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Secretion by *Trypanosoma cruzi* of a peptidyl-prolyl *cis–trans* isomerase involved in cell infection

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Macrophage infectivity potentiators are membrane proteins described as virulence factors in bacterial intracellular parasites, such as *Legionella* and *Chlamydia*. These factors share amino acid homology to eukaryotic peptidyl-prolyl *cis–trans* isomerases that are inhibited by FK506, an inhibitor of signal transduction in mammalian cells with potent immunosuppressor activity. We report here the characterization of a protein released into the culture medium by the infective stage of the protozoan intracellular parasite *Trypanosoma cruzi*. The protein possesses a peptidyl-prolyl *cis–trans* isomerase activity that is inhibited by FK506 and its non-immunosuppressing derivative L-685,818. The corresponding gene presents sequence homology with bacterial macrophage infectivity potentiators. The addition of the protein, produced heterologously in *Escherichia coli*, to cultures of trypomastigotes and simian epithelial or HeLa cells enhances invasion of the mammalian cells by the parasites. Antibodies raised in mice against the *T.cruzi* isomerase greatly reduce infectivity. A similar reduction of infectivity is obtained by addition to the cultures of FK506 and L-685,818. We concluded that the *T.cruzi* isomerase is involved in cell invasion.

Key words: host cell invasion/PPIase/signal transduction/ *Trypanosoma cruzi*

Introduction

The protozoan parasite *Trypanosoma cruzi* is the etiological agent of Chagas' disease, or American trypanosomiasis, which afflicts millions of people in Central and South America. The life cycle of the parasite comprises several distinct developmental stages (De Souza, 1984). Amastigotes and epimastigotes are replicative forms found, respectively, in the cytoplasm of mammalian cells and in the midgut of the insect vectors (Family Reduviidae). Trypomastigotes are non-dividing forms of the parasite responsible for its infectivity to a wide spectrum of mammalian cells. Like all members of the Family Trypanosomatidae, *T.cruzi* exhibits a number of distinct biochemical and cellular properties. Polycistronic transcription (González *et al.*, 1985; Muhich and Boothroyd, 1988), *trans*-splicing (Murphy *et al.*, 1986) or mitochondrial RNA editing (Simpson and Shaw, 1989) are phenomena that, together with the presence of glycosomes (Michels and Opperdoes, 1991) and a unique kinetoplast-containing mitochondrion (Brener, 1973), attest to the ancient evolutionary divergence of these organisms.

The process of entry of intracellular parasites into their host cells involves complex interactions between them (reviewed in Bliska *et al.*, 1993). The target cell is rendered susceptible to penetration, be it passive or active, by a series of events that start with the production by the parasite of so-called virulence factors. The active entry of *T.cruzi* into target cells within its mammalian host requires the recruitment of lysosomes from the perinuclear area, a crucial step in the formation of a parasite-containing vacuole (Schenkman *et al.*, 1991; Tardieux *et al.*, 1992). Active invasion implies the mobilization of Ca^{2+} in the parasite (Moreno *et al.*, 1994) and the target cell (Tardieux *et al.*, 1994). However, the molecules that mediate these events remain to be characterized.

FK506 binding proteins (FKBP; Standaert *et al.*, 1990; Tropschug *et al.*, 1990) are peptidyl-prolyl *cis–trans* isomerases, and this activity is inhibited by the macrolide antibiotic FK506. They belong to the group of the immunophilins, rotamases that accelerate protein folding (Schmid, 1993). The FK506–FKBP complex has immunosuppressive activity, but this is unrelated to the concomitant inhibition of the isomerase activity (Schreiber, 1991). The FK506–FKBP complex interacts with calcineurin, a calmodulin-regulated phosphatase necessary for a variety of signal transduction mechanisms (Liu *et al.*, 1991). A class of FKBPs is bacterial membrane proteins, generically known as macrophage infectivity potentiators (MIP; Fischer *et al.*, 1992; reviewed in Hacker and Fischer, 1993), that have been shown to be virulence factors in the bacterial intracellular pathogens *Legionella* and *Chlamydia* (Cianciotto *et al.*, 1989; Lundemose *et al.*, 1991). Although the mechanisms of action of the MIPs remain to be clarified, they must contribute to the invasion of the host; deletion of the corresponding gene in *Legionella* causes a significant reduction of *in vitro* infectivity, but does not affect the rate of intracellular replication (Cianciotto *et al.*, 1989).

We have found that *T.cruzi* trypomastigotes secrete a MIP protein (TcMIP), cloned the corresponding gene, expressed TcMIP in *Escherichia coli*, characterized its enzymatic activity and demonstrated its role as an invasion factor.

Results

Cloning, sequence, transcription and genomic organization of the TcMIP gene

Whilst sequencing the upstream region of a *T.cruzi* gene homologous to cystatin an additional open reading frame

