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Disruption of the Zinc Finger Motifs in the *Leishmania tarentolae* LC-4 (=TbMP63) L-complex Editing Protein Affects the Stability of the L-complex*

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The uridine insertion/deletion editing complex, which we have termed the L-complex, is composed of at least 16 polypeptides stabilized entirely by protein-protein interactions. Three L-complex proteins contain zinc finger motifs that could be involved in these interactions. In *Leishmania* these proteins are labeled LC-1, LC-4, and LC-7b, and the orthologs in *Trypanosoma brucei* are labeled MP81, MP63, and MP42. Overexpression of TAP-tagged LC-4 in *Leishmania tarentolae* led to a partial localization of the protein in the L-complex together with the endogenous LC-4 protein, suggesting at least a dimeric organization. Disruption of zinc fingers 1 or 2 (ZnF-1 and ZnF-2) in the tagged LC-4 protein was performed by mutation of the two zinc-binding cysteines to glycines. Disruption of ZnF-1 led to a partial growth defect and a substantive breakdown of the L-complex, whereas disruption of ZnF-2 had no effect on cell growth and caused a partial breakdown of the L-complex. A close interaction of LC-4 with 2–4 proteins, including REL1 (RNA ligase) and LC-3, was suggested by chemical crosslinking and co-immunoprecipitation experiments. Our results suggest that both ZnF-1 and ZnF-2 in LC-4 play a role in protein-protein interactions and indicate that the LC-4 subcomplex may be required for formation or stability of the entire L-complex.

RNA editing of mitochondrial maxicircle mRNA transcripts in trypanosomatid protozoa involves the insertion and deletion of uridine residues at specific sites within coding regions through a series of enzymatic cleavage-ligation steps mediated by base pairing with small guide RNAs (1). A macromolecular complex, which has been termed the L-complex[1] because it contains the two RNA ligases, REL1 and REL2, has been isolated and characterized from both *Leishmania tarentolae* (2) and *Trypanosoma brucei* (3–6). At least 16 polypeptides are present in this complex, the stability of which is determined entirely by protein-protein interactions (2, 7, 8). Two additional complexes were shown to interact with the L-complex via RNA linkers (2, 9, 10). Three L-complex proteins, LC-1 (MP81), LC-4 (MP63, Band III), and LC-7b (MP42) contain zinc finger motifs that could be involved in protein-protein interactions or protein-RNA/DNA interactions (2, 5). Monoclonal antibodies specific for MP63 immunoprecipitated *in vitro* RNA editing activities (5), and conditional down-regulation of expression of this protein resulted in a growth defect apparently resulting from an effect on RNA editing due to the breakdown of the L-complex (11).

The classical C2H2 zinc finger motifs are found in many regulatory proteins (12). Zinc finger motifs interact with DNA and RNA and with proteins. C2H2 zinc finger proteins can be divided into three groups based on the number and pattern of the fingers, namely triple C2H2, multiple-adjacent C2H2, and separated-paired C2H2. LC-4 belongs to the latter group, in which each finger pair behaves independently in the binding reaction (13).

We have investigated the role of the two zinc finger motifs in the *Leishmania* LC-4 protein by overexpression of mutated proteins *in vivo* and show that these are involved in protein-protein interactions and stability of the L-complex.

EXPERIMENTAL PROCEDURES

Cloning—The *LC-4* gene (2) was amplified from *Leishmania major* genomic DNA, and the product was cloned in the pCR2.1-TOPO vector (Invitrogen). The primers for the amplification of the LC-4 from *L. tarentolae* genomic DNA, which were designed from the upstream and downstream flanking regions of the *L. major LC-4* gene (2), are the following: 5′-primer, 5′-GGATCCATGACGATGAAGAGGCGGATCGGAG-3′; and 3′-primer, 5′-CATGCCAGTTGGAAGACCCACGT-3′. Multiple PCR products were obtained, and the correct one was selected by Southern hybridization using the cloned *L. major LC-4* gene as a probe. The product was cloned into the pCR2.1-TOPO vector. The following oligonucleotides were used to amplify LC-4 from the pCR2.1-TOPO clone: 5′-primer, 5′-GGATCCATGACGATGAAGAGGCGGATCGGAG-3′; and 3′-primer, 5′-GGATCCATTATCCGTGAACGGTAAAATGCGC-AGCGAGC-3′. The PCR product was inserted into the BamHI site of the pX-derived p26 (MRP1)-TAP vector (9). For expression in *Escherichia coli*, the *LC-4* gene was excised with *Eco*RI from pCR2.1-TOPO and inserted into the EcoRI site of pMAL-c2X. (New England Biolabs) (9). *E. coli* cells were grown at 37 °C to A_{600} of ~0.5, isopropyl-1-thio-β-D-galactopyranoside was added to final concentration of 0.5 mM, and the cells were incubated at 15 °C overnight. Amylose resin (New England Biolabs) was used for affinity isolation of the recombinant fusion protein. After elution from the column with 10 mM maltose, LC-4-MBP was separated by SDS-PAGE, and the protein band was excised and used for generation of polyclonal antibodies in mice (Covance Inc.).

