THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 22, Issue of June 1, pp. 19294–19300, 2001

Printed in U.S.A.

Lack of Abundance of Cytoplasmic Cyclosporin A-binding Protein Renders Free-living *Leishmania donovani* Resistant to Cyclosporin A*

Received for publication, October 13, 2000, and in revised form, February 21, 2001
Published, JBC Papers in Press, February 21, 2001, DOI 10.1074/jbc.M009379200

Madhuri Dutta‡, Praveen Delhi‡, Krishna M. Sinha, Rahul Banerjee§, and Alok K. Datta¶

From the Leishmania Group, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Rd., Kolkata 700 032, and §Saha Institute of Nuclear Physics, Bidhan Nagar, Kolkata, India

The majority of the effects of cyclosporin A (CsA) on cells is caused by the inhibition of phosphatase activity of calcineurin (CN) by the cyclophilin A (CyPA)-CsA complex formed in the cytoplasm. Although CsA inhibits the proliferation of a large number of parasites, not all are susceptible. The presence of structurally altered CyPA with lower affinity for CsA had been suggested to be the cause of resistance. We report here the identification and cloning of a high affinity CsA-binding protein (LdCyP) from *Leishmania donovani*, a trypanosomatid parasite that is naturally resistant to CsA. The translated LdCyP consists of 187 amino acids with a cleavable 21-amino acid hydrophobic NH_2-terminal extension. Modeling studies confirmed that all the residues of human CyPs responsible for interaction with CsA are sequentially and conformationally conserved in LdCyP. The purified recombinant protein displayed biochemical parameters comparable to human CyPs. Reverse transcription-polymerase chain reaction analysis confirmed that LdCyP was abundantly expressed. Immunoblot experiments and direct CsA binding studies revealed that LdCyP located in the subcellular organelles constituted the bulk of the CsA binding activity present in *L. donovani*, whereas the level of binding activity in the cytosol was conspicuously low. CsA selectively facilitated the secretion of LdCyP in the culture medium. Based on these results, it is concluded that the insensitivity of *L. donovani* to CsA is probably due to the paucity of CsA binding activity in the cytoplasm of the parasite. We suggest that LdCyP, located in the secretory pathway, may function as a chaperone by binding to membrane proteins rather than as the mediator of CN inhibition.

Studies on mechanisms of drug action and drug resistance have often led to the discovery of new biological phenomena. Cyclosporin A (CsA),[1] a lipophilic cyclic undecapeptide, was originally discovered as an antifungal agent (1). Its diverse effects on various cells, which range from immunosuppression to inhibition of cell proliferation, have kindled great interest in the studies related to its mode of action. Most, if not all, of the effects of CsA are exerted through the formation of a specific complex with cyclophilins (CyPs), a multigenic family of proteins. The CyP·CsA complex formed in the cytoplasm binds to calcineurin and inhibits its serine-threonine protein phosphatase activity, which leads to immunosuppression (2). The formation of such a binary complex between CyP and CsA is thus the first crucial step for CsA action.

Of the five isoforms of CyPs (CyPA, CyPB, CyPC, CyPD, and CyP40) that have been identified in mammalian tissues, CyPA and CyPB are well studied (3–5). All CyPs are known to exhibit CsA-sensitive peptidylprolyl *cis-trans* isomerase activity; however, the inhibition of peptidylprolyl *cis-trans* isomerase activity is unrelated to immunosuppression. Due to their multiple cellular localization, this group of proteins exhibits other biological functions such as signal transduction, protein-protein interaction, protein folding, and several cellular processes besides immunosuppression (6–9). CyPA, the most abundant and ubiquitously expressed cytosolic CsA-binding protein, is known to play a direct role in CsA-mediated inhibition of immune function and cell proliferation (2). In contrast, CyPB located in the secretory pathway plays the role of chaperone to proteins destined for plasma membranes (9, 10). A dual localization of CyP encoded by the same gene has been found both in the cytosol and mitochondria of *Neurospora crassa* (11).

Leishmania donovani, a dimorphic parasitic protozoan, is the causative agent of kala azar in humans. This protozoan exists as a flagellated promastigote (extracellular form) in the sand fly vector and is transformed into amastigote (intracellular form) in the mammalian macrophages. CsA has a wide range of effects on various parasitic protozoa both *in vivo* and *in vitro* (12). Despite the fact that CsA is highly toxic to many parasites *viz. Plasmodium*, *Schistosoma*, tapeworms, and *Toxoplasma gondii*, there are others that are insensitive to this drug (13). The cause of resistance in some of these parasites has been attributed to the absence of the concensus CsA binding motif in their CyPs. Despite normal uptake of the drug, the *in vivo* and *in vitro* effects of CsA on various *Leishmania* parasites are conflicting. Cutaneous lesions in susceptible mice caused by infection of *Leishmania major* or *Leishmania tropica* could be prevented with the administration of CsA or its non-immunosuppressive analogs. In contrast, CsA distinctly exacerbated intracellular *L. donovani* (amastigote) parasite load in murine cells, suggesting possible differences in the status of CyPs in these two species of *Leishmania* (13).

Rascher *et al.* (14) suggest that due to the occurrence of a single amino acid change (Arg → Asn with respect to Arg^{69} of human CyPA) in the CyP of *L. major*, the CyP·CsA complex fails to interact with the calcineurin (CN) in the cell. In contrast, both *T. gondii* and *Plasmodium falciparum* are known to

* This work was supported by Department of Biotechnology, Government of India Grants BT/PRO493/Med/09/096/96 and BT/PRO888/HRD/15/O93/98. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipients of individual C. S. I. R. (government of India) fellowships. Contributed equally to this work.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF 158368.

¶ To whom correspondence should be addressed. Tel.: 91-33-473-0284; Fax: 91-33-473-5197; E-mail: alokdatta@iicb.res.in.

[1] The abbreviations used are: CsA, cyclosporin A; CyP, cyclophilin; LdCyP, CyP from *L. donovani*; RT-PCR, reverse transcription-polymerase chain reaction; CN, calcineurin; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

harbor Asn in the same position of their cytosolic CyPs, yet they are highly sensitive to the drug (15, 16). This raises the question of the importance of this particular Arg residue in CN binding. Moreover, the recent report that the CN-associated serine-threonine phosphatase activity present in the whole cell extract of *L. donovani* can be effectively inhibited *in vitro* by the sole addition of exogenous CsA suggests that CsA resistance in *L. donovani* probably occurs by an alternative mechanism (17).

In an effort to understand the mechanism by which *L. donovani* becomes resistant to CsA, we set out to analyze the status of CyPs in this organism. This article deals with identification, sequencing, structural analysis, and expression of a CyP homologue distinctly different from that reported in *L. major*. Our results show that *L. donovani* lacks a threshold level of high affinity CsA-binding protein (CyPA) in the cytosol. The observation that *L. donovani* is deficient in cytosolic CyP is novel and provides a potential explanation for CsA resistance of the parasite, supporting the growing notion that a balance in the concentrations of cytoplasmic immunophilins and CN play an important role in determining the effectiveness of immunosuppressants. A possible biological role of this CyP in *L. donovani* is also suggested.