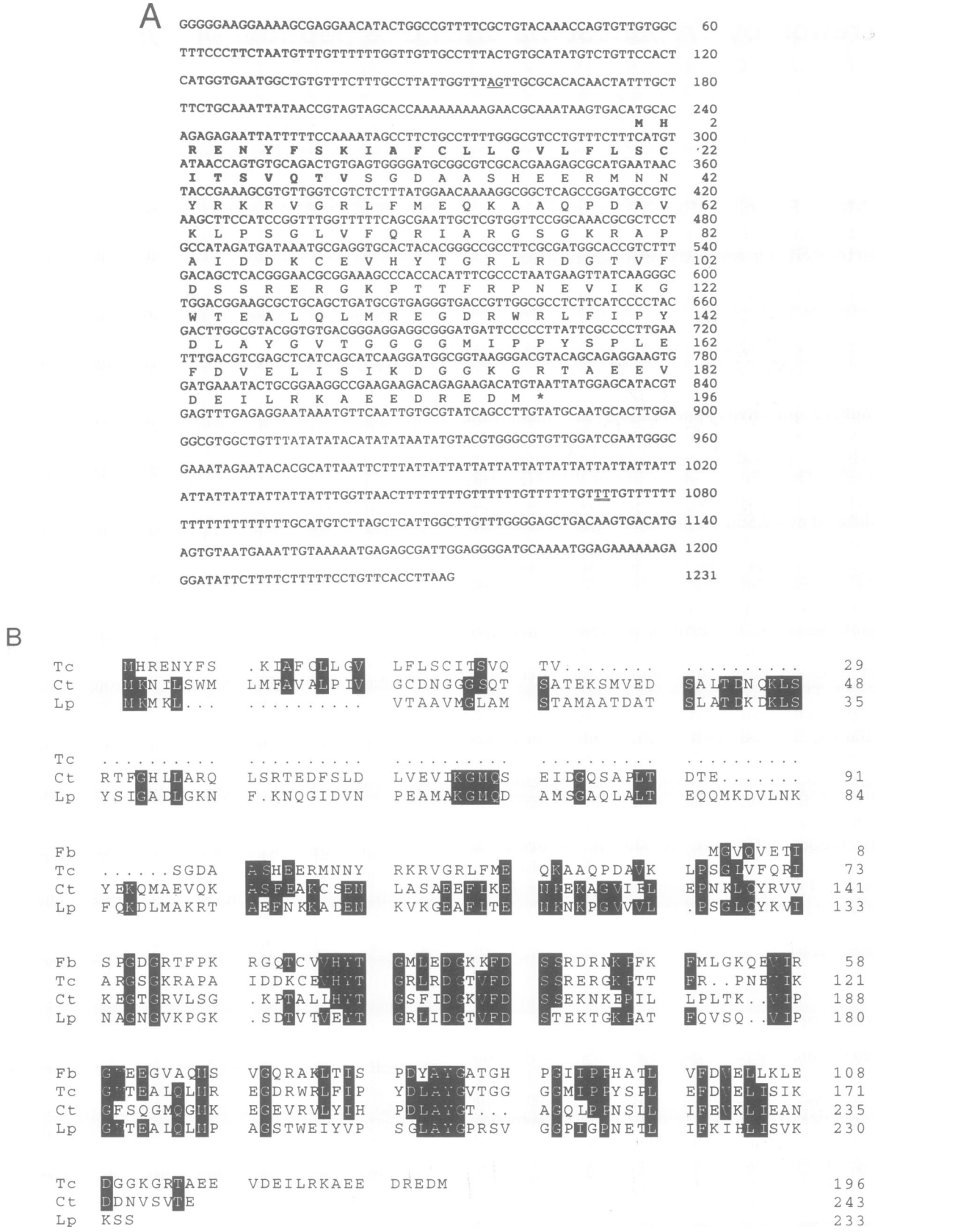

Fig. 1. (**A**) DNA and derived amino acid sequence of the TcMIP gene. *Trans*-splicing acceptor and polyadenylation sites appear underlined and double underlined respectively. Computer-predicted leader peptide sequence is marked in bold. (**B**) Amino acid homology of TcMIP (Tc) to *Legionella pneumoniae* (Lp), *Chlamydia trachomatis* (Ct) MIPs and human FKBP-12 (Fb). Boxes indicate identical residues; dots indicate gaps generated for best fit.

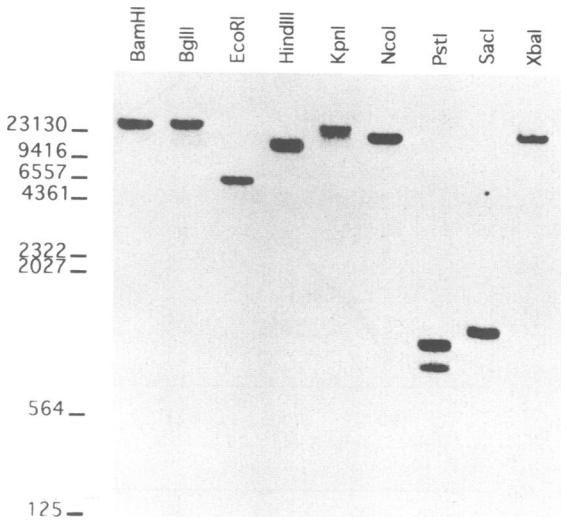

Fig. 2. Southern blot of *T.cruzi* DNA with a probe specific for TcMIP. Size markers are λ phage DNA digested with *Hin*dIII (Promega).

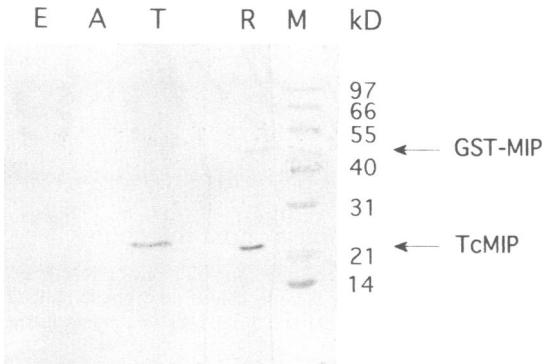

Fig. 3. Western blot of supernatants of epimastigote (E), 48 h amastigote (A) and trypomastigote (T) forms of *T.cruzi* with TcMIP affinity-purified anti-GST–TcMIP. Lane R, recombinant TcMIP, as well as the uncut fusion product GST–TcMIP. Protein molecular weight markers are shown in the right-hand lane (M) and their sizes are indicated in kDa.