The *LC-2* gene was amplified from *L. major* genomic DNA (2) using the following oligonucleotides: 5′-primer, 5′-TCTCCTTTCCTCGATGC-GGGGTGCG-3′; and 3′-primer, 5′-CACACGGAGCACGTCGGTCACA-

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[1] The abbreviations used are: L-complex, ligase-containing complex; LC, L-complex protein; DMS, dimethyl suberimidate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IPG, immobilized pH gradient; MS, mass spectroscopy; wt, wild type; TEV, tobacco etch virus; ZnF, zinc finger motif.

GGA-3′. The PCR product was cloned into the pCR2.1-TOPO vector. The following oligonucleotides were used to amplify the *L. major LC-2* gene from a pCR2.1-TOPO clone for construction of the *L. tarentolae* TAP-expression vector: 5′-primer, 5′-TCCCCCGGGATGCGGGGTGCGCTGGCGCG-3′; and 3′-primer, 5′-TCCCCCGGGCAGGACTTGGAACTGCATGC-3′. The PCR product was inserted into the XmaI site of the pX-derived p26 (MRP1)-TAP vector (9).

The *LC-3* gene was amplified from *L. major* genomic DNA using the following oligonucleotides: 5′-primer, 5′-CATATGATGTACGGCGTGACACGGTACTTGCG-3′; and 3′-primer, 5′-GGATCCTCAGCCGGGCATGCCACTGC-3′. The product was cloned into the pCR2.1-TOPO vector (Invitrogen). The gene was sub-cloned into the pET15b vector (Novagen) using NdeI and BamHI and expressed as a fusion protein with an N-terminal His_6 tag. The LC-3 formed inclusion bodies in *E. coli* and was purified under denaturing conditions by metal affinity chromatography on Talon resin (BD Biosciences) according to the manufacturer's protocol. Polyclonal antibodies were raised against recombinant LC-3 (Covance Inc.).

The *L. major REL1* gene was identified in the GenBank™ data base using the *T. brucei REL1* sequence (CAB95523) (14). Oligonucleotides from conserved regions of the *L. major REL1* gene were used to amplify fragments of the *L. tarentolae* ortholog (5′-primer, 5′-GAGAAGGTGCACGGCACAAACTT-3′; and 3′-primer, 5′-GGCCGATCTTCGACAGCACGT-3′). A 4-kb XbaI-KpnI *L. tarentolae* genomic fragment containing the entire *LtREL1* gene was cloned from a mini-library. The following oligonucleotides were used to amplify REL1 from this plasmid: 5′-primer, 5′-GGCGAATTCATGCGTCGACTGGCACTGCG-3′; and 3′-primer, 5′-GGCGGATCCTCACTGCGCCTCTGCCTTCT-3′. This PCR product was inserted into the EcoRI and BamHI sites of the pGAD T7 expression vector (Clontech).

LC-4 Zinc Finger Mutations—The QuikChange multi site-directed mutagenesis kit (Stratagene) was used for the mutation of the two zinc fingers of LC-4 in the LC-4-TAP vector. The following oligonucleotides were 5′-phosphorylated and simultaneously used in mutagenesis reactions: 5′-CGTTTCCACgGCAGCGTTgGCAAGAAGTCG-3′; 5′-CGCATGTAgGCATCGTTGgCGAGAAGAAC-3′ (T to G substitutions are indicated by a lowercase g). Colonies were selected and screened for the desired mutations.

Cell Transfection—L. tarentolae cells were harvested by centrifugation, washed in electroporation buffer (21 mM HEPES, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO4), and resuspended at a density of 100×10^6 cells/ml. DNA (10 μg) was added to 400-μl cells in an electroporation cuvette (BTX; 2-mm gap), and the cells were incubated on ice for 10 min. After electroporation (BTX, Electro Cell Manipulator 600) using conditions described previously (15), the cells were cooled on ice, transferred into brain heart infusion medium, and incubated overnight at 27 °C. Cells were harvested by centrifugation and plated onto brain heart infusion agar plates containing 200 μg/ml G418 (Invitrogen).

Glycerol Gradient Fractionation and Adenylation—Freshly isolated mitochondria (100 mg wet weight) were resuspended in lysis buffer (25 mM HEPES, pH 8.1, 10 mM KCl, 5 mM MgCl, and 0.5% Nonidet P-40) containing Complete proteinase inhibitors (Roche Applied Science) and incubated on ice for 30 min. After sonicating for 3 s, the mixture was centrifuged at 90,000 rpm for 10 min in a Beckman Optima centrifuge. The supernatant was loaded on a glycerol gradient (10–30% glycerol) and centrifuged at 30,000 rpm for 20 h in the SW41 rotor. Fractions of 750 μl were collected from the top using an Isco density gradient fractionator. For adenylation of REL1, 0.5 μl of [α-^{32}P]ATP were added to 10 μl of each fraction, and the aliquots were incubated for 30 min at 27 °C. REL2 is pre-charged with AMP and, therefore, does not adenylate well in this reaction (16).

Chemical Crosslinking—Mitochondrial extract was fractionated on a glycerol gradient, the 20 S fraction was incubated with [α-^{32}P]ATP to label REL1, and then dimethyl suberimidate (DMS) (Pierce) or formaldehyde was added to a final concentration of 10 mM or 0.1–0.5%, respectively. The mixture was incubated on ice for 30 min and subjected to SDS-PAGE. After transfer to a membrane and PhosphorImager analysis, the membrane with the formaldehyde-crosslinked proteins was subjected to Western analysis using the LC-4 polyclonal antiserum. The low amount of protein crosslinked by DMS precluded Western analysis of that blot.