MATERIALS AND METHODS

Reagents, Cells, and Libraries—All chemicals used were of analytical grade. *L. donovani* promastigote (D1700), a clone of Sudanese 1S strain and its genomic library made in bacteriophage λGEM-11, were kindly provided by Prof. Buddy Ullman (18). Cells were grown at 26 °C under mild shaking in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with fetal calf serum (10%), hemin (5 mg/liter), xanthine (0.1 mM), 0.3% bovine serum albumin fraction V (Sigma), biotin (1 mg/liter), and Tween 80 (40 mg/liter) along with $NaHCO_3$ (3.7gm/liter) and 20 mM Hepes, pH 7.3. Exponentially grown cells were used in all experiments. [^{3}H]CsA (7Ci/mmol) was purchased from Amersham Pharmacia Biotech. CsA and D-Ala(3-amino)8-CsA were gifts from Drs. G. Engel and B. P. Richardson respectively of Sandoz Pharmaceuticals, Basel, Switzerland.

Isolation of LdCyP-encoding Sequence—Filters containing 150,000 plaques of an *L. donovani* genomic DNA library constructed in λGEM-11 bacteriophage were screened with ^{32}P-labeled degenerate antisense oligonucleotide (5′-GGATCCRAAIACIACRTGICGICCRTC-3′) synthesized from the most conserved part (DGRHVVF) of the CsA binding sequence (WLDGRHVVF) found in most CyPs from a variety of sources. Hybridization was carried out in 6× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) buffer at 40 °C overnight according to standard published protocol. After washing in 6× SSC, 0.1% SDS at room temperature, filters were autoradiographed. DNA isolated from six positive plaques were cleaved with *Pst*I-*Xho*I and Southern-hybridized with the same oligonucleotide. An ~4.2-kilobase hybridized band was eluted from a duplicate gel and ligated into the pBS vector. Automated DNA sequencing was carried out, and the analysis of the sequence was done using DNASIS program.

Characterization of the LdCyP Transcript —Total RNA was prepared according to the procedure published elsewhere (18). The integrity of the RNA was checked by undegraded ribosomal RNA bands on ethidium bromide-stained gels. To detect and characterize the LdCyP transcript in *Leishmania*, the following reaction was carried out: initial reverse transcription reaction (25 μl) contained total RNA (1 μg), 2.5 pmol of oligo(dT)$_{18}$, 200 μM dNTPs, 5 mM DTT, 5 units of RNasin, 1.5 mM $MgCl_2$, 5% (v/v) Me_2SO, and 1 μl of enzyme (Titan™). The reaction was carried out at 37 °C for 5 min followed by 1 h at 42 °C. To amplify the LdCyP-specific cDNA, 20 pmol each of the sense primer, 5′-CTCGGAATTCCAACGCTATATAAGTATCAGTTTCTGTACTTTATTG-3′, containing a short leader sequence with an *Eco*RI site followed by 36 of the 39-nucleotide mini-exon (19), and an antisense primer, 5′-GCCGAAAACCACATGGCGG-3′ (438–456), designed from a part (GRHVVFG) of the conserved CsA binding motif (WLDGRHVVFG), were added to the reverse transcription mixture. The PCR reaction was continued for 35 cycles at 94, 55, and 68 °C for 1, 1, and 2 min, respectively. Products were separated on 1.2% agarose gel, probed with a reverse internal oligonucleotide, 5′-GCCGCCCTGGATCATGAAG-3′ (247–265), designed from the peptide sequence ^{83}FMIQGG^{88} (to confirm the product), and subcloned into pBS vector at *Eco*RI/*Sma*I sites, and sequenced.

Expression and Purification of Recombinant LdCyP—The coding sequence (amino acid 22–187) without the 21-amino acid amino-terminal signal sequence was amplified by PCR and ligated in-frame at the *Nde*I/*Bam*HI sites of pET3a expression plasmid (Stratagene). The 5′-PCR primer (sense), 5′- CTCGAATTCATATGGAGCCGGAGGTGACCGCG-3′ with an *Nde*I site and 3′-primer (antisense), 5′- CGACCAGGATCCCTACAGCTCACCACTGGCA-3′ containing a *Bam*HI site were used. The PCR product, after digestion with *Nde*I/*Bam*HI, was cloned in pET3a and transformed into *Escherichia coli* BL21(DE3)pLysS cells. The cells containing the chimeric plasmid were induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) at 37 °C for 4–8 h, and induced protein (molecular mass 17.7 kDa) was purified following a published procedure (20). Using a similar strategy, another expression plasmid was constructed by ligating the same insert (coding amino acid 22–187) at the *Bam*HI/*Sal*I sites of the expression vector pQE32 (Qiagen), resulting in isopropyl-1-thio-β-D-galactopyranoside-inducible expression of the LdCyP as a histidine-tagged fusion protein. LdCyP from *E. coli* M15 cells harboring the plasmid was purified to homogeneity using a nickel-nitrilotriacetic acid-agarose column as per the manufacturer's suggested procedure. For all the biochemical experiments, imidazole-eluted His-tagged LdCyP that was extensively dialyzed against a buffer containing 10 mM Tris, pH 7.5, and 0.1 mM DTT was used.

Peptidylprolyl cis-trans Isomerase and CsA Binding Assays—Peptidylprolyl *cis-trans* isomerase activity in the purified recombinant LdCyP was measured using a synthetic tetrapeptide substrate *N*-succinyl-AAPF-*p*-nitroanilide. The procedure of assay and calculations was essentially the same as described by Kofron *et al.* (21). For CsA binding studies, both Sephadex LH-20 column assay using ^{3}HCsA (7Ci/mmol) and fluorescence enhancement assay of CyPs, caused due to binding of CsA, were used (22, 23).

Antisera Production and Immunoblotting—Polyclonal rabbit antisera against SDS-polyacrylamide gel electrophoresis-purified recombinant LdCyP, was raised, and immunoblotting of the sample was carried out using ^{125}I-labeled protein A as the secondary ligand (24).

Isoelectric Focussing—The sample for isoelectric focussing was made by mixing the total extract with 3× buffer (30% glycerol, 10% ampholines, pH 3.5–10). Tube gel was prepared using 4% acrylamide and 2% ampholine. Electrophoresis was carried out for 3 h at 200 volts using 0.01 M NaOH and 0.02 M orthophosphoric acid at the anode and cathode, respectively. After the first-dimension run, the tube was placed horizontally on a SDS-polyacrylamide slab gel, and electrophoresis was carried out.

Preparation of Whole Cell Extract and Subcellular Fractions—For the preparation of whole parasite extract, cells were directly suspended in buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 10 μg/ml each leupeptin and aprotinin) containing 0.5% CHAPS. The sample was sonicated and clarified by centrifugation at 10,000 × *g* for 30 min. For cellular subfractionation, the procedures of High *et al.* (15) and Harris *et al.* (25) were essentially followed. Briefly, the cells were harvested and washed with ice-cold buffer (150 mM NaCl, 20 mM glucose, 20 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl). The washed cell pellet was suspended at 1.2×10^9 cells/ml in 1 mM Tris-HCl, pH 8.0, 1 mM EDTA, briefly homogenized in a Dounce homogenizer, and gently lysed by passing through a 26-gauge needle. Sucrose (60%) was immediately added to a final concentration of 0.25 M. The mixture was centrifuged at 100,000 × *g* for 2 h. The resultant supernatant was called the "cytosol fraction." The pellet, containing mostly different organelles and membranes, referred to as "particulate fraction," was suspended in 0.5% CHAPS-containing buffer, sonicated, and dialyzed extensively against buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA,100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT.

