. congolense is stored in the DDBJ database under accession no. AB096859.

RESULTS

MTP gene cloning and sequence determination. Immunoscreening of the T. congolense PCF cDNA expression library with MAbs 10F9 yielded a cDNA clone of 1,987 bp. It showed a high degree of homology to the mitochondrial HSP70 of T. cruzi (DDJ database accession no. M73627) but lacked homology at the 5’ end. By using a partial sequence of this truncated cDNA clone and the consensus sequence of the spliced leader RNA as primers, we obtained full-length MTP cDNA from the cDNA library. This cDNA clone was 2,805 bp in length with an open reading frame of 1,977 bp encoding a polypeptide of 659 amino acids. The similarities of the predicted amino acid sequence to the amino acid sequences of the MTPs of T. cruzi, Leishmania major, and humans were 88, 76, and 57%, respectively (Fig. 1). However, the similarity to the Bip homologue (or HSP70) of T. congolense was only 46% (Fig. 2). The N-terminal region of MTP contained a sequence highly homologous to the mitochondrial transportation signal peptide present in other trypanosomatid mitochondrial proteins (Fig. 1). Interestingly, there is a glutamine-rich region near the C terminus of MTP (Fig. 1).

Southern blotting analysis. Southern blotting analysis was performed to determine the copy number of the MTP gene. Treatment with enzymes that do not cut within the sequence

noglobulin G (IgG; Amersham Biosciences) for 1 h. The results were visualized by using 3,3-diaminobenzidine and H₂O₂.

Epitope mapping of MTP and expression of recombinant protein. Four truncated DNA fragments of the MTP gene were amplified by PCR with primers to which the EcoRI site was added (see Fig. 7A). These fragments were cloned into the EcoRI site of Escherichia coli expression vector pSET15 (Invitrogen, Carlsbad, Calif.). The vector was introduced into E. coli BL21(DE3)pLysS (Invitrogen), and the cells were incubated in SOB medium (2% [vol/vol] Bacto-tryptone, 0.5% [vol/vol] Bacto-yeast extract, 8.6 mM NaCl, 2.5 mM KCl) at 37°C in the presence of isopropyl-β-D-thiogalactopyranoside to express the partial MTP. The truncated recombinant MTP was purified by using the Pre Lloyd purification system (Invitrogen).

Detection of anti-MTP IgG in infected mouse sera by enzyme-linked immunoassay (ELISA). Serum samples from 10 female ICR mice (age, 12 weeks; CLEA Japan Inc.) experimentally infected with T. congolense IL-3000 and noninfected serum samples from healthy ICR mice were prepared as follows. Four mice were each infected with 1,000 cells of metacyclic forms (MCFs) by intraocular injection, and six mice were each infected with 1,000 cells of MCFs by subcutaneous injection. Infected mice were repeatedly treated with pentamidine (25 μg/dose/mouse) when parasitemia levels increased to >10⁷ parasites/ml. Each mouse was challenged with 10,000 BSFs at 30 days postinfection (DPI). Serum samples were collected from the tail vein at 30 and 60 DPI.

ELISA. The purified recombinant MTP was diluted with carbonate-bicarbonate buffer (pH 9.6; 10 μg/ml) and dispensed into the wells of flat-bottom 96-well microplates (Maxisorp; Nalge Nunc International, Rochester, N.Y.) in 100-μl aliquots. After incubation at 4°C for 24 h, unabsorbed MTP was discarded and 350 μl of blocking solution (TBS containing 5% skim milk) was added. After incubation at 37°C for 1 h, the blocking solution was discarded. The plates were washed four times with a washing solution (TBS with 0.05% Tween 20) and 100 μl of diluted serum (diluted 1:100 with TBS) was added to each well. After incubation at room temperature for 2 h, the plates were washed four times with the washing solution, and 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:2,000 with TBS) was added to each well. After incubation at room temperature for 30 min, 25 μl of stop solution (2 M NaOH) was added. Then, the absorbance at 415 nm was read.

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FIG. 1. Comparison of predicted amino acid sequences of MTP genes. Tco, Tcr, Lm, and Hu, MTP genes of T. congolense, T. cruzi, L. major, and humans, respectively; arrow, mitochondrial transportation signal peptide; double-headed arrow, glutamine-rich region in MTP.
consistent with the probe produced three bands (Fig. 3, lanes 1 and 2), and five or six bands were observed after treatment with enzymes that cut at a single position within the probe sequence (Fig. 3, lanes 3 and 4). These results indicate that there are at least three copies of the MTP gene in the haploid genome and that two of the genes are tandemly arranged.