Two-dimensional Gel Electrophoresis—ReadyStrip IPG strips (Bio-Rad) were used for isoelectric focusing separation. Samples were concentrated by trichloroacetic acid precipitation and prepared in rehydration sample buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, and 0.2% Bio-Lyte ampholytes (Bio-Rad)) and loaded into the rehydration/equilibration tray. Strips were placed gel side down onto the sample and overlaid with 1 ml of mineral oil to prevent evaporation during the rehydration process. The tray was covered and placed into the PROTEAN IEF cell (Bio-Rad) at 20 °C overnight to rehydrate the IPG strips and load the protein sample. After rehydration, a wet paper wick was placed at both ends of the channels covering the wire electrodes, and the IPG strips were transferred into the focusing tray (gel side down) and covered with 1 ml of mineral oil. The focusing tray was then placed into the PROTEAN IEF cell (Bio-Rad) for focusing (250 V for 15 min, linear ramping to 4000 V for 2 h, and 12,000–20,000 V for 20 h). Prior to running the second dimension, the IPG strips were equilibrated in SDS-PAGE equilibration buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20 glycerol, and 2% dithiothreitol) for 10 min and then loaded onto an SDS-PAGE gel for the second dimension.

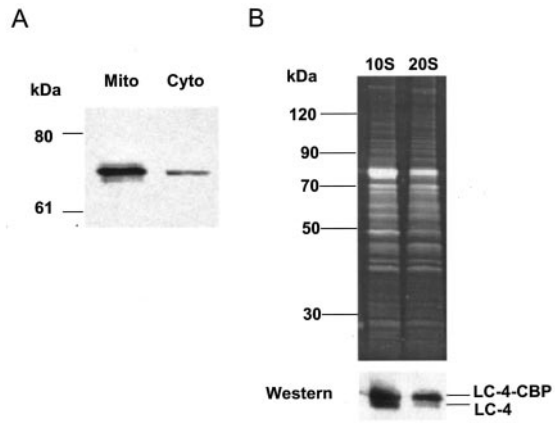

FIG. 1. **Targeting of the TAP-tagged LC-4 protein to the mitochondrion and TAP isolation of tagged LC-4.** *A*, equivalent amounts of cytosol (*Cyto*) and mitochondrial extract (*Mito*) were separated in an 8–16% polyacrylamide SDS gel. The gel was blotted and probed with the peroxidase anti-peroxidase (PAP) reagent (Sigma) to detect the TAP-tagged LC-4. *B*, mitochondrial extract was fractionated in a 10–30% glycerol gradient, and the 10 S fractions and the 20 S fractions were subjected to LC-4-TAP affinity isolation. The gel was SYPRO-stained (Molecular Probes) and blotted, and a Western blot was performed using anti-LC-4 antiserum. *CBP*, calmodulin-binding petide.

Protein Sequencing—Mass spectrometric analysis of proteins was performed as described in detail previously (2). After electrophoresis, the protein spots of interest were excised and digested with trypsin *in situ*. The recovered peptides were purified using νC_{18} ZipTips (Millipore). Mass spectrometric measurements were performed on an Applied Biosystems 4700 proteomics analyzer, which is a tandem time-of-flight instrument (TOF/TOF) with a matrix-assisted laser desorption/ionization (MALDI) ion source (17). Peptide sequences were determined by manual interpretation of the MS/MS spectra. The inferred sequences were searched using Protein Prospector (University of California, San Francisco) against the NCBInr data base as well as the parasite genome databases (www.genedb.org).

Co-Immunoprecipitation of LC-4, LC-3, and REL1—The *L. tarentolae LC-4* gene was excised from the pMal-c2X construct with SalI and EcoRI, and the fragment was inserted into the pGBK T7 expression vector (Clontech). *L. tarentolae* REL1 was cloned in the pGAD T7 expression vector, and the *L. major LC-3* gene was cloned in the PET15b T7 expression vector as described above. Each plasmid was transcribed and translated using the TnT coupled transcription/translation system (Promega). The LC-3 and REL1 proteins were labeled by inclusion of [^{35}S]methionine (Easytag express protein labeling mix, PerkinElmer Life Sciences) in the reaction mix. The reactions were clarified for 30 min in the Microfuge, and 10 μl of each were mixed together and incubated for 1 h at 25 °C. Then, 5 μl of LC-4 antiserum was added, and the reaction was incubated for 1 h at 25 °C. After clarification, protein G beads (γ-binding, G-Sepharose; Amersham Biosciences) were added and incubated for 1 h at 25 °C. The beads were pelleted, washed multiple times in 0.01 M sodium phosphate, 0.15 M NaCl, 0.01 M EDTA (pH 7) with 0.1% Nonidet P-40, and the bound protein was released by boiling with SDS loading buffer for gel electrophoresis.