Amino-terminal Sequencing of CsA Binding Proteins—For amino-terminal sequencing, the CsA-binding proteins were enriched from different sources using CsA analog (D-Ala(3-amino)8-CsA) affinity column as per the published procedure (26). In brief, the extracts from different sources (rat liver or different subcellular fractions of the parasite) were loaded onto the column. After extensive washing of the column with buffer (20 mM Tris-HCl, pH7.5, 1 mM EDTA, and 100 mM NaCl), the bound proteins were eluted with the same buffer containing 10 μg/ml CsA. The eluate containing CsA-binding proteins was pooled and precipitated with trichloroacetic acid, separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and immunoblotted using LdCyP antibodies. Immunopositive protein bands from a duplicate polyvinylidene difluoride paper were cut out and subjected to amino-terminal sequencing.

Modeling Studies—Modeling was performed on an Indigo II silicon graphics work station using human CyPA as the template (27–30). Several rounds of energy minimization using the CHARM force field

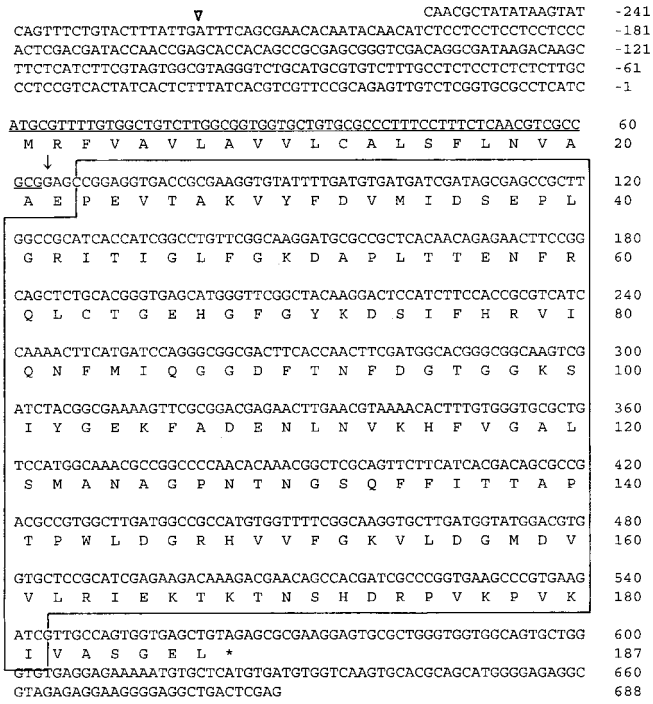

FIG. 1. **Nucleotide and deduced amino acid sequence of LdCyP.** The *solid underline* indicates the signal peptide-like sequence. The *down arrow* (↓) shows the predicted site for signal-peptide cleavage. The *closed box* represents the sequence homologous to CyPs from other sources, and the symbol (▽) indicates the site of transplicing of the mini-exon.

parameters in X-PLOR coupled with local manual model building using FRODO was performed (31, 32). The final model was superposed onto human CyPA in its complex structure with CsA and subjected to additional cycles of energy minimization (33).

LdCyP Secretion Experiment—Exponentially grown *L. donovani* (D1700) promastigotes were harvested and washed twice with Dulbecco's modified Eagle's medium depleted of fetal bovine serum and bovine serum albumin as described before. The cell pellet was then suspended in the same medium at a density of 10^7cells/ml and divided into three equal parts. One part was allowed to grow as such, whereas another was grown in the presence of noninhibitory concentrations of CsA (2 μM). To the third part, sodium azide (50 mM) was included along with CsA. Growth was continued at 22 °C for 24 h. At the indicated times, a measured aliquot of the cell suspension from each of the three cultures were withdrawn and harvested. The cell pellets obtained were boiled in SDS-mercaptoethanol containing buffer (cell extract). The spent media from each culture after passing through GF/C filters were dialyzed against buffer (10 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT) and concentrated. Cell extracts and the corresponding processed media were subjected to Western blot analysis using LdCyP antisera.

RESULTS

Sequence Analysis of LdCyP—Screening of an *L. donovani* (D1700) genomic library made in bacteriophage λGEM-11 with a degenerate oligonucleotide, based on a highly conserved amino acid sequence, led to the isolation of six positive chimeric phage clones. Sequencing of a portion (a 4.2-kilobase *Pst*I/*Xho*I fragment) of the insert from all six phage clones and translation of the sequences in all possible reading frames predicted the presence of an open reading frame consisting of 187 amino acids, corresponding to a polypeptide of 20,643 daltons (Fig. 1). A 159-amino acid stretch of this encoding sequence (spanning amino acids 22–181) of LdCyP showed 67% homology with human CyPB (Fig. 2). Several of the sequence stretches found in CyPs from other sources, *viz.* the consensus CsA-docking site [WLDG(K or R)HVVFG] and sequences like SMANAG and FM(C/I)QGGDF, were also present in the LdCyP sequence (Fig. 2). Primary structure analysis of LdCyP and its modeling revealed that the entire set of 13 residues in human CyPA known to interact with CsA are sequentially and conformationally conserved in LdCyP, making it quite capable of equivalent interactions with the ligand molecule (Figs. 2 and 3) (1). The root mean square deviation between their C^{α} atoms was calculated to be 0.85 Å. The three charged residues (Arg^{69}, Lys^{125}, and Arg^{148}) of human CyPA implicated in the electronic interaction with CN were, however, replaced in the LdCyP by Asn, Arg, and Asn, respectively (Fig. 2). It is to be mentioned here that the complex of CsA with human CyPB, which harbors Arg, Lys, and Asp in positions corresponding to Arg^{69}, Lys^{125}, and Arg^{148} of CyPA, is 14-fold more effective as a CN inhibitor than the CyPA·CsA complex (34). Furthermore *T. gondii* and *P. falciparum*, despite having Asn, Lys, Ser and Asn, Lys, Lys in the corresponding positions of their cytosolic isoforms, are highly sensitive to CsA *in vivo* (15, 16). Therefore, factors other than these three amino acids of CyPs are probably responsible for the differential affinity of these molecules with CN (34). The PSIGNAL program of the PCGENE sequence analysis software package further predicted that the encoded LdCyP contained a typical endoplasmic reticulum-directed signal sequence at the amino-terminal end of the protein, with the predicted cleavage occurring between the amino acids 21 and 22 (35).