Interference of MTP gene RNA in T. congolense PCFs. MTP gene expression was transiently knocked out to determine its function in T. congolense PCFs. T. congolense IL-3000:29-13 cells were transfected with p2T7Ti/MTP by electroporation. As controls, vectors with the /H9251-tubulin (DDBJ database accession no. AH001117) or the enhanced green fluorescent protein (DDBJ database accession no. AF323988) gene or without an insert were prepared. Cells transfected with p2T7Ti/H9251-tubulin were microscopically examined for the FAT phenotype due to the loss of a normal cytoskeletal structure in order to determine that the RNA interference system was working correctly (data not shown) (13). Cells transfected with the control vectors showed normal growth. In 48 h the number of cells increased more than 3.3-fold compared with the initial number of cells and gradually decreased after that. Cells that interfered with the expression of MTP were not able to increase in number at all (Fig. 4B).

Quantitative change of MTP expression during transformation from BSF to PCF in vitro. Northern blotting analysis was carried out to determine changes in the sizes and the quantities of the transcripts between BSFs and PCFs. The probe for MTP mRNA recognized a 2.4-kb transcript in the total RNA (Fig. 5), which indicated that there were no qualitative and quantitative changes in mRNA expression between BSFs and PCFs.

Western blotting analysis was performed to compare the protein expression levels between the two developmental stages of the parasite. MAb 10F9 recognized a single band of 70 kDa, which corresponded to the predicted size of the protein in BSFs. The relative intensities of the bands of 70 kDa were almost the same between the two life cycle stages, supporting the results of Northern blotting analysis. Interestingly, the number of bands increased after the transformation from BSF to PCF in vitro (Fig. 6). The sizes of the newly detected bands were 66, 55, 41, and 39 kDa. We speculated that these bands were due to proteolytic cleavage of the 70-kDa polypeptide. In order to confirm the instability of MTP, the following experiment was performed. A PCF extract was incubated with or without protease inhibitors at 4, 27, and 37°C for 30 to 120 min and was then subjected to Western blotting analysis. As a result, even in the presence of protease inhibitors, the small
fragments were observed after 2 h of incubation at all temperatures tested. However, the epitope of MTP recognized by MAb 10F9 was conserved even after incubation at 37°C for 2 h without protease inhibitors (data not shown).

Epitope mapping of MTP. Four truncated versions of MTP, named ΔMTP1, ΔMTP2, ΔMTP3, and ΔMTP4, were expressed in *E. coli* (Fig. 7A). Western blotting analysis with MAb 10F9 showed that the MAb recognized ΔMTP4, which was a C-terminal polypeptide consisting of 206 amino acids (Fig. 7B). Therefore, ΔMTP4 was purified for use as an antigen in the antibody capture ELISA detection system.

Detection of anti-ΔMTP4 antibodies in infected mouse sera. To evaluate whether ΔMTP4 can be a suitable diagnostic antigen for the detection of *T. congolense* infection, purified ΔMTP4 was used as an antibody capture antigen for ELISA and Western blotting. As shown in Fig. 8, the absorbances of all serum samples from mice experimentally infected with *T. congolense* were significantly higher than those of serum samples from healthy mice (*P* < 0.01). The anti-ΔMTP4 antibody was not detected by Western blotting analysis (data not shown), probably due to the lower sensitivity of Western blotting compared with that of the ELISA.

DISCUSSION

We have cloned the *T. congolense* MTP gene and determined its entire nucleic acid sequence. Southern blotting analysis demonstrated that the haploid genome contains at least three copies of the MTP gene and that two of the genes may be closely arranged on the same chromosome. The open reading frame encodes a polypeptide of 659 amino acids that is highly homologous to a series of MTPs in eukaryotes and DnaK of *E. coli* (5, 7, 17). The N-terminal region of MTP contained a sequence highly homologous to the mitochondrial transport signal sequences demonstrated in other trypanosomatid mitochondrial proteins (Fig. 1). The transportation signal sequence for some mitochondrial matrix proteins in trypanosomes appears to be relatively short (7 to 9 amino acids or less, according to a recent report [9]), but the length of the signal peptide of *T. congolense* MTP is as long as that of *T. brucei* MTP (15 to 20 amino acids). Interestingly, there is a glutamine-rich region near the C terminus of MTP that is not present in *L. major* and human MTPs. Although the function and biological importance of the glutamine-rich region are not yet understood, the region is unique in *T. congolense*. Therefore, the region may be the parasite-specific epitope in MTP.