RESULTS

Overexpression of TAP-tagged LC-4 Protein in L. tarentolae—The LC-4 protein (the ortholog in *T. brucei* is MP63) is a

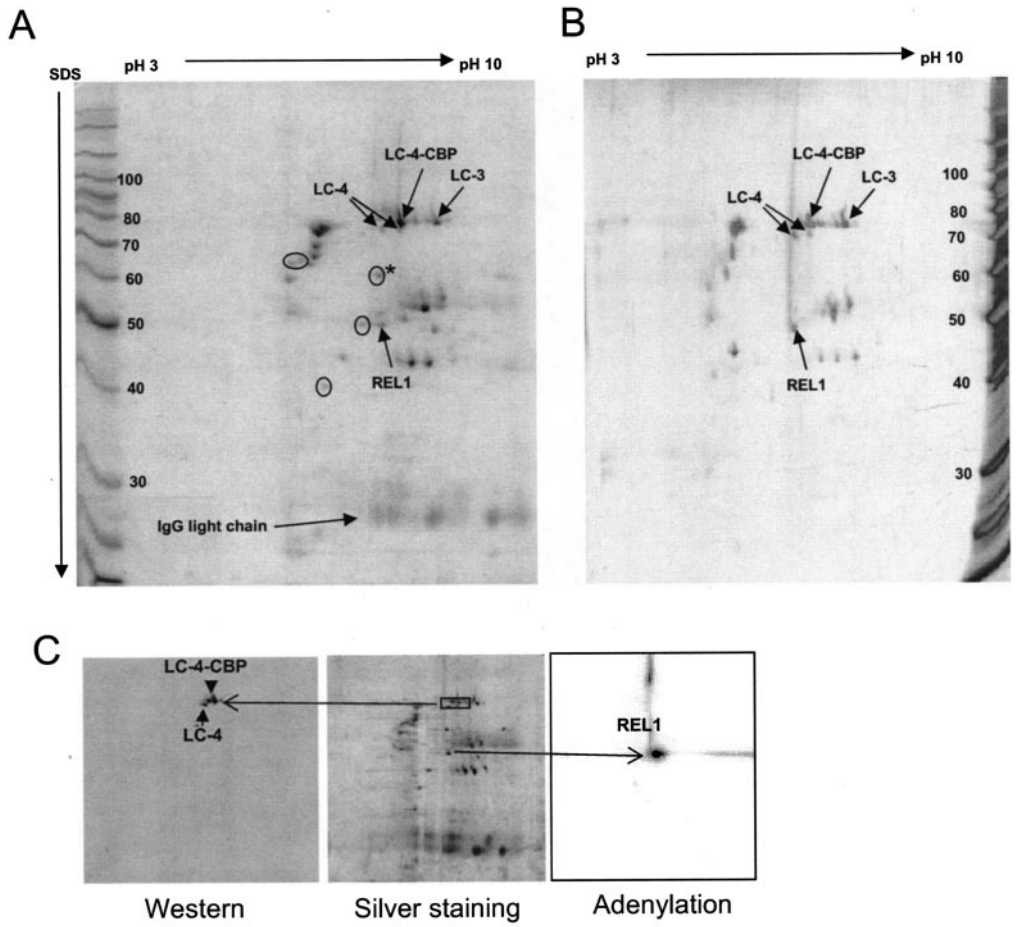

FIG. 2. **Two dimensional gel electrophoresis of LC-4-TAP isolated proteins.** *A*, TEV-released material, Coomassie Blue stained. The identified spots are indicated by *arrows*, and the spots missing in *panel B* are *circled*. The IgG light chain was identified by a control transfection with a plasmid containing the mitochondrial target signal fused to the TAP tag (not shown). *B*, EGTA-released material. *C*, identification of spots by Western analysis with antisera for LC-4 and by adenylation for REL1. *CBP*, calmodulin-binding protein.

component of the ~16 polypeptide L-complex (7). The *L. tarentolae LC-4* gene was inserted into the pX expression vector (18) as a fusion with the calmodulin-binding and protein A motifs (TAP tag) (19), and the plasmid was used to transfect *L. tarentolae* cells. The majority of the tagged LC-4 protein was targeted to the mitochondrion (Fig. 1*A*). Mitochondrial extract from transfected cells was fractionated on a glycerol gradient, and the 10 S fractions and the 20 S fractions were subjected to the tandem affinity isolation procedure (19). The polypeptide profiles for the TAP-isolated 10 and 20 S material were the same (Fig. 1*B*) and essentially identical to that reported previously for the LC-4-TAP isolation from total mitochondrial extract (2). The gel was blotted and Western analysis performed. As shown in Fig. 1*B*, the tagged LC-4 protein was relatively more abundant than the endogenous LC-4 in the TAP pull-downs from both the 20 S fractions and the 10 S fractions.

Two-dimensional Gel Electrophoresis of the LC-4-TAP Purified L-complex—The TAP isolation procedure involves two affinity purification steps. In the first step, the C-terminal protein moiety of the LC-4-TAP is bound to an IgG matrix and then eluted after digestion with TEV protease, which cleaves between the protein A motif and the calmodulin binding peptide. The second step involves binding to calmodulin agarose and elution with EGTA. Both the TEV-released and the EGTA-released materials were fractionated by two-dimensional gel electrophoresis. As shown in Fig. 2, ~15–20 spots can be detected using the TEV-released material. The REL1 spot was identified by adenylation, and the LC-4 and tagged LC-4 spots were identified by Western analysis (Fig. 2*C*). At least four proteins from the TEV-released material (*circled* in Fig. 2*A*) are missing in the EGTA-released material (Fig. 2*B*). A control experiment using a plasmid containing only the mitochondrial target signal fused to the TAP tag yielded only the expected IgG heavy and light chain contaminants in the TEV-released material (not shown).

