The sorting of membrane-bound proteins from the trans-Golgi network to lysosomal/endosomal compartments is achieved by preferential inclusion into clathrin-coated vesicles. Contained within the cytoplasmic domains of such proteins, specific sequence motifs have been identified (tyrosine-based and/or di-leucine-based) that are essential for targeting and are recognized by a family of heterotetrameric adaptor complexes, which then recruit clathrin. These cytosolic protein complexes, which have been found in a wide variety of higher eukaryotic organisms, are essential for the development of multicellular organisms. In trypanosomatids, the adaptin-mediated sorting of proteins is largely uncharacterized. In order to identify components of the adaptor-complex machinery, this study reports the cloning and characterization of \( \alpha \)-and \( \mu \)-adaptin gene homologues from the eukaryotic protozoan parasite, \textit{Leishmania mexicana}. Generation of \( \alpha \)- and \( \mu \)-adaptin gene deletion mutants shows that these promastigote parasites are viable in culture, but are unable to establish infection of macrophages or mice, indicating that adaptin function is crucial for pathogenesis in these unicellular organisms.

Several coat proteins have been described that are involved in the formation of carrier vesicles at different points in the secretory and endocytic trafficking pathways (1–3). Clathrin-coated vesicles (CCVs),\(^1\) which were the first coated transport vesicles to be identified, belong to one of the major classes of transport vesicles for the trafficking of proteins from the trans-Golgi network (TGN) and plasma membrane (PM) to the endosomal/lysosomal system (4). Essential to vesicle trafficking is the initiating step of cargo recognition by heterotetrameric adaptor protein (AP) complexes in association with regulatory molecules, followed by the recruitment of clathrin to the membrane for budding and vesicle formation (5, 6). Each heterotetramer of the TGN- and PM-associated AP complexes, AP-1 and AP-2 respectively, consist of two large adaptins (\( \gamma \) and \( \alpha \) are found together with \( \beta 1 \) and \( \beta 2 \), respectively, \( \sim 100 \text{ kDa} \)), one medium-sized adaptin (\( \mu 1 \) or \( \mu 2 \), \( \sim 50 \text{ kDa} \)) and one small adaptin (\( \alpha 1 \) or \( \alpha 2 \), \( \sim 20 \text{ kDa} \)) (2, 6, 7). The corresponding subunits of each AP complex are homologous to one another (25–84\% amino acid identity), which suggest functional similarity, and each adaptin has been shown to fulfill a different function. Their predominant role is as follows: the \( \beta \) subunits are important for clathrin binding (8, 9); \( \gamma \) and \( \alpha \)-adaptors target the AP complexes to specific membranes (10, 11); \( \mu \)-adaptors recognize and bind cargo for selection into CCVs via distinct sorting signals found in the cytoplasmic domains of certain transmembrane proteins: tyrosine-based motifs YXX\( \Phi \) (where \( \Phi \) is a bulky hydrophobic residue) or NPXY and di-leucine/acidic residues (12). To date there is no function assigned to the \( \sigma \) subunits. Recently, two structurally related AP complexes, AP-3 and AP-4 have been identified (2, 3). Previously, the functional roles of the AP complexes were based on biochemical and morphological experiments; however, more recent investigations have used targeted disruptions or naturally occurring mutants for the study of the physiological roles of these adaptor proteins (13). In contrast to \textit{Saccharomyces cerevisiae} where AP-1 and AP-2 are not essential for cell viability, it has been demonstrated that AP-1, although not required for cell viability in culture, is essential for the development of \textit{Caenorhabditis elegans} and mice, and AP-2 is necessary for \textit{C. elegans} embryonal development (14–17).