Biochemical Characterization of LdCyP—The purified recombinant 6×His-LdCyP without the signal sequence catalyzed the isomerization of the prolyl-peptide bond in the synthetic substrate with a K_{cat}/K_m of 6.3×10^6 $M^{-1}s^{-1}$ at 10 °C. The activity was drastically inhibited at a nearly 1:1 molar ratio of 6×His-LdCyP to CsA (Fig. 4*A*). Binding of CsA to recombinant 6×His-LdCyP, as determined by a fluorescence enhancement assay, followed a typical saturation kinetics (Fig. 4*B*). From the Scatchard analysis, the stoichiometry of association was determined to be 1:1 with a K_d of 135 nM (Fig. 4*B*, *inset*). These values are close to the published values for CyPs isolated and characterized from various other sources (9, 20, 22).

Characterization of the LdCyP Transcript in L. donovani Promastigotes—To ascertain the level of expression of LdCyP gene, Northern blot analysis was carried out with total promastigote RNA. However, even with >30 μg of RNA and a very high specific activity randomly labeled LdCyP gene probe, the transcript signal was barely visible. Thus, to characterize the transcript, an RT-PCR approach was adapted. Due to the phenomenon of transplicing in *Leishmania*, the 5′ termini of all mature mRNAs are known to contain an identical 39-nucleotide-long mini-exon sequence (19). It was therefore reasoned that the use of this common mini-exon sequence as a sense primer along with the antisense internal oligonucleotide primer designed from the conserved CsA binding region (WLDGRHVVFG) should amplify at least one (LdCyP) if not all of the isoforms of the CyP gene family present in the total RNA. A Southern blot of the RT-PCR reaction products probed with an oligonucleotide designed against another conserved internal sequence (^{83}FMIQGG^{88}) detected only one hybridizable band (Fig. 5*b*). Cloning and sequencing of the RT-PCR product revealed a 713-base pair cDNA that originated from transcription of the cognate LdCyP gene. The mini-exon was transpliced at −221 base pairs upstream of the predicted translation initiation site (Fig. 1). These results clearly indicated that in *Leishmania* promastigotes, LdCyP gene, along with its hydrophobic amino-terminal extension, was indeed transcribed to certain abundance to be analyzed by RT-PCR, whereas the other CyP transcripts (if at all present) could not be detected under this condition.

Subcellular Distribution of LdCyP—The failure to detect other isoforms of CyP transcripts in the free-living *L. donovani* could be due to (*a*) the paucity of respective concentrations of transcripts,

```
L.d        ---------MRFVAVLAVVLCALSFLNVAAEPEVTAKVYFDVMIDSEPLGRITIGLFGKD 51
L.m        -------------------PYTPHYPVVESNP----KVWMDIDIGGKPAGRVTMELFKDA 37
Hu.A       --------------------------MVNP----TVFFDIAVDGEPLGRVSFELFADK 28
Hu.B       MKVLLAAALIAGSVFFLLLPGPSAADEKKKGPKVTVKVYFDLRIGDEDVGRVIFGLFGKT 60
P.f19      -------------------------MSKRS----KVFFDISIDNSNAGRIIFELFSDI 29
B.m        ------------------------MSKKDRR----RVFLDVTIDGNLAGRIVMELYNDI 31

                                          *     ** *       #   *
L.d        APLTTENFRQLCTGE--------HGFGYKDSIFHRVIQN FMIQGGDF T N FDGTGGKSIYG 103
L.m        VPQTAENFRALCTGE--------KGFGYANSPFHRVIPD FMCQGGDF T N GNGTGGKSIYG 89
Hu.A       VPKTAENFRALSTGE--------KGFGYKGSCFHRIIPG FMCQGGDF T R HNGTGGKSIYG 80
Hu.B       VPKTVDNFVALATGE--------KGFGYKNSKFHRVIKD FMIQGGDF T R GDGTGGKSIYG 112
P.f19      TPRTCENFRALCTGEK--IGSRGKNLHYKNSIFHRIIPQ FMCQGGDI T N GNGSGGESIYG 87
B.m        APRTCNNFLMLCTGMAGTGKISGKPLHYKGSTFHRVIKN FMIQGGDF T K GDGTGGESIYG 91

                                  ***       * *       **  #*
L.d        EKFADENLK---VKHF-VGAL SMANAG PNTNGSQFFITTAPTP WLDGRHVVFG KVLDGMD 159
L.m        SKFADESFLGKAGKHFGPGTL SMANAG PNTNGSQFFLCTAPTS WLDGKHVVFG QVLEGYE 149
Hu.A       EKFEDENFI---LKHTGPGIL SMANAG PNTNGSQFFICTAKTE WLDGKHVVFG KVKEGMN 137
Hu.B       ERFPDENFK---LKHYGPGWV SMANAG KDTNGSQFFITTVKTA WLDGKHVVFG KVLEGME 169
P.f19      RSFTDENFN---MKHDQPGLL SMANAG PNTNSSQFFITLVPCP WLDGKHVVFG KVIEGMN 144
B.m        GMFDDEEFV---MKHDEPFVV SMANKG PNTNGSQFFITTTPAP HLNNIHVVFG KVVSGQE 148

                                #
L.d        VVVRIEKTKT N SHDRPVKPVKIVASGEL---------- 187
L.m        VVKAMEAVGS R S-GTTSKPVRVSACGQL---------- 176
Hu.A       IVEAMERFGS R N-GKTSKKITIADCGQLE---------- 165
Hu.B       VVRKVESTKT D SRDKPLKDVIIADCGKIEVEKPFAIAKE 208
P.f19      VVREMEKEGA K S-GYVKRSVVITDCGEL---------- 171
B.m        VVTKIEYLKT N SKNRPLADVVILNCGELV---------- 177
```

FIG. 2. **Comparison of the predicted amino acid sequence of LdCyP with CyPs from *L. major* (*L.m*) (14), human (*Hu.A* and *Hu.B*) (3, 9), *P. falciparum* (*P.f19*) (16), and *Brugia malayi* (*B.m*) (53).** The sequences were aligned by CLUSTAL W (54). *Dashed lines* were introduced to achieve maximum homology. Amino acid residues predicted to be involved in CsA binding and calcineurin docking are represented by *asterisks* (*) and number signs (#), respectively. *Dark boxes* represent sequences found in most CyPs, whereas the *large open box* represents the conserved sequence against which the internal oligonucleotide was designed. *L.d*, *L. donovani*.

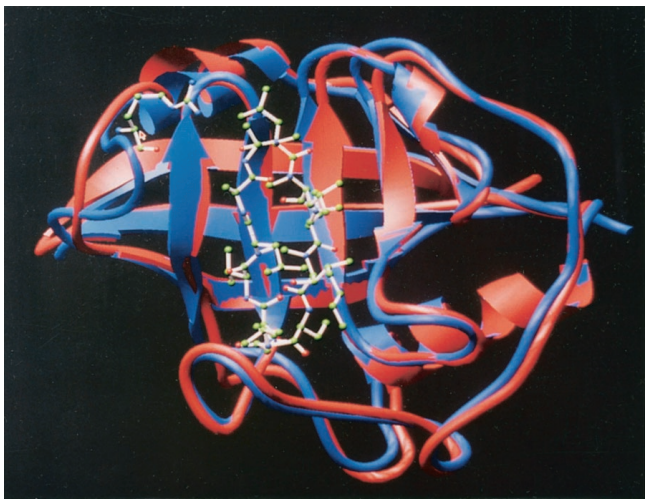

FIG. 3. **Ribbon diagram of LdCyP.** Modeled LdCyP (*red*) was superposed on human CyPA (*blue*) CsA (*ball and stick*) complex. Arg^{148} of human CyPA is shown on the top left-hand side of the figure, indicated by an *arrow*.