Very similar two-dimensional gel patterns, including the four spots present in the TEV-released material and absent in the EGTA-released material, were obtained using LC-2-TAP isolated material (not shown). The spot labeled LC-3 in Fig. 2 (but obtained from the LC-2-TAP pull-down) was excised, subjected to tryptic digestion, and analyzed by tandem mass spectrometry. Three peptides were obtained (EEHNAAAPSAAQNLLR, GHTLPAGAAADITQTR, and (AL)SGAHVGLPAGNRPTPH) (the parenthesis around AL indicates that their order was not determined by MS/MS), which all mapped to the *L. major* LC-3 protein sequence. We also analyzed one of the spots (* in Fig. 2*A*) present only in the TEV-released material. Three tryptic peptides were obtained (FTEVGFHGR, HQVPSDIREEIAPK, (VL)EEISFEGPER), which mapped to an *L. major* protein (LmjF15.0090) with an "AAA" PFAM domain that contains an ATP-binding site. It is possible that this and the other three unidentified proteins represent L-complex components that are less

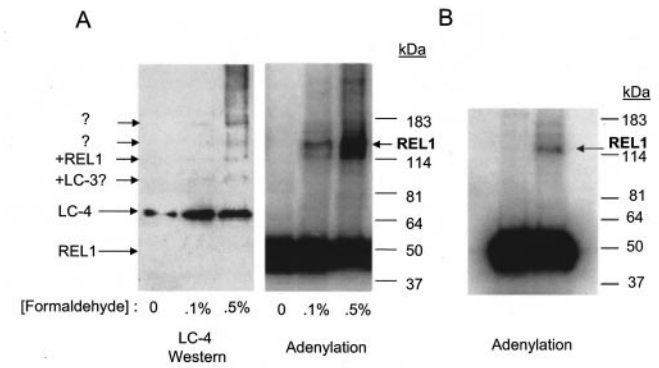

FIG. 3. **Chemical crosslinking of proteins interacting with LC-4.** *A*, formaldehyde crosslinking of an isolated 20 S glycerol gradient fraction from wild type cells. The fraction was incubated with [α-^{32}P]ATP prior to crosslinking to label mainly the REL1 ligase protein. Formaldehyde was added to final concentrations of 0 and 0.5%, The proteins were separated in a SDS gel that was blotted for LC-4 Western analysis and autoradiography to detect REL1. *B*, DMS crosslinking of a 20 S gradient fraction. After adenylation, the DMS was added to a concentration of 10 mM, and the solution was incubated on ice for 30 min. The proteins were separated in an SDS gel and blotted and exposed to the PhosphorImager for detection of adenylated REL1.

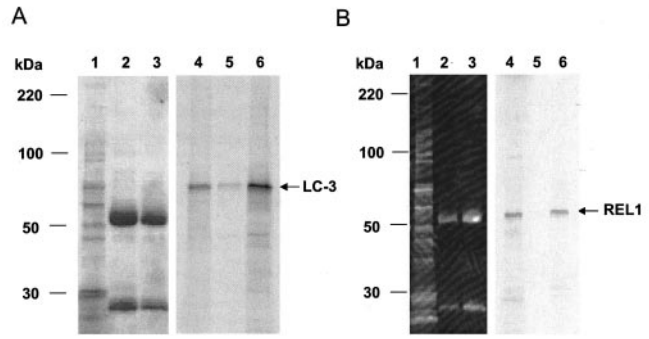

FIG. 4. **Co-immunoprecipitation of *in vitro* transcribed and translated LC-4, LC-3, and REL1.** See "Experimental Procedures" for details. The LC-3 and REL1 proteins were labeled with [^{35}S]methionine. *A*, interaction of LC-4 and LC-3. *Lane 1*, Ponceau Red-stained blot of SDS gel of clarified supernatant from the LC-3 [^{35}S]methionine *in vitro* transcription/translation reaction. *Lane 2*, proteins released with SDS from protein G beads mixed with ^{35}S-labeled LC-3 plus LC-4 antiserum. *Lane 3*, proteins released with SDS from protein G beads mixed with unlabeled LC-4, ^{35}S-labeled LC-3, and LC-4 antiserum. *Lane 4*, autoradiograph of *lane 1*. *Lane 5*, autoradiograph of *lane 2*. *Lane 6*, autoradiograph of *lane 3*. *B*, interaction of LC-4 and REL1. *Lane 1*, Sypro Ruby-stained blot of SDS gel of clarified supernatant from the REL1 [^{35}S]methionine *in vitro* transcription/translation reaction. *Lane 2*, proteins released with SDS from protein G beads mixed with ^{35}S-labeled REL1 plus LC-4 antiserum. *Lane 3*, proteins released with SDS from protein G beads mixed with unlabeled LC-4, ^{35}S-labeled REL1, and LC-4 antiserum. *Lane 4*, autoradiograph of *lane 1*. *Lane 5*, autoradiograph of *lane 2*. *Lane 6*, autoradiograph of *lane 3*.

tightly bound and lost during the calmodulin binding step or that they are merely contaminants. The former possibility is consistent with the fact that sub-stoichiometric amounts of associated RET1 and MRP1/2 are greater in the TEV-released material than in the final calmodulin-released material (2) (data not shown).