\textit{Leishmania} are kinetoplastid protozoan parasites that are responsible for several important human diseases, ranging from mild skin ulcers to fatal visceral disease. These organisms lead a digenetic life style, where several forms of extracellular, flagellated, motile promastigotes colonize the digestive tract of vector sandflies. Upon transmission to the mammalian host during bloodfeeding by the insect, the promastigotes transform to non-flagellated, intracellular amastigotes, which reside in the phagolysosomes of macrophages (18). Extensive studies have shown that the cell surface of both life cycle stages of these pathogens are coated with high levels of varying GPI-anchored glycoprotein, glycoconjugates, and glycolipids, which, depending on the \textit{Leishmania} species studied, are vital for survival and virulence in the harsh environments that are encountered by the parasite (19–21).

\textit{Leishmania} cells are highly polarized structures with an elongated shape in most life cycle stages. A microtubular corset lines the plasma membrane maintaining the morphology of the cell and appears to prohibit membrane fusion. All endocytosis and exocytosis occurs at an anterior specialized invagination of the cell surface membrane at the point of the emerging flagellum, termed the flagellar pocket (22, 23). Organelles involved in the secretory/endocytic pathways are located between the flagellar pocket and the nucleus (24). These morphological features make \textit{Leishmania} and related organisms, such as \textit{Trypanosoma brucei}, interesting models for protein trafficking.

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\( 1 \) The abbreviations used are: CCVs, clathrin-coated vesicles; TGN, trans-Golgi network; PM, plasma membrane; AP, adaptor protein; DIG, digoxigenin; ORF, open reading frame; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; WT, wild type; PV, parasitophorous vacuole.

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Adaptin Homologues of Leishmania mexicana

29401

studies. For both Leishmania and T. brucei, several transmembrane proteins have been identified that have the potential to be used in protein trafficking studies (23). In particular, a membrane-bound acid phosphatase of L. mexicana contains within its cytoplasmic domain both tyrosine-based and diisoleucine sorting signals (25) and in T. brucei, p67, a lysosomal membrane glycoprotein contains within its carboxy terminus two di-leucine motifs (26). Both of these proteins localize to endosomal/lysosomal compartments of each respective organism, but little is known about their intracellular trafficking. The p67 protein contains a sorting motif that suggests that the molecular machinery for adaptin-mediated sorting maybe conserved in these highly divergent group of unicellular eukaryotes. With the recent advent of the genome sequencing projects for both L. major and T. brucei, several sequences encoding potential components of the secretory/endocytic pathways have been identified. Homologues for several Rab proteins and clathrin of T. brucei are now being used as markers for the identification of subcellular compartments involved in protein trafficking (27, 28).

In order to identify components of the adaptor-complex machinery of L. mexicana, a PCR-based homology approach was used. This article describes the cloning and initial characterization of two AP complex subunits of L. mexicana that are potential α- and µ-adaptins, due to their significant homology to other adaptins of these classes.

EXPERIMENTAL PROCEDURES

Parasite Maintenance and Transfections—L. mexicana promastigotes (MNYC/B2/62/M379 strain) and derived gene deletion mutant lines were maintained in vitro at 27 °C in semidefined medium 79 (SDM-79) supplemented with 5% heat-inactivated fetal calf serum (Invi-trogen, Inc.) and 8 µg/ml hemin (Sigma). Transfections were performed as described previously (29), and recombinant clones were isolated by limiting dilution on 96-well plates in SDM medium containing the appropriate drug for the selectable markers, used at the following concentrations: 32 µg/ml hygromycin (Sigma), 2.5 µg/ml phleomycin (Sigma), and 80 µm puromycin (Sigma).

DNA Techniques—Restriction enzyme digests, DNA ligations, transformation of Escherichia coli, isolation of λ-phage and colony lifts, agarose gel electrophoresis, Southern blotting were performed according to standard methods (30). Large- and small-scale parasite genomic DNA were purified according to protocols previously described (31, 32). Large- and small-scale parasite genomic DNA: TA-cloning vector (Invitrogen) and sequenced. This DIG-labeled PCR product was used to screen a 3-µg DNA fragment, to introduce Clal and XbaI restriction enzymes sites for subcloning of the ORF into the pX6SPAC epozeral vector (34).