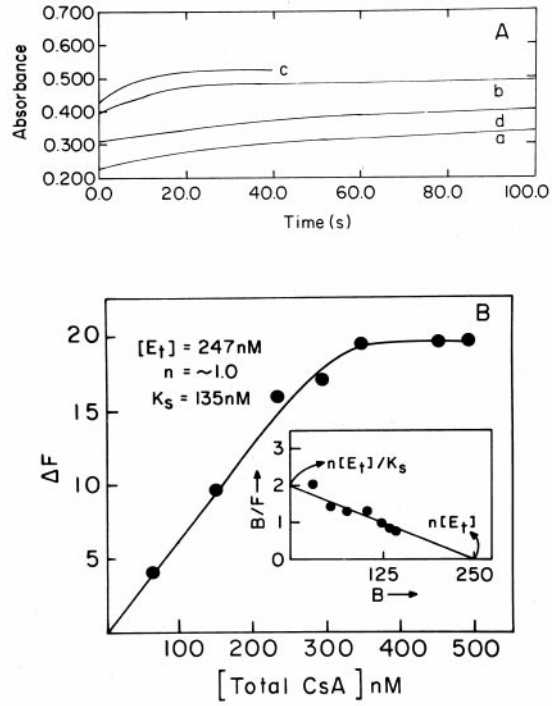

FIG. 4. **Biochemical characterization of recombinant LdCyP.** *A*, progress curves from the chymotrypsin-coupled assay for 6×His-LdCyP peptidylprolyl *cis-trans* isomerase. Shown are nonenzymatic isomerization without 6×His-LdCyP (*a*), 7.8 nM 6×His-LdCyP (*b*), 11.8 nM 6×His-LdCyP (*c*), and 11.8 nM 6×His-LdCyP plus 300 nM CsA (*d*). *B*, Binding of CsA to 6×His-LdCyP using fluorescence enhancement assay. To recombinant 6×His-LdCyP (247 nM) increasing concentrations of CsA was added, and the change in absolute fluorescence was monitored. The *inset* shows a Scatchard analysis of binding.

(*b*) the use of LdCyP-specific reverse primer rather than its degenerate form in both RT-PCR and southern hybridization, or (*c*) analysis of fewer clones generated from the RT-PCR reaction product. To sort this out and to determine the number of different CyP proteins expressed in *Leishmania*, immunoblot analysis of the whole parasite extract and its different subcellular fractions was carried out using polyclonal antisera raised against SDS-polyacrylamide gel electrophoresis-purified recombinant LdCyP. Due to extensive amino acid homology, the antigenic cross-reactivity among different CyP isoforms within the same species and across different species is well established (3, 36, 37). This was confirmed by cross-reactivity of the LdCyP antisera with several of the well characterized CyP isoforms (CyPA, CyPB, and CyP40) from rat liver, cytosolic recombinant CyP from *P. falciparum* (PfCyP19) (Fig. 6*A*), and even with *E. coli* CyP (data not shown). Western blot analysis of different subcellular fractions of *L. donovani* revealed that the immunoreactive protein detected in the total extract was mostly sequestered in the particulate fraction of the cell (Fig. 6*B*, *upper panel*). Contrastingly, the cytosol fraction did not display any additional band except the one seen in other two extracts. The faint band observed in the cytosol fraction was clearly due to leaching of the same protein from the particulate fraction. Futhermore, the immunoreactive protein in all of the fractions appeared to be equal in size to that of the recombinant LdCyP (M_r 17.7 kDa) that lacked the putative signal sequence (Fig. 6*B*, *upper panel*). A control immunoblot of

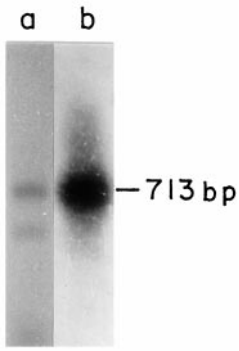

FIG. 5. **Analysis of *L. donovani* CyP transcript(s) by RT-PCR.** Shown are an ethidium bromide-stained agarose gel containing the PCR products (*a*) and an autoradiogram of the same gel probed with a ^{32}P-labeled internal reverse oligonucleotide (corresponding to ^{83}FM-IQGG^{88}) (*b*). *bp*, base pair.

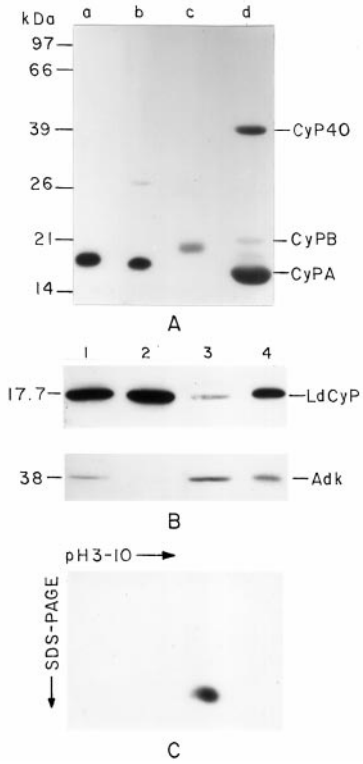

FIG. 6. **Immunoblot of CyPs from various sources.** *A*, reactivity of the LdCyP antisera with purified recombinant LdCyP (*a*), *L. donovani* total extract (*b*), purified recombinant *P. falciparum* cytoplasmic CyP19 (*c*), and CsA column eluate of rat liver total extract (*d*). *B*, immunoblot analysis of different subcellular fractions of *L. donovani*. *Lanes 1–3* (*upper* and *lower panels*), total extract (25 μg), particulate extract (25 μg), cytosol extract (25 μg), respectively; *lane 4*, purified recombinant LdCyP (*upper panel*) and purified recombinant Adk (*lower panel*). *Upper* and *lower panels* were probed with LdCyP and Adk antibodies, respectively. *C*, after two-dimensional gel electrophoresis of total CHAPS extract, the protein was transferred from the gel to nitrocellulose paper and probed with LdCyP antibodies. *PAGE*, polyacrylamide gel electrophoresis.

all the fractions with antibodies to *L. donovani*-specific adenosine kinase, an enzyme known to be present exclusively in the cytosol of *L. donovani*, confirmed that the particulate fraction was free of unlysed cells and cytosolic proteins (Fig. 6*B*, *lower panel)* (24, 38). The immunoblot of a two-dimension-run gel with total CHAPS extract further confirmed that the 17.7-kDa band was indeed due to a single immunoreactive protein (Fig. 6*C*). It is to be noted that the molecular mass of the immunoreactive protein (17.7 kDa) detected in both total extract and the particulate fraction is