Table I. ELISA of culture supernatants for TcMIP

Supernatant	OD_{405} ± SD		
	Anti-TcCMIP	Anti-TC-TOX	Rib.-prot.[a]
Epimastigotes	0.082 ± 0.001	0.060 ± 0.005	0.032 ± 0.008
Epimastigotes + 50 mM NaN_3	0.097 ± 0.003	0.030 ± 0.002	0.040 ± 0.001
Trypomastigotes	0.414 ± 0.008	0.210 ± 0.006	0.007 ± 0.067
Trypomastigotes + 50 mM NaN_3	0.247 ± 0.001	0.100 ± 0.013	0.008 ± 0.001

ELISA detection of TcMIP, TC-TOX and ribosomal proteins (as control of parasite integrity) in supernatants of trypomastigotes and epimastigotes in the presence or absence of 50 mM sodium azide.
[a]Rat anti-ribosomal protein (total) from *T.cruzi*. Values are corrected for background readings.

was identified. Computer analysis revealed homology with MIPs from *Legionella* (Cianciotto and Fields, 1992) and *Chlamydia* (Lundemose *et al.*, 1991), as well as human FKBP (Standaert *et al.*, 1990). The identities were 25.5%

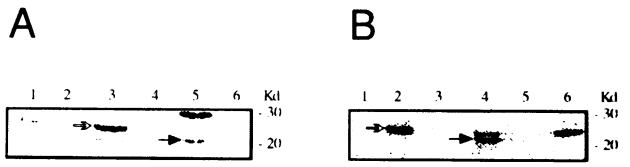

Fig. 4. Processing of TcMIP. (**A**) A Western blot (developed with mouse anti-GST–TcMIP). (**B**) An autoradiogram of the same filter, in which *in vitro*- and *in vivo*-synthesized TcMIP was run. Lane 1, uninduced *E.coli* transformants; lane 2, *in vitro*-synthesized TcMIP without canine microsomes (B, white arrow); lane 3, induced transformants (A, white arrow); lane 4, *in vitro*-synthesized TcMIP in the presence of canine microsomes [note the drop in molecular weight (B, black arrow), probably corresponding to the cleavage of the signal peptide]; lane 5, GST–TcMIP fusion protein cut with thrombin (A, black arrow; the upper band is GST and the lower is TcMIP without the leader peptide); lane 6, same as lane 2.

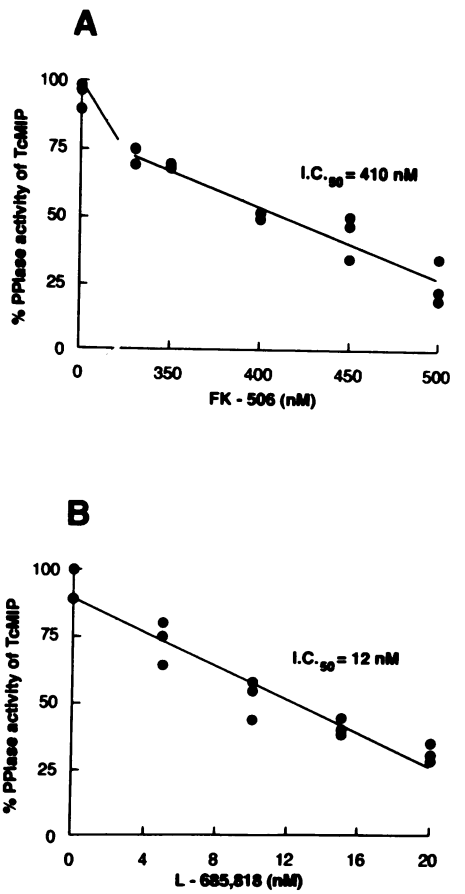

Fig. 5. Percent inhibition of *cis–trans* isomerization of the Leu–Pro bond by increasing concentrations of (**A**) FK506 and (**B**) L-685,818. First-order rate constants (K_{obs}) were calculated by monitoring the time course of the absorbance at 390 nm (as in Fischer *et al.*, 1989). TcMIP concentration in both assays was 33.1 nM, 100% activity corresponding to K_{obs} = 0.03949/s. The 50% inhibitory concentrations (IC_{50}) for FK506 and L-685,818 are indicated on the plots.

with MIP from *Chlamydia* and human FKBP12, and 29.6% with MIP from *Legionella*.

The new open reading frame starts 1498 nucleotides upstream of the cystatin-like gene, contains 196 codons (Figure 1A) and shares some of the features of MIP proteins. Computer analysis predicted a 29-amino acid signal peptide. A distinctive structural feature of MIPs is an intermediate sequence between the prokaryotic signal peptide and the region homologous to eukaryotic FKBPs.