Chemical Crosslinking of L-complex Proteins to LC-4 and Co-Immunoprecipitation Evidence for Interaction of LC-4, LC-3, and REL1—Proteins interacting with LC-4 were detected by chemical crosslinking of the 20 S L-complex gradient fraction and also by co-immunoprecipitation with anti-LC-4 antiserum. As shown in Fig. 3*A*, several crosslinks are visible in the LC-4 Western blot of the formaldehyde-crosslinked material. The second crosslink from the bottom (Fig. 3*A*, *left panel side*) was identified as REL1 by correspondence with the major adenylated band. There is a third crosslink after REL1. A similar pattern of crosslinking was seen using DMS in Fig. 3*B*. In this experiment, the extent of crosslinking was too low to allow Western analysis, but crosslinking of the adenylated REL1 in the DMS+ lane is clear.

Direct evidence for interactions of LC-4 and LC-3 and LC-4 and REL1 was obtained by co-immunoprecipitation. The TNT coupled transcription/translation system was used to obtain ^{35}S-labeled LC-3 and REL1 and unlabeled LC-4 proteins, which were used in this experiment. As described under "Experimental Procedures," the unlabeled LC-4 protein and the labeled LC-3 or REL1 proteins were incubated together, and then the LC-4 antibody was added, followed by protein G-Sepharose beads. The proteins bound to the beads were separated in an SDS gel, which was blotted, and the blot was stained and exposed to the PhosphorImager to detect the labeled protein. As shown in Fig. 4*A*, labeled LC-3 protein was only co-immunoprecipitated with anti-LC-4 antibody when unlabeled LC-4 was also present, indicating an interaction of LC-4 and LC-3. Similar results showing an interaction of LC-4 and REL1 are shown in Fig. 4*B*. These results confirm our previous identification of a subcomplex apparently containing LC-4, LC-3, and REL1, which was detected after overexpression of REL1-TAP in *L. tarentolae* (2) and also the co-immunoprecipitation and yeast two-hybrid results in the *T. brucei* system (20). These data do not eliminate the possibility that there may be additional proteins associated with the REL1 subcomplex.

Effect of Mutagenesis of the Two Zinc Fingers in the LC-4-TAP Protein on Cell Growth—The location of the two C2H2 zinc fingers is shown in the alignment of the LC-4 proteins from *L. major*, *L. tarentolae*, and the MP63 homologue from *T. brucei* (Fig. 5). The plasmid-encoded *L. tarentolae* LC-4-TAP protein was mutagenized by changing the two metal-binding cysteines to glycines in each motif. Cells were transfected and clonal lines selected that expressed the mutated tagged LC-4 proteins. As shown in Fig. 6, the ZnF-1 mutation was not lethal but did produce an increase in the cell division time from 6 to 8 h. The ZnF-2 mutants showed no change in growth as compared with wild type cells.

The ZnF-2 Mutation Causes a Partial Breakdown of the L-complex, and the ZnF-1 Mutation Causes a Major Disruption of the L-complex—Glycerol gradient sedimentation of mitochondrial extract from wt cells (Fig. 7, *panels 1–4*) and ZnF-2 mutant cells (Fig. 7, *panels 5–8*) showed that the wt LC-4-TAP protein was incorporated mainly into the 10 S L-complex but also into the 20 S L-complex as defined by the presence of the adenylated REL1 and REL2 ligase markers. Almost all of the untagged LC-4 was present in the 20 S L-complex in an ~1:1 ratio with the LC-4-TAP protein. The ZnF-2 mutation caused a partial breakdown of the REL1-containing 20 S L-complex into the 10 S L-complex and possibly a slight decrease in the Svedberg value of the remaining larger L-complex. This minor perturbation of the structure of the L-complex is consistent with the absence of a cell growth phenotype.

TAP isolation of the TAP-tagged LC-4 and associated proteins from the 10 and 20 S gradient fractions from ZnF-2 mutant cells yielded polypeptide profiles identical to that obtained with wt cells. The relative abundance of tagged and endogenous LC-4 proteins in both fractions (not shown) was also very similar to that of wt cells in Fig. 1*B* (data not shown).

Glycerol gradient sedimentation of mitochondrial extract from ZnF-1 cells indicated a major disruption of the L-complex in that the adenylated REL1 marker ligase sedimented in the 5–10 S region (Fig. 8, *panel 2*); in addition, the relative amount