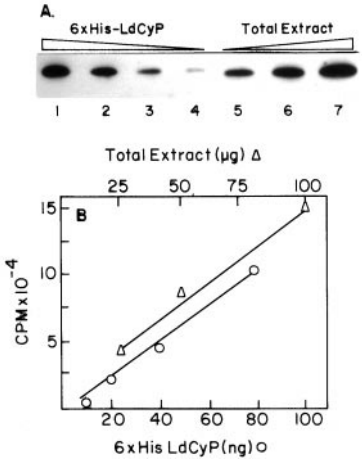

FIG. 7. **Quantitation of CyP in total *L. donovani* promastigote extract by immunoblot analysis.** *A*, various amounts of CHAPS-extracted total *L. donovani* promastigote protein along with known amounts of recombinant LdCyP were immunoblotted using LdCyP antisera. Autoradiography was performed, and each of the bands was subjected to phosphoroimaging analysis. *Lanes 1–4* contained LdCyP (80, 40, 20, and 10 ng) as the standard, whereas *lanes 5–7* contained 25, 50, and 100 μg of total promastigote protein. *B*, total counts obtained under each band shown in *A* after subtraction from the background were plotted against the amount of protein loaded on the gel, and CyP content was determined.

significantly smaller (by ~2.9 kDa) than the size predicted from the open reading frame (20.6 kDa). To confirm that the 17.7-kDa protein was indeed the *bona fide* LdCyP gene product, extracts from all three fractions (total, cytosolic, and particulate) were passed through a CsA analog affinity column. Bound proteins, after elution with buffer containing CsA, were resolved on SDS-polyacrylamide gel electrophoresis (in duplicate). One of the gels was subjected to immunoblotting with LdCyP antisera, whereas the other gel was processed for NH_2-terminal sequencing. Expectedly, the CsA column eluate of the cytosol extract failed to react on the Western blot. However, a single immunopositive band (17.7 kDa) was prominently visible with equivalent amounts of CsA column eluate from both total and particulate extracts (data not shown). Amino-terminal sequencing of these two immunoreactive bands from either fractions revealed that their first 12 amino acids, EPEVTAKVYFDV, were identical and matched with the predicted amino acid sequence (amino acids 22–33) of the cloned LdCyP (Fig. 1). These results unequivocally proved that (i) the immunopositive protein is indeed the product of the cloned LdCyP gene and is concentrated mostly in the particulate fraction of the cell, and (ii) the 21-amino acid NH_2-terminal hydrophobic extension gets cleaved at the computer-predicted peptide cleavage site.

Level of CsA Binding Activity in Leishmania Promastigotes—Studies of the cytosolic CyP (CyPA) gene product, both at the level of transcript and protein, have shown that it is the most abundant among all the CyP isoforms and constitutes about 75–80% of total CyPs present in almost all cell types studied to date (9, 22, 23). Despite this, our inability to detect unambiguously this isoform of CyP (CyPA) in the cytosol of *L. donovani* was puzzling. To investigate whether the failure to detect appreciable signal in the Western blot of the cytosol extract was at all due to nonreactivity of the polyclonal antisera with other isoforms of CyPs or the lack of their abundance in the cytosol, the CyP content in the total cell extract was monitored by two independent methods and compared. In the first method, the total extract was subjected to quantitative Western blot analysis using purified recombinant LdCyP as the standard (Fig. 7), whereas the second method consisted of direct measurement of [^{3}H]CsA binding capacity of the extract (Table I). CsA binding

TABLE I
Cyclophilin content in total L. donovani extract determined by both immunoblotting and CsA binding assay

CHAPS-extractable soluble protein was prepared from *L. donovani* promastigotes according to the procedure described under "Materials and Methods." The dialyzed extract was used for both quantitative immunoblotting (Fig. 7) and [^{3}H]CsA binding. Assuming 1:1 stoichiometry of binding of CsA to CyP, the CyP content was calculated from the Sephadex LH-20 column binding assay using an equation described previously (22, 23). Protein concentration was estimated using Bio-Rad method.

Fraction	Total protein	Total CyP content	Specific activity
	mg	*μg*	*μg/mg*
Total extract	9.42	Immunoblotting, 11.3	1.2
		CsA binding, 14.13	1.5

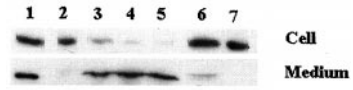

FIG. 8. **CsA-mediated secretion of LdCyP in the medium.** After incubation of *L. donovani* promastigotes in defined media (with or without CsA and CsA plus sodium azide), cells and the corresponding utilized media were collected ("Materials and Methods") and immunoblotted using LdCyP antisera. *Lane 1* in both *panels* represents standard recombinant LdCyP. *Lanes 2–5* (*upper panel*) contained equal amounts of protein extracts from cells grown for 0, 6, 10, and 24 h in the presence of CsA, whereas *lanes 2–5* (*lower panel*) contained equal volumes of the corresponding utilized media. *Lanes 6* (*upper panel*) contained CsA plus sodium azide-treated cell extract (24-h sample), whereas the lower *panel* had the corresponding medium. *Lane 7*, *upper* and *lower panels*, represents 24 h grown normal cell extract and the corresponding medium, respectively.

capacity by definition would be expected to detect all the high affinity CyPs. The results obtained by these two methods are in good agreement and show that LdCyP in the whole cell extract constitutes about 0.12–0.16% of the total soluble cellular protein (Fig. 7 and Table I). This correlation is consistent with the interpretation that (i) LdCyP, associated with the particulate fraction, is the major constituent of the high affinity CsA binding proteins present in *L. donovani* promastigotes, and (ii) the CsA-binding activity, which normally is known to be most abundant in the cytoplasm of almost all the cells studied to date, is conspicuously low in *L. donovani* promastigotes.

LdCyP Secretion in the Culture Medium—The absence of the endoplasmic reticulum signal sequence from the matured LdCyP coupled with its predominant localization in the particulate fraction of the cell indicated that LdCyP may be one of the proteins residing in the secretory pathway. Since LdCyP, like other CyPs, binds CsA with high affinity, these findings led us to study the fate of the *in vivo* LdCyP after exposure of the cells to CsA. The result showed that CsA (2 μM) facilitated the secretion of the protein into the medium (Fig. 8). With increased time of exposure to CsA, the amount of LdCyP secreted in the medium gradually increased with its concomitant loss from the intracellular pool. Adenosine kinase and gp63, known to be cytosolic and membrane-located enzymes of *L. donovani*, could not be detected in the medium with their respective antisera, confirming that the CsA-induced secretion of LdCyP in the medium was not due to parasite disintegration or generalized effect in the presence of CsA (data not shown). Furthermore, the fact that CsA, in combination with sodium azide (50 mM), failed to release comparable amounts of LdCyP suggests that the cells need to be metabolically active to be able to secrete LdCyP. The low level of LdCyP released in presence of sodium azide was possibly due to mechanical lysis of some parasites during experimental procedure (Fig. 8). A plausible explanation of these findings could be that CsA mimics the interaction of LdCyP with some unknown proteins or ligands.