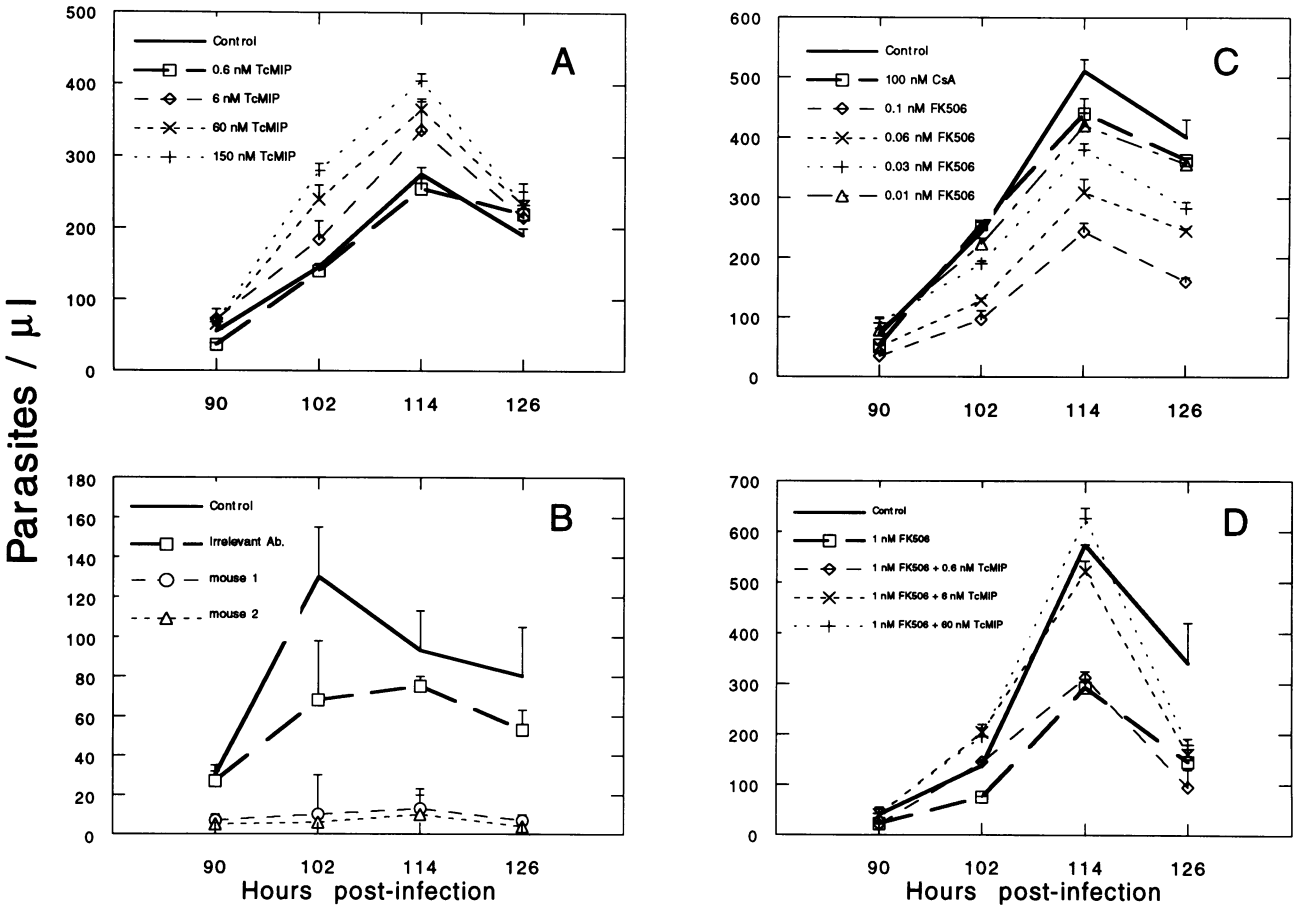

Fig. 6. Release of *T.cruzi* cells by infected monolayers of simian epithelial cells. (**A**) Dose–response of increasing concentrations of TcMIP on infection of monolayers. (**B**) Effect of mouse anti-TcMIP antibodies on parasite production. Trypanosomes were incubated for 1 h with antibodies (1:20 dilution) in DMEM and then infection was carried out for 3 h (in the presence of antibodies). Mouse 1 and 2 indicates sera obtained from two mice. (**C**) Effect of cyclosporin A and FK506. (**D**) Antagonistic effect of TcMIP and FK506.

The TcMIP sequence between the leader peptide and the beginning of homology to human FKBP (amino acid 66) is shorter than, and not homologous to, the bacterial intermediate sequence (Figure 1B). The exclusive C-terminus of TcMIP is very polar (12 charged amino acids out of 19).

The TcMIP transcript was fully characterized. The mini-exon added by *trans*-splicing at the 5′ end of all trypanosomatid mRNAs (Agabian, 1990) is added to the TcMIP transcript at position 159 (Figure 1A, single underlined AG). Polyadenylation takes place at position 1070 or 1071 (double underlined TT). The untranslated 3′ end of the transcript includes the triplet UUA repeated 17 times, a motif found at the 3′ end of *T.cruzi* hsp85 mRNA (Dragon *et al.*, 1987), as well as runs of U residues that might be involved in the regulation of mRNA abundance.

A Southern blot (Figure 2) showed the presence of a single copy of the MIP gene in the *T.cruzi* genome. Most known genes of this organism are repeated in clusters of contiguous copies (García-Salcedo *et al.*, 1994).

TcMIP production in the culture medium of T.cruzi

To explore the expression of TcMIP, Western blots of epimastigote, amastigote and trypomastigote culture media were assayed with mouse antibodies raised against a chimeric protein of glutathione-*S*-transferase (GST) and

Table II. Effect of TcMIP, FK506 and L-685,818 on penetration of trypomastigotes into HeLa cells

Treatment of parasites	Intracellular parasites per 200 cells ± SD
Ringer's	88.5 ± 5.7
Ringer's + methanol	87.9 ± 25.1
2 nM FK506	37.9 ± 5.6
10 nM TcMIP	158.9 ± 30.8
2 nM L-685,818	67.0 ± 18.4