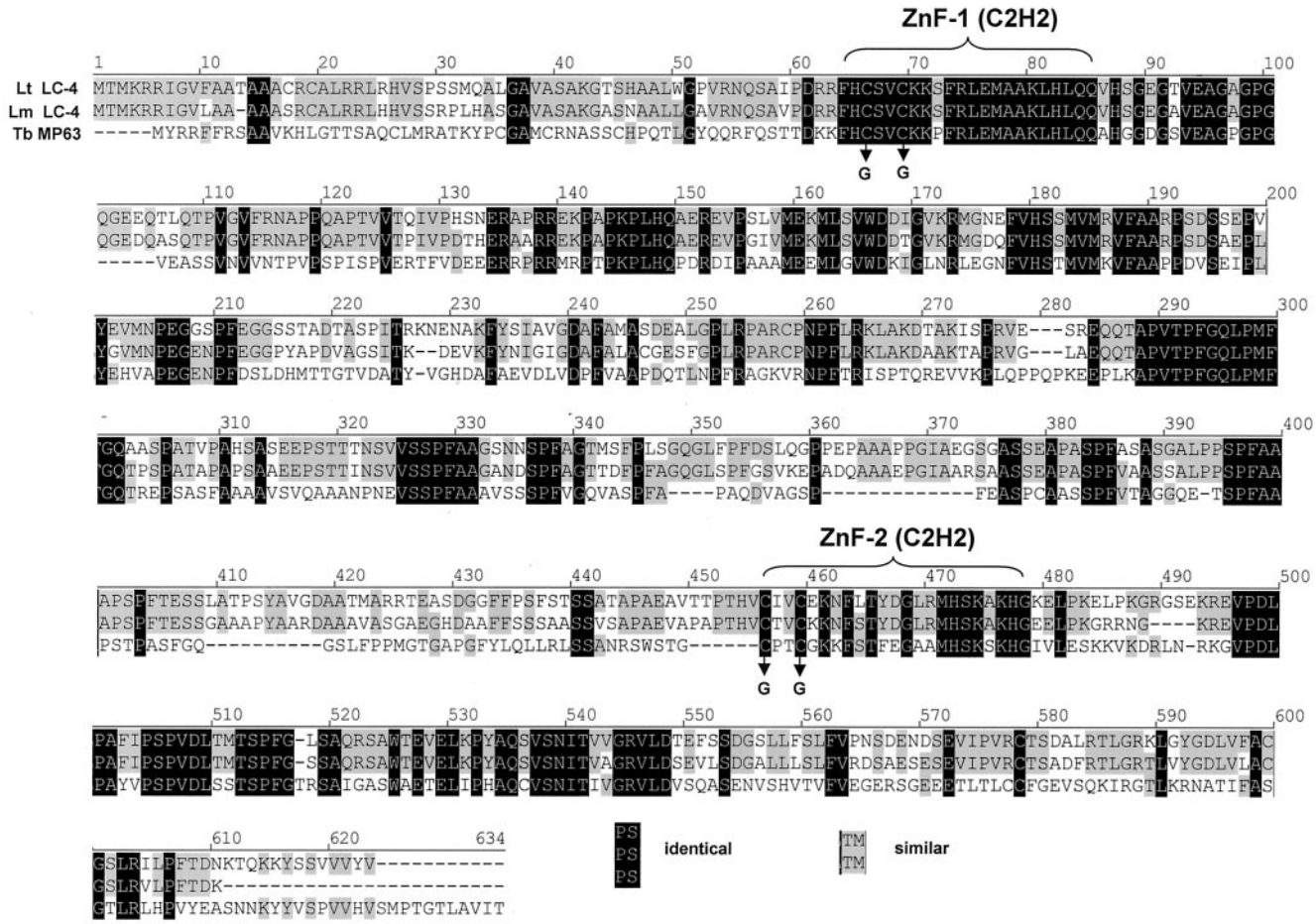

FIG. 5. **Alignment of LC-4 from *L. tarentolae* and *L. major* and the MP63 homologue from *T. brucei*.** The two zinc finger motifs are indicated as are the ZnF-1 and ZnF-2 mutations.

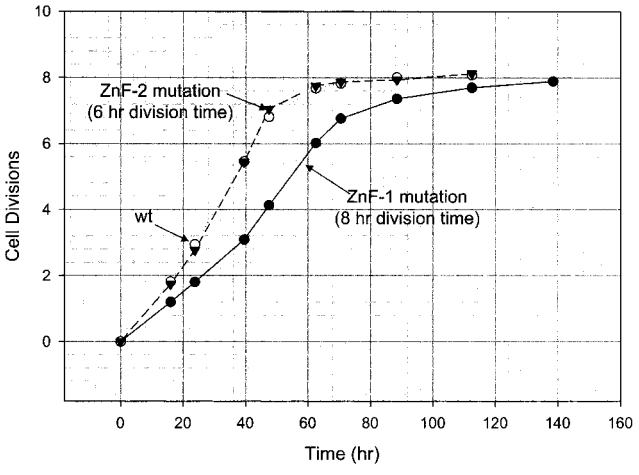

FIG. 6. **Growth curves of wt cells, ZnF-1 mutants, and ZnF-2 mutants.**

of LC-4-containing complexes decreased at least 1000-fold, and the LC-4-TAP decreased to below detection levels even using the femto-substrate for Western analysis (Fig. 8, *panel 1*). The remaining LC-4 containing material showed a broad distribution in the 5–10 S region and also a peak sedimenting several fractions slower than the 20 S L-complex peak obtained with the wt and LC-4 TAP cells, possibly suggesting a loss of the REL1 subcomplex prior to complete disruption.

DISCUSSION

The LC-4 (MP63) L-complex protein is one of three related L-complex proteins containing zinc finger motifs (2, 5). LC-4 has been shown previously to form a subcomplex with REL1 and LC-3 (2, 20), and the coimmunoprecipitation and chemical crosslinking results in this paper support this conclusion. Our crosslinking analysis also suggests that there may be a third, yet unidentified protein interacting with LC-4 in addition to REL1 and LC-3.

TAP-tagged LC-4 expressed from a plasmid was targeted to the mitochondrion and partially incorporated into the 20 S L-complex. ~80% of the LC-4-TAP protein was incorporated into a 10 S complex that also contained the REL1 ligase marker and was associated with the same set of proteins as the LC-4-TAP in the 20 S region. The endogenous LC-4 protein was found almost entirely in the 20 S L-complex in wt cells (21) and in the LC-4-TAP cells, but ~30% of endogenous LC-4 appeared in the 10 S region in the LC-4-TAP-ZnF-2 mutant cells.