DISCUSSION

The status of CyPs in *L. donovani* has remained an enigma ever since it was discovered, that these cells, unlike other parasitic protozoa, were relatively resistant to CsA. Using degenerate oligonucleotide, designed against the conserved CsA binding motif (WLDGRHVVF), a CyP gene (LdCyP) was isolated from the genomic library of the parasite. Although this result by no means ruled out the absence of other CyP genes in *L. donovani*, it prompted us to study the status of CyP proteins in the free-living form of this parasite. While analyzing the 5′-end of the LdCyP-encoded sequence, which distinctly differs from that reported in *L. major*, an alternative in-frame ATG upstream (−90 base pair) of the predicted translation initiation site was detected. However, several features of the DNA sequence suggested that this in all probability is not the physiological translation initiation site. This was borne out by the smaller size of the *in vivo* protein product. Second, the predicted translation initiation is preceded by a sequence closer to the eucaryotic translation initiation signal (39). Third, the occurrence of the multiple polypyrimidine stretches, typical of all the *Leishmania*-encoded genes, was clearly prevalent upstream of the predicted translation initiation site, and finally, the sequence upstream of the predicted translation site exhibited only 60% preference for G/C at the wobble position, far lower than the high G/C bias (≥80%) invariably observed in most genes, including LdCyP, cloned from *L. donovani* (18, 40, 41).

The most important finding of this work, however, relates to the levels of expression of CyP proteins in the free-living *L. donovani*. Although the RT-PCR amplification studies indicated the expression of only the cognate LdCyP gene, the combined results of immunochemical analysis and direct [^{3}H]CsA binding assays firmly established that LdCyP, associated with the particulate fraction of the cell, was indeed the most abundant CsA-binding protein present in the free-living promastigotes. Interestingly, none of these assays detected any appreciable levels of CsA-binding protein in the cytoplasmic fraction of the cell. Because gene expression in *Leishmania* is thought to be predominantly regulated post-transcriptionally by *cis*-acting elements (*e.g.* by RNA decay and RNA processing and translation), it remains to be seen whether the lack of abundance of cytosolic CyP in *L. donovani* is due to lack of its expression in the promastigote stage or due to other causes like message instability (42).

There is mounting evidence to believe that an appropriate balance in the intracellular concentrations of CyP or FK506 binding protein and CN play a significant role in determining the dose-response curves of respective ligand inhibitors, CsA and FK506 (43, 44). In view of this scenario, the extremely low concentration of high affinity CsA-binding protein in the cytosol of free-living *L. donovani* promastigotes becomes relevant with regard to CsA resistance of the organism. In the cellular context, the IC_{50} of ligand inhibitors is governed not only by intrinsic affinities but also by effective concentrations of interacting agents. Therefore, it is most likely that the lack of threshold level of high affinity cytosolic CsA-binding protein limits the formation of adequate amounts of intracellular immunophilin-CsA complex in the cell cytosol simply due to laws of mass action. This allows cytosol-located CN to carry out its required function, resulting in relative resistance of the *L. donovani* promastigotes (45). LdCyP, located in the particulate fraction of the cell, clearly cannot participate in this process. Observations that (i) yeast mutants lacking in CyPA are CsA-resistant but can be made sensitive by the introduction of the CyPA gene, (ii) the ring-stage form of *P. falciparum*, expressing the highest amount of CyP during its growth cycle, are most susceptible to CsA inhibition, and (iii) the CsA-resistant human fibroblastic MRC cell line shows low level

cytosolic cyclophilin support such a contention (46–48). Resistance to FK506 has also been found to be associated with a deficiency in FK506-binding protein 12 (49). An independent report published recently further suggests that the low abundance of immunophilins can indeed restrict CN inhibition by CsA and FK506 in human peripheral blood leukocytes and mouse spleen cells (50).

The abundance of CsA binding activity in the particulate fraction of the cell raises questions as to the function of LdCyP. Besides mediating the inhibition of calcineurin phosphatase, CyPs have been implicated in folding and maturation of proteins *in vivo* (7, 8). There can be two ways by which CsA can affect these molecular processes: either by inhibiting the peptidylprolyl *cis-trans* isomerase activity of CyPs or by destabilizing the interaction (chaperoning function) of putative proteins with immunophilins. Because the process of protein folding and interactions among proteins can be either partially or totally prevented by CsA, it is conceivable that in the absence of these functions, cells might be more sensitive to conditions that place extra stress on the capacity of the cell to carry out its growth process (51, 52). Therefore, the CsA-induced secretion of intracellular LdCyP in the culture medium leads us to suggest that the drug, because of its high affinity for LdCyP, probably disrupts the interaction of LdCyP with some naturally occurring slow-folding protein substrates that under normal conditions help LdCyP to remain anchored to the secretory pathway. The fact that LdCyP lacks the endoplasmic reticulum-anchoring signal sequence (KDEL) and the concentration of CsA (>7 μM) required to have an inhibitory effect on *L. donovani* growth is much higher than the concentration necessary for inhibition of peptidylprolyl *cis-trans* isomerase activity provides support to this proposal. It is likely that at higher concentrations of CsA, the inhibitory effect of the drug becomes pronounced, probably due to increased depletion of LdCyP from the intracellular pool. Similar mechanisms have been proposed while studying the trafficking of human CyPB and *ninaA*-rhodopsin interaction in *Drosophila* (8, 10). However, the identification of such proteins in *L. donovani* remains to be determined.

In summary, we have identified and characterized a noncytosolic high affinity CyP homologue that is predominantly expressed in free-living *L. donovani*. Furthermore, the evidence for extremely low abundance of CsA binding activity in the cytosol of *Leishmania* provides an explanation for CsA resistance of the organism. This has opened up a new opportunity to carry out detailed studies on the physiological function(s) of this noncytosolic CyP in a cell that is naturally deficient in cytoplasmic CsA binding activity. Gene knock-out experiments and identification of physiological partner(s) of LdCyP would be steps toward understanding such a function(s).

Acknowledgments—We thank Dr. Sailen Barik and Prof. Alan H. Fairlamb for supplying the recombinant PfCyP19. We are also indebted to Prof. Buddy Ullman for providing *L. donovani* (D1700) promastigotes and its genomic library. Dr. Rabindra Roy of the Sealy Center for Molecular Sciences, Galveston, TX, deserves special thanks for carrying out amino-terminal sequencing of proteins. Editorial assistance from Prof. A. N. Bhaduri and Dr. P. K.Sarkar of this institute is gratefully acknowledged.