TcMIP. Trypomastigote culture medium contained a protein of the expected molecular weight of ~22 kDa that was recognized by these antibodies (Figure 3). Similarly, in an ELISA, rabbit anti-GST-TcMIP antibodies detect TcMIP in trypomastigote-, but not epimastigote-, conditioned medium (Table I). Both results clearly indicate that TcMIP is secreted by the infective stage of the parasite. The treatment of parasites with 50 mM NaN_3 markedly diminishes the levels of TcMIP in the supernatants, indicating that metabolically active parasites are necessary for TcMIP release into the culture medium, just as for the release of the hemolysin TC-TOX (Andrews and Whitlow, 1989). On the other hand, ribosomal proteins are not detected by the corresponding antiserum in NaN_3-treated or untreated culture supernatants, revealing that TcMIP

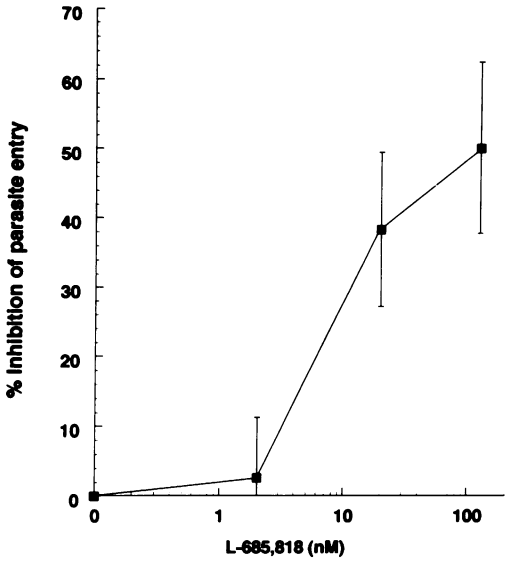

Fig. 7. Inhibitory effect of increasing concentrations of L-685,818 on trypomastigote penetration of HeLa cells.

appearance is not a consequence of parasite rupture under culture conditions (Table I).

Brefeldin A, an inhibitor of vesicle transport and secretion (Lippincot-Schwartz *et al.*, 1991), has no effect on the levels of TcMIP in supernatants (results not shown). This indicates that the protein is either shed or secreted by mechanisms not affected by the drug. The release of TcMIP suggests that a putative target molecule for the rotamase activity is outside the parasite, i.e. on the host cell membrane.

The signal peptide predicted by computer analysis is likely to be processed *in vivo*. TcMIP produced *in vitro* in the presence of canine microsomes was slightly smaller than that produced in *E.coli* or translated *in vitro* in the absence of microsomes (Figure 4).

Determination of PPIase activity of TcMIP

The PPIase activity of the recombinant protein was determined by the isomer-dependent proteolytic release of 4-nitroanilide from the peptide Suc-Ala-Leu-Pro-Phe-4-nitroanilide by α-chymotrypsin (Fischer *et al.*, 1989). In the presence of an excess of α-chymotrypsin to perform rapid cleavage of the *trans* substrate, the *cis* isomer remains uncleaved due to the inability of α-chymotrypsin to split off the 4-nitroanilide moiety in the *cis* conformer. The *cis* form undergoes slow spontaneous isomerization; in the presence of TcMIP, the slow kinetic phase of the appearance of 4-nitroanilide following *trans* cleavage is accelerated and the reaction is a composite of the uncatalyzed (K_{uncat}) and the PPIase-catalyzed (K_{enz}) *cis–trans* interconversion. Thus, first-order kinetics can be described in terms of the rate equation $v = K_{obs}(cis)$, where $K_{obs} = K_{uncat} + K_{enz}$. Under conditions where TcMIP (PPIase) is at high concentration, the slow phase of the reaction is strictly first order and the slope of the plot K_{obs}/PPIase is K_{cat}/K_m. The particular conditions of the assay result in a K_{cat}/K_m value of 745/mM/s for the *cis–trans* isomerization of the Leu–Pro bond, activity which exceeds the corresponding value for human FKBP12 (Fischer *et al.*, 1992).

As it has been shown that the enzymatic activity of FKBPs can be inhibited by nanomolar concentrations of FK506 (Siekierka *et al.*, 1990; Tropschug *et al.*, 1990), we examined the effect of increasing concentrations of FK506 as well as L-685,818, an antagonist of FK506 which does not suppress T-cell activation (Dumont *et al.*, 1992), on the PPIase activity of TcMIP. The IC_{50} (50% inhibitory concentration) calculated from the data in Figure 5 is 410 nM for FK506 and 12 nM for L-685,818, indicating that the K_i value for the latter may be even lower.

Table III. Effect of pretreatment of LLC–MK_2 cells with 150 nM TcMIP prior to infection with trypomastigotes

Pretreatment of cells	Infection	Intracellular parasites per 200 cells ± SD
Ringer's solution	parasites	180 ± 4.9
Ringer's solution	parasites + 150 nM TcMIP	305 ± 22
150 nM TcMIP	parasites	310 ± 23

Cells were incubated for 30 min at 37°C with Ringer's solution or 150 nM TcMIP in Ringer's solution, washed and incubated with trypomastigotes as described in Materials and methods.

Biological activity of TcMIP

Exogenous TcMIP facilitated the productive infection of simian epithelial cells by trypomastigotes (Figure 6A). Antibodies against TcMIP had the opposite effect (Figure 6B). Low concentrations of FK506 were sufficient to significantly inhibit the process, while high concentrations of cyclosporin A, an inhibitor of cyclophilins, had no effect (Figure 6C). The addition of exogenous TcMIP reverts the effect of FK506 (Figure 6D).