The simplest explanation for these results is that there is a 20 S dimer that breaks down into a 10 S monomer due to the incorporation of the ZnF-2 mutated LC-4 into the complex. This model is consistent with the evidence that TAP-isolation of the 10 S gradient fractions yielded the same polypeptide profile as from the 20 S fractions. Also, the presence of the endogenous LC-4 in the TAP-isolated material suggests that at least two copies of LC-4 are present in the 20 S complex. However, the fact that the ratio of LC-4-TAP and endogenous LC-4 in the 20 S L-complex region was ~1:1, whereas the ratio in the TAP-isolated 20 S L-complex was ~9:1, suggests that the incorpo-

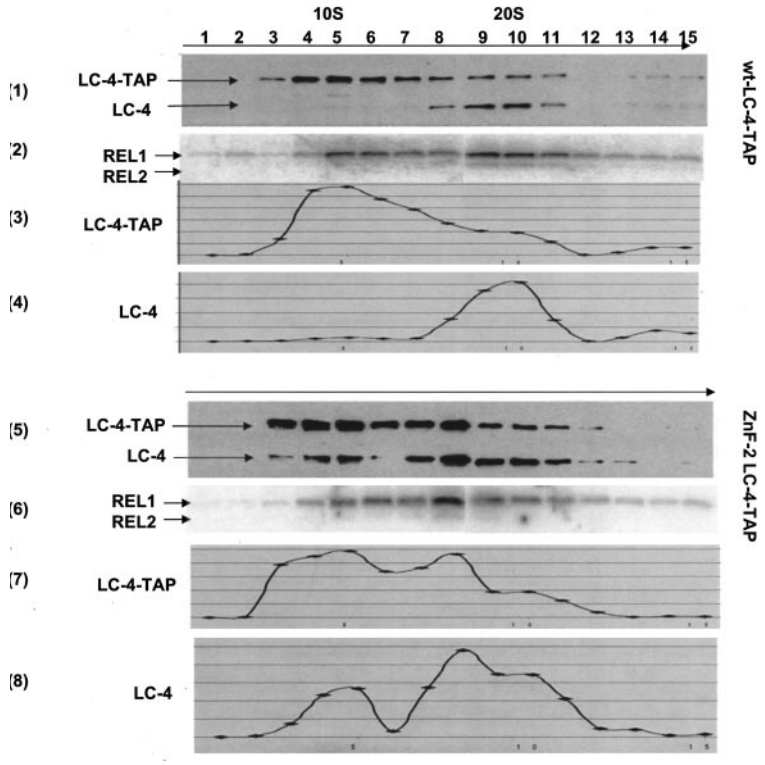

FIG. 7. **Glycerol gradient sedimentation of mitochondrial extract from LC-4-TAP-transfected wt cells (*panels 1–4*) and from the ZnF-2 mutant cells (*panels 5–8*).** Each fraction was adenylated with [α-^{32}P]ATP to label the RNA ligases and separated in an SDS gel, which was blotted for Western analysis with the anti-LC-4 antiserum using the SuperSignal West Pico chemiluminescent substrate (Pierce). Densitometer scans of the LC-4 and LC-4-TAP bands are shown in panels *3–4* and *7–8*.

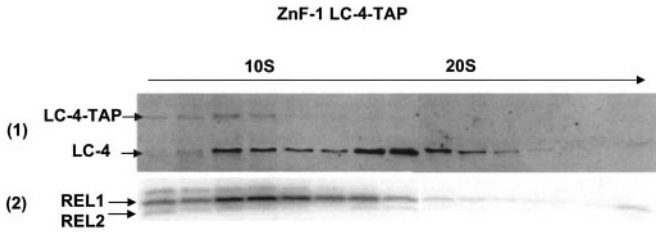

FIG. 8. **Glycerol gradient sedimentation of mitochondrial extract from LC-4-TAP-transfected ZnF-1 mutant cells.** See Fig. 7 legend for details. Identical amounts of mitochondrial extract were used as in Fig. 7, but the blot had to be developed using the SuperSignal West Femto maximum sensitivity substrate (Pierce) due to the low amount of protein.

ration of the LC-4-TAP protein into the L-complex specifically affects dimer formation with the wt L-complex. It is clear that the dimer-monomer model is suggestive but not conclusive and that the precise quaternary structure of the L-complex remains to be determined. In any case, the LC-4 protein appears to play a major role in determining the stability of the 20 S L-complex. Similar conclusions were reached by Huang *et al.* in 2002 (11), who showed that conditional RNA interference down-regulation of TbMP63 (=Band III) expression is lethal and causes a disruption of the L-complex.

It has been speculated that the three related zinc finger proteins in the L-complex serve a structural role in interacting with other proteins or with RNA (5). In this paper we show that both zinc finger motifs in LC-4 are functional in stabilizing the L-complex. Disruption of ZnF-1 produced a growth defect and an almost complete breakdown of the L-complex, and disruption of ZnF-2 showed no phenotype but did produce a partial disruption of the L-complex. It is tempting to speculate that ZnF-1 is involved with stability of the entire L-complex and that ZnF-2 is involved with dimerization, but further work must be performed to substantiate this. Our data indicates that these two motifs are at least involved in protein-protein interactions, but an additional role in RNA binding cannot be ruled out.

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