One of the striking characteristics of trypanosomes is the flexibility of their energy metabolism in response to the vari-
able environmental changes encountered during their life cycle. It has been reported that the levels of expression of ATP synthase subunits α, β, and 9, which are nucleus-encoded mitochondrial proteins, are regulated during the life cycle by differential mRNA stability. The levels of expression of the genes for these proteins are lower in BSFs than in PCFs, reflecting the switching of the metabolic system (4, 18). Since MTP is also one of the mitochondrial matrix proteins, the MTP expression level might be lower in BSFs if the level of expression of the MTP gene is regulated during the life cycle. Northern blotting and Western blotting analyses were carried out to determine the level of MTP expression in the BSF stage. The

![FIG. 5. Northern blotting analysis of MTP transcript. Five micrograms of total RNA from PCFs (lanes P) and BSFs (lanes B) was electrophoresed and transferred to a nylon membrane. (A) Comparison of MTP transcripts of PCFs and BSFs. The membrane was probed with the 593-bp fragment of the MTP gene. (B) The membrane was probed with the 590-bp fragment of 18S rRNA gene as a control.](http://cvi.asm.org/)

![FIG. 6. Western blotting analysis of the quantitative change in MTP expression during parasite transformation. Protein samples were collected at the indicated times after the initiation of the in vitro culture (day 0 [BSF], day 1 [D1], day 2 [D2], day 3 [D3], day 7 [D7], and day 14 [PCF]). Eight micrograms of protein samples was electrophoresed on an SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed with MAb 10F9.](http://cvi.asm.org/)

![FIG. 7. Epitope mapping of MTP. (A) Schematic diagram of the locations of four DNA fragments inserted and expressed for determination of the epitope. 1, 2, 3, and 4, ΔMTP1, ΔMTP2, ΔMTP3, and ΔMTP4, respectively. The numbers in parentheses indicate the nucleic acid positions in the MTP gene sequence. (B) Western blotting analysis of recombinant ΔMTPs. ΔMTPs (lanes 1, 2, 3, and 4, ΔMTP1, ΔMTP2, ΔMTP3, and ΔMTP4, respectively) were electrophoresed on an SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed with MAb 10F9. Whole trypanosome extract was electrophoresed in lane C. ORF, open reading frame.](http://cvi.asm.org/)
results showed that there were no significant differences in transcription and translation levels between BSFs and PCFs. This indicates that MTP is equally required by both PCFs and BSFs, although cytochrome-mediated respiration and oxidative phosphorylation are not active in the latter form. Since the level of MTP expression in BSFs is the same level as that in PCFs, host immune responses could be directed to MTP.

Specific antibodies against MTP might not effectively inhibit the MTP function because MTP is not accessible to the antibody. However, if MTP could induce antibody production in the infected host, it would be a candidate as a diagnostic antigen. Therefore, we examined an antigenic epitope of MTP in infected mouse sera by ELISA. Four mice were each infected with 1,000 MCFs by the intra-peritoneal route (circles), and six mice were each infected with 1,000 MCFs via the subcutaneous route (triangles). When parasitemia levels increased to >10^7 parasites/ml, the mice were treated two to seven times with pentamidine (25 μg/dose/mouse). At 30 DPI, each mouse was challenged with 10,000 MCFs via the same route by which primary infection was initiated. Negative control sera were obtained from three mice (squares).

![Graph](http://cvi.asm.org)  
**FIG. 8.** Detection of anti-ΔMTP4 IgG in infected mouse sera by ELISA. Four mice were each infected with 1,000 MCFs by the intra-peritoneal route (circles), and six mice were each infected with 1,000 MCFs via the subcutaneous route (triangles). When parasitemia levels increased to >10^7 parasites/ml, the mice were treated two to seven times with pentamidine (25 μg/dose/mouse). At 30 DPI, each mouse was challenged with 10,000 MCFs via the same route by which primary infection was initiated. Negative control sera were obtained from three mice (squares).

of antibody to ΔMTP4 seemed to be lower at 60 DPI than at 30 DPI, but in six mice the antibody levels increased from 30 to 60 DPI, while the antibody levels decreased in the other four mice. The mice were infected with *T. congolense* MCFs by either the intraperitoneal or the subcutaneous route. Subcutaneous injection of MCFs mimics natural infection by a tsetse fly bite, while intraperitoneal injection is commonly used to induce experimental infection. However, the site of injection did not make a difference in the antibody response pattern. Although we could not clearly demonstrate in this study how early during the infection the antibody to ΔMTP4 became detectable, the result clearly indicated that ΔMTP4 was useful for detection of the primary stage of *T. congolense* infection by ELISA. As the sera used in the test were obtained from experimentally infected mice, further evaluation of ΔMTP4 with sera from naturally infected animals is required.

ACKNOWLEDGMENTS

We thank John E. Donelson (University of Iowa, Ames) for providing plasmids pLEW13, pLEW29, and pZT75 and for advice on the experiment. We are grateful to Kim Jeong-Yon and Warren Baticados of the National Research Center for Protozoan Diseases (NRCPD) for technical support. We also thank Naoki Yokoyama of NRCPD for helpful suggestions and criticism.

This work was supported by a Grant-in-Aid for Scientific Research (grant 13356048) to N.I. from the Japan Society for the Promotion of Science.

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