The results in Figure 6 did not provide information about the specific step of the infection process that is affected by TcMIP and its antagonists. A short-term invasion assay revealed that TcMIP facilitates entry of the parasites into the host cells. The number of trypomastigotes found inside the cells 30 min after infection was increased in the presence of TcMIP and decreased in the presence of its inhibitors FK506 and L-685,818 (Table II and Figure 7). It is apparent from these data that, as opposed to the *in vitro* inhibition of rotamase activity, FK506 is a more active inhibitor of invasion than L-685,818. When comparing Figures 5 and 7, there is a good correlation between the inhibition of rotamase activity and the inhibition of invasion for L-685,818. FK506, however, is a much better inhibitor of invasion than one would expect from the enzyme inhibition data (Table II and Figure 5A).

TcMIP acted on the cells but not on the parasites: cells pretreated with TcMIP and washed out prior to incubation with the parasites were more efficiently invaded than the controls (Table III). This suggests that the target for the rotamase lies on the host cell membrane.

Discussion

Here we show that a trypomastigote-secreted protein (TcMIP) is involved in the process of entry of *T.cruzi* into the host cell. The expression of this molecule is observed mainly in the invasive form, as demonstrated by Western blots and ELISAs of stage-specific culture supernatants. A motif found in the 3′ end of the TcMIP mRNA, akin to that of *T.cruzi* hsp85, may be a regulatory element

of expression, consistent with the fact that it is the trypomastigote which must endure the transition from the insect vector to the mammalian host (Rondinelli, 1994). The blocking of metabolic activity with 50 mM sodium azide diminishes the secretion of TcMIP, as well as control TC-TOX. Also, *in vitro* synthesis of TcMIP in the presence of canine microsomes suggests that the protein is post-translationally processed, possibly losing the predicted signal peptide. Taken together, these results show that TcMIP is actively secreted by the parasite.

A possibility is that TcMIP interacts with some protein(s) of the host cell membrane, contributing, in an as yet undetermined way, to the rendering of the host cell to be more susceptible to penetration via conformational changes through *cis–trans* isomerization of peptidyl-prolyl bonds. A common property to *Legionella*, *Chlamydia* and *T.cruzi* is their ability to invade a wide range of host cells, from macrophages to non-professional phagocytes (Moulder, 1985). It is worth noting that such evolutionary distant pathogens make a similar use of MIPs in cell invasion. Hence, it is tempting to speculate on a direct relationship between MIP activity and wide host cell range. This hypothesis might be supported by searching for MIP activity in additional intracellular parasites of wide host cell range, such as *Toxoplasma*.

The mode of action of the immunosuppressants cyclosporin A and FK506 is well documented (Schreiber, 1991; Fruman *et al.*, 1994). However, little is known about the natural roles for their binding proteins, cyclophilins and FKBPs, except their general role as aids in slow protein folding reactions. Moreover, their protein substrates remain largely unknown.

The relationship between infection and rotamase activity deserves some discussion. FK506 inhibits infection much better than L-685,818, but it is a worse inhibitor of the enzyme. Our present hypothesis is that FK506 acts by disrupting some signaling pathway at the level of the target cell, possibly related to the Ca^{2+} increase necessary for an effective invasion. If this is the case, then it is an important functional difference between the macrolide and cyclosporin A as both, when complexed to their ligands, are capable of inhibiting signal transduction in other biological systems (Liu *et al.*, 1991). Rotonda *et al.* (1993) demonstrated the structural and functional differences of the FKBP–L-685,818 and FKBP–FK506 complexes, and showed that the former are not inhibitors of calcineurin function. Keeping this in mind, we conclude that the observed differences in parasite entry when the infection cultures are treated with L-685,818 (Figure 7) are exclusively due to the inhibition of TcMIP rotamase activity and not by interference with any known signaling mechanism in the target cell, as was possible with FK506.

Cytoplasmic regulatory activities have been described for human FKBP, which is associated to the ryanodine receptor of the sarcoplasmic reticulum in skeletal muscle where it has been shown to modulate the activity of the calcium release channel (Jayaraman *et al.*, 1992; Timerman *et al.*, 1993; Brillantes *et al.*, 1994). It has also been proposed that FKBP may be the endogenous protein kinase C inhibitor 2 (PKC I2; Goebl, 1991), and a FK506 binding domain has been identified in a proteolytic fragment of chicken hsp56 (Yem *et al.*, 1993). In the case of *Legionella*, MIP may inhibit host cell protein kinase C (Hurley, 1993). If comparable activities could be assigned to TcMIP, alone or in conjunction with other invasion factors acting from the outside of the target cell, then another fragment of the puzzle constituted by the events necessary for the invasion of cells by *T.cruzi*, as well as other intracellular pathogens, would be elucidated. The findings reported suggest that a widespread mechanism of signal transduction is in operation for intracellular pathogen invasion, in which *cis–trans* isomerization of proline bonds plays an important role.

The role of TcMIP in the invasion process is clear in that the inhibition of parasite entry also occurs when TcMIP is blocked by specific antibodies, and that exogenous TcMIP significantly enhances invasion (as measured by parasite production) in a dose-dependent manner at nanomolar concentrations. Also, a 20 nM concentration of L-685,818 suffices to significantly decrease the number of parasites which can access the cell in a 30 min infection period.

Immune response is a key step in the control of parasite proliferation in *T.cruzi* infections (Hoff and Boyer, 1985). Therefore, attempts to reduce parasite burden by treatment with FK506 seem futile because of the accompanying immunosuppressive effects of the antibiotic. However, there are functionally related substances, such as L-685,818, that, while lacking immunosuppressive capacity, are still able to inhibit the rotamase activity of MIPs/FKBPs (Dumont *et al.*, 1992). Our results suggest the potential use of these compounds of reduced toxicity as therapeutic agents against Chagas' disease and, possibly, Legionnaires' disease and chlamydiasis.