REFERENCES

1. Dreyfuss, M., Harri, E., Hofmann, H., Kobel, H., Pache, W., and Tscherter, H. (1976) *Eur. J. Appl. Microbiol.* **3,** 125–133
2. Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Albers, D. G., Belshaw, P. J., Cohen, P., Mackintosh, C., Klee, C. B., and Schreiber, S. L. (1992) *Biochemistry* **31,** 3896–3901
3. Bergsma, D. J., Eder, C, Gross, M., Kersten, H., Sylvester, D., Appelbaum, E., Cusimano, D., Livi, G. P., McLaughlin, M. M., Kasyan, K., Porter, T. G., Silverman, C., Dunnington, D., Hand, A., Prichett, W. P., Bossard, M. J., Brandt, M., and Levy, M. A. (1991) *J. Biol. Chem.* **266,** 23204–23214
4. Friedman, J., Trahey, M., and Weissman, I. (1993) *Proc. Nat. Acad. Sci. U. S. A.* **90,** 6815–6819
5. Kieffer, L. J., Seng, T. W., Li, W., Osterman, D. G., Handschumacher, R. E., and Bayney, R. M. (1993) *J. Biol. Chem.* **268,** 12303–12310
6. Schreiber, S. L. (1991) *Science* **251,** 283–287
7. Fischer, G., and Schmid, F. X. (1990) *Biochemistry* **29,** 2205–2221
8. Baker, E. K., Colley, N. J., and Zuker, C. S. (1994) *EMBO J.* **13,** 4886–4895
9. Price, E. R., Zydowski, L. D., Jin, M., Baker, C. R., McKeon, F. D., and Walsh, C. T. (1991) *Proc. Nat. Acad. Sci, U. S. A.* **88,** 1903–1907
10. Price, E. R., Jin, M., Lim, D., Pati, S., Walsh, C. T., and McKeon, F. D. (1994) *Proc. Nat. Acad. Sci. U. S. A.* **91,** 3931–3935
11. Tropschug, M., Nicholson, D. W., Hartl, F-U., Kohler, H., Pfanner, N., Wachter, E., and Neupert, W. (1988) *J. Biol. Chem* **263,** 14433–14440
12. Page, A. P., Kumar, S., and Carlow, C. K. S. (1995) *Parasitol. Today* **11,** 385–388
13. Chapell, L. H., and Wastling, J. M. (1992) *Parasitology* **105,** S25–S40
14. Rascher, C., Pahl, A., Pecht, A., Brune, K., Solbach, W., and Bang, H. (1998) *Biochem. J.* **334,** 659–667
15. High, K. P., Joiner, K. A., and Handschumacher, R. E. (1994) *J. Biol. Chem.* **269,** 9105–9112
16. Berriman, M., and Fairlamb, A. H (1998) *Biochem. J.* **334,** 437–445
17. Banerjee, C., Sarkar, D., and Bhaduri, A. N. (1999) *Parasitology* **118,** 567–573
18. Hanson, S., Adelman, J., and Ullman, B. (1992) *J. Biol. Chem.* **267,** 2350–2359
19. Wilson, K., Hanson, S., Landfear, S. M., and Ullman, B. (1991) *Nucleic Acids Res.* **19,** 5787
20. Hasel, K. W., Glass, J. R., Godbout, M., and Sutcliffe, J. G. (1991) *Mol. Cell. Biol.* **11,** 3484–3491
21. Kofron, J. L., Kuzmic, P., Kishore, V., Colon-Bonilla, E., and Rich, D. H. (1991) *Biochemistry* **30,** 6127–6134
22. Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., and Speicher, D. W. (1984) *Science* **226,** 544–547
23. Koletsky, A. J., Harding, M. W., and Handschumacher, R. E. (1986) *J. Immunol.* **137,** 1054–1059
24. Bhaumik, D., and Datta, A. K. (1989) *J. Biol. Chem.* **264,** 4556–4561
25. Harris, M. E., Moore, R., and Hajduk, S. L. (1993) *J. Biol. Chem.* **265,** 11368–11376
26. Kieffer, L. J., Thalhammer, T., and Handschumacher, R. E. (1992) *J. Biol. Chem.* **267,** 5503–5507
27. Ke, H., Zydowsky, L. D., Liu, J., and Walsh, C. T. (1991) *Proc. Nat. Acad. Sci. U. S. A.* **88,** 9483–9487
28. Jones, T. A. (1982) in *Computational Crystallography*, (Sayer, D., ed) pp. 303–317, Oxford University Press, Oxford
29. Briinger, A. T., Kuriyan, J., and Kurplus, M. (1987) *Science* **235,** 458–460
30. Ke, H., Moyrose, D., Belshaco, P. J., Alberg, D. G., Schreiber, S. L., Chang, Z. Y., Etzkorn, F. A., Ho, S., and Walsh, C. T. (1994) *Structure* (*Lond.*) **2,** 33–44
31. Brooks, B., Bruccoleri, R., Olafson, B., States, D., Swaminathan, S., and Karplus, M. (1983) *J. Comput. Chem.* **4,** 187–217
32. Subba Rao, N., and Haneef, I. (1991) *Protein Eng.* **4,** 877–884
33. Carson, M. (1991) *J. Appl. Crystallogr.* **24**, 958–961
34. Mikol, V., Kallen, J., and Walkinshaw, M. D. (1994) *Proc. Nat. Acad. Sci. U. S. A.* **91,** 5183–5186
35. Pelham, H. R. B. (1989) *Annu. Rev. Cell Biol.* **5,** 1–23
36. Harding. M. W., Handschumacher, R. E., and Speicher, D. W. (1986) *J. Biol. Chem.* **261,** 8547–8555
37. Allain, F., Boutillon, C., Mariller, C., and Spik, G. (1995) *J. Immunol. Methods* **178,** 113–120
38. Datta, A. K., Bhaumik, D., and Chatterjee, R. (1987) *J. Biol. Chem.* **262,** 5515–5521
39. Kozak, M. (1984) *Nucleic Acids Res.* **12,** 857–872
40. Beverley, S. M., Ellenberger, T. E., and Cordingley, J. S. (1986) *Proc. Nat. Acad. Sci. U. S. A.* **83,** 2584–2588
41. Meade, J. C., Shaw, J., Lemaster, S., Gallagher, G., and Stringer, J. R. (1987) *Mol. Cell. Biol.* **7,** 3937–3946
42. Argaman, M., Aly, R., and Shapiro, M. (1994) *Mol. Biochem. Parasitol.* **64,** 95–110
43. Klee, C. B., Ren, H., and Wang, X. (1998) *J. Biol. Chem.* **273,** 13367–13370
44. Crabtree, G. R. (1999) *Cell* **96,** 611–614
45. Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996) *Nature* **382,** 370–373
46. Breuder, T. Hemenway, C. C., Movva, N. R., Cardenas, M. E., and Heitman, J. (1994) *Proc. Nat. Acad. Sci. U. S. A.* **91,** 5372–5376
47. Reddy, G. R. (1995) *Mol. Biochem. Parasitol.* **73,** 111–121
48. Foxwell, B. M. J., Woerly, G., Husi, H., Mackie, A., Quesniaux, F. J., Hiestand, P. C., Wenger, R. M., and Ryffel, B. (1992) *Biochim. Biophys. Acta* **1138,** 115–121
49. Fruman, D. A., Bierer, B. E., Benes, J. E., Burakoff, S. J., Austen, K. E., and Katz, H. R. (1995) *J. Immunol.* **154,** 1846–1851
50. Jung, L., and Halloran, P. F. (2000) *Transplantation* **70,** 327–335
51. Lodish, H. F., and Kong, N. (1991) *J. Biol. Chem.* **266,** 14835–14838
52. Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993) *Cell* **73,** 1067–1078
53. Page, A. P., Landry, D., Wilson, G. G., and Carlow, C. K. S. (1995) *Biochemistry* **34,** 11545–11550
54. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680