Materials and methods

Cells and media

Trypomastigotes from *T.cruzi* Y strain (Silva and Nussenzweig, 1953) were cultured in monolayers of LLC–MK_2 simian epithelial cells as described previously (Andrews and Colli, 1982). Epimastigote forms were grown in liver infusion tryptose (LIT) medium supplemented with 10% fetal calf serum (FCS; Gibco-BRL), as described previously (Castellani *et al.*, 1967). Extracellular amastigotes were obtained by incubation of recently released trypomastigotes in LIT medium with 10% FCS at 37°C, 5% CO_2 for 24 or 48 h (Andrews *et al.*, 1987). HeLa and LLC–MK_2 cells were routinely maintained in DMEM supplemented with 10% FCS.

Bacterial strains and plasmids

A genomic DNA library in λ-EMBL3 (González *et al.*, 1990) was used in this study. For subcloning, we used Bluescript (Stratagene), Geminis (Promega) and the expression system pGEX-2T (Pharmacia). The bacterial strain used was *E.coli* XL1-Blue, made competent by the $CaCl_2$ method (Morrison, 1979).

Recombinant DNA techniques

Phage DNA was isolated by the Lambdasorb™ Phage Adsorbent (Promega) protocol. Plasmid DNA was purified by the alkaline-SDS method (Birnboim and Doly, 1979). Subclones were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase (USB). The *trans*-splicing acceptor was determined by AMV reverse transcription (Boehringer) of trypomastigote poly(A)$^+$ RNA using the oligonucleotide 5′-CCCGACCTGCCTTCCGGAC, followed by PCR amplification using a second primer 5′-CGCTATTATTGATACAGTTT-CTG, corresponding to the *T.cruzi* spliced leader sequence (De Lange *et al.*, 1984). The polyadenylation site was determined using the S1 nuclease (Boehringer) method (Berk and Sharp, 1977). Briefly, 3′ end-labeling at the *Sac*I site (position 724) was carried out using cordycepin [α-^{32}P]triphosphate and terminal transferase (Boehringer) in a genomic clone spanning the whole intergenic sequence. Upon denaturation and hybridization to polyadenylated RNA, S1-protected fragments were electrophoresed in parallel to chemical degradation sequencing reactions

(Maxam and Gilbert, 1980). The EMBL accession number for this sequence is X69655. Computer sequence analysis was carried out using the University of Wisconsin GCG software package, Version 7.3-AXP.

Southern blot

DNA from *T.cruzi* was digested with *Bgl*II (Boehringer), *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I (Promega), *Bam*HI, *Nco*I, *Sac*I and *Xba*I (Pharmacia), loaded onto a 1% agarose gel and, after electrophoresis, blotted by capillarity onto a nylon membrane (Zeta-Probe, Bio-Rad) in 20× SSC. The blot was prehybridized with 50% formamide, 5× SSC, 0.2% SDS, 0.05 M Na_2HPO_4, pH 6.5, and 5× Denhardt's solution at 42°C. Hybridization was performed with the *Nru*I (Pharmacia)–*Sac*I fragment from the TcMIP gene, labeled with [α-^{32}P]dCTP by nick translation (Sambrook *et al.*, 1989).

Isolation and purification of recombinant TcMIP protein

The coding sequence corresponding to amino acids 30–196 (i.e. without the putative signal peptide) was ligated into the expression vector PGEX-2T (PGEX-2T–MIP) and expressed as a fusion protein with *Schistosoma japonicum* glutathione-*S*-transferase (GST) in *E.coli* XL1-Blue (Stratagene). Cells from 1 l cultures of PGEX-2T–MIP-transformed *E.coli* induced with 0.1 mM IPTG (Boehringer) for 5 h were pelleted by centrifugation at 8000 r.p.m. in a GSA rotor (Sorvall) at 4°C for 10 min, resuspended in cold PBS + 1% Triton X-100 and lysed by sonication. The lysate was pelleted by centrifugation at 10 000 r.p.m. for 10 min, the supernatant filtered through a 0.45 µm syringe filter (Millipore), loaded onto a Sepharose–glutathione affinity column (Pharmacia) and washed with ice-cold PBS + 1% Triton X-100. Elution of GST–TcMIP was carried out with 50 mM Tris–HCl, pH 8.0, and 5 mM reduced glutathione. Elution of TcMIP and simultaneous removal of the GST domain was performed by the addition to the column of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% (w/w) human thrombin (Sigma). This purified protein was used for PPIase and biological activity determinations. The recombinant fusion protein was used for antibody production in mice or rabbits.

ELISAs

Washed epimastigotes or trypomastigotes of *T.cruzi* were incubated for 24 h in DMEM (Gibco-BRL) without FCS at a density of 10^8 parasites/ml with or without 50 mM NaN_3. After centrifugation, supernatants were dialyzed against carbonate buffer, pH 9.6. ELISA microtiter plates (Nunc) were incubated with the dialyzed, conditioned medium, blocked with 5% non-fat dry milk in Tris-buffered saline, 0.5% Tween 20 (TBST), incubated with mouse anti-GST–TcMIP fusion protein, rabbit anti-TC-TOX or a rat anti-ribosomal protein antisera, washed and incubated with rabbit anti-mouse IgG alkaline phosphatase (or goat anti-rat IgG alkaline phosphatase, or sheep anti-rabbit alkaline phosphatase, all from Sigma). Wells were developed with *p*-nitrophenyl phosphate (Sigma) and read at 405 nm in a Titertek ELISA reader. For the anti-ribosomal protein antiserum production, Wistar rats were immunized with purified *T.cruzi* ribosomal proteins purified as described for yeast (González *et al.*, 1981). Epimastigotes were broken by sonication, and the lysate was clarified by centrifugation at 30 000 *g*. The S-30 fraction was centrifuged at 175 000 *g* for 2 h. The pellet was resuspended, clarified and centrifuged through a 20–40% discontinuous sucrose gradient for 12 h at 150 000 *g*. The pelleted ribosomes were resuspended in a buffer containing 0.2 M magnesium acetate and the rRNA was precipitated by the addition of two volumes of glacial acetic acid and removed by centrifugation. The supernatant, containing the ribosomal proteins, was dialyzed and used for immunizations. The antiserum reacted with a crude *T.cruzi* lysate at a 1:5000 dilution.

Western blot

Supernatants (0.2 ml) obtained as above were dialyzed against 100 mM ammonium acetate, lyophilized, resuspended and loaded onto a 15% SDS–PAGE gel. After electrophoresis, proteins were electroblotted onto an Immobilon-P (Millipore) membrane, blocked with 5% non-fat dry milk in TBST, and incubated successively with MIP affinity-purified rabbit-anti-GST–TcMIP and with sheep-anti-rabbit IgG alkaline phosphatase (Boehringer). The blot was developed with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride (BCIP/NBT; Sigma).

In vitro transcription and translation of TcMIP and processing by canine microsomes

The coding sequence of TcMIP with the leader peptide included (as opposed to that used for the generation of the GST–TcMIP chimera) was amplified by PCR using the following primers: 5′-GAGT-**TAA**CaggagTGAC***ATG***CACAGA and 5′-CTTGAATTCCTCTCAAA-CTCACG, of which the first contains the sequence elements necessary for *in vivo* translation in bacteria upon cloning manipulation (see below) and induction with IPTG: a terminator for *lacZ* (bold), a ribosomal binding site (Shine and Dalgarno, 1974; lower case) and the start codon for the TcMIP coding sequence (bold italic). The amplified fragment was digested with *Eco*RI and *Hpa*I (Boehringer), ligated to *Hin*dIII-(blunt-ended with Klenow polymerase) and *Eco*RI-digested pBluescript KS(+), and used to transform XL1-Blue cells. The recovered plasmid was transcribed *in vitro* with T3 RNA polymerase, using 0.4 mM ATP, UTP and TTP, 0.05 mM GTP and 0.5 mM m^7GpppG (Boehringer). The RNA obtained was used for *in vitro* translation by a rabbit reticulocyte lysate (Promega) with [^{35}S]L-methionine (New England Nuclear), with or without canine microsomes (Promega). The reaction mixtures were loaded onto a 15% SDS–PAGE gel, electrophoresed, electroblotted onto nitrocellulose, blocked with 5% non-fat milk, developed with rabbit-anti-GST–TcMIP as in the Western blot, and autoradiographed to compare the *in vitro*-synthesized product with that produced in bacteria induced with IPTG. Transformed *E.coli* (100 µl; cultured in LB medium to OD 0.8 and induced for 3 h) was centrifuged, resuspended in Laemmli (1970) sample buffer and loaded onto the SDS–PAGE gel. The composite picture (Figure 4) was generated using Adobe Photoshop™ software.

PPIase assay

Enzymatic activity was measured in 0.035 M HEPES buffer, pH 7.8, at 15°C using the protease-coupled assay described by Fischer *et al.* (1989). The PPIase assay was performed by adding 1 µl of the peptide succinyl-Ala-Leu-Pro-Phe-4-nitroanilide (Bachem) dissolved in DMSO at 30 mg/ml to a cuvette containing buffer, TcMIP and 100 µl α-chymotrypsin (Sigma) solution at 14 mg/ml in a final volume of 1 ml. The concentration of TcMIP was calculated from absorbance at 280 nm using the extinction coefficient E_{280} = 17 780/M/s calculated from the aromatic amino acid content (Gill and von Hippel, 1989). For the kinetic runs, a Hewlett-Packard 8452A diode-array UV/VIS spectrophotometer was used for the measurement of absorbance at 390 nm for 200 s at 0.1 s intervals. First-order rate constants (K_{obs}) were calculated using 200 data points. Under these reaction conditions, the uncatalyzed rate of isomerization (K_{uncat}) was determined to be 0.0134/s.

Assay of parasite infection

Confluent monolayers of LLC–MK_2 cells were infected with purified trypomastigotes for 3 h at a multiplicity of five parasites per cell. Routinely, 10^6 parasites were used in 1 ml to infect ~2×10^5 cells in a p24 well (Nunc). Released parasites were counted at the indicated times post-infection. Every experiment represents the average of four countings of triplicated wells (±SD of the mean). Cyclosporin A, FK506 (Fujisawa), affinity-purified mouse anti-TcMIP or TcMIP were incubated with trypomastigotes for 30 min before infection and maintained throughout the infection time. After washing twice with pre-warmed fresh medium, monolayers were incubated at 37°C, 5% CO_2, in DMEM supplemented with 10% FCS.

Assay of parasite entry

The assay was performed essentially as described (Tardieux *et al.*, 1992) with the following modifications. The treatment of cells or parasites and infection were performed for 30 min each [200× stocks of FK506, L-685,818 (Merck) in methanol, and TcMIP in PBS]. Immunofluorescence was carried out with a rabbit anti-*T.cruzi* serum which detects trypomastigotes with high affinity at a dilution of 1:500 in PBS + 2% bovine serum albumin. Microscopic observation was carried out using a Zeiss Axiophot equipped for epifluorescence.

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