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Adaptin Homologues of Leishmania mexicana

the control of the rRNA promoter this blunt-ended Lmx1l-ADAPTIN ORF-containing DNA fragment was subcloned into CLoUl-Digested ends-filled-in pSSU-int plasmid (35). The integration cassette was excised by digestion with PacI and PmeI for transfection of L. mexicana promastigotes.

Antibodies—For high level expression and purification of L. mexicana ωl- and μ1-ADAPTIN recombinant proteins the pQE-8 vector (Qiagen) was used. In order to subclone the full-length ORFs into the BamHI/HindIII sites of this plasmid, PCR was carried out to introduce these restriction sites using the primer pairs: CCGGGATCCGATTT-CAGTTTCCTGCGG and AAGCTTCCAACCCCTGATGCGCGGT for Lmx1l-ADAPTIN and GGAATTCGATGGCGCGCGGTG and AAGCTTCCAACCCCTGATGCGCGGT for Lmx3-ADAPTIN. To avoid sequencing the entire amplified ORF, full-length ORF PCR product was replaced by the same DNA fragment excised from the genomic subclone. The sequences of these constructs were verified and used for transformation of competent E. coli M15 cells. Cell culture, induction of recombinant protein expression, and batch purification of solubilized inclusion bodies (8 mℓ/mL) by Ni-nitrilotriacetic acid agarose chromatography were performed according to the manufacturer’s instructions (Qiagen). Rabbit antisera were immunized with 200 μg of each purified recombinant protein or with 300 μg of a 15-residue peptide corresponding to the carboxyl terminus of Lmx1l-ADAPTIN (coupled to KLH (Calbiochem) via an inserted amino-terminal cysteine residue as described previously, Ref. 30), emulsified with 50% (v/v) complete Freund’s adjuvant for primary immunizations and with 50% (v/v) incomplete Freund’s adjuvant for all subsequent boosts. Polyclonal antisera were collected 14 days after each booster immunization. Anti-Lmx1l-ADAPTIN peptide antibodies were affinity-purified using as the ligand the peptide immobilized to SulfoLink™ coupling gel (Pierce) according to the supplier’s instructions. In order to remove unspecific antibodies, antisera raised against the L. mexicana ω1- and μ1-ADAPTIN full-length ORFs were absorbed using the respective L. mexicana-null mutant cell lines. Late logarithmic phase promastigotes were harvested, followed by a fixation step (rotating for 1 h at room temperature in PBS containing 0.05% glutaraldehyde and 2% formaldehyde), centrifuged at 30,000 × g for 1 h, permeabilized (rotating for 1 h at room temperature in PBS containing 1% skimmed milk powder, 0.5% bovine serum albumin, and 0.1% saponin) and centrifuged as before. The pellets were resuspended in the permeabilization buffer, and an aliquot of this was added to antiserum, rotated for 1 h at room temperature or overnight at 4 °C, followed by centrifugation. This latter step was repeated several times using a fresh portion of permeabilized cells added to the resulting supernatant. The final supernatant was ultracentrifuged at 140,000 × g for 30 min.

Analytic Procedures—To obtain protein for Western blot analysis, L. mexicana parasites were washed twice in PBS, followed by extraction of lipids by addition of CHCl3-CH3OH-H2O at a ratio of 1:2:0.8. Cells were immediately vortexed, incubated at room temperature for 30 min then centrifuged at 10,000 rpm in a benchtop microcentrifuge at room temperature. Solvents were removed from pellets by evaporation at 30 °C. This protein was then resuspended at the equivalent of 5 × 106 cells/ml in 50 mM Tris-HCl pH 8.0 containing 5 mM MgCl2, 0.5 mM phenylmethanesulfonyl fluoride, 20 μM leupeptin, 5 μM o-phenanthroline, and 100 units/ml benzonase (Merck). After incubation at 37 °C for 30 min to digest nucleic acids, one-fifth volume of 5× sample buffer (30) was added. Discontinuous SDS-PAGE on 4% stacking gels over 7.5–20% resolving gradient gels, electrophoresis of proteins onto polyvinyldene difluoride membranes (Millipore) and incubations of the membranes with primary and secondary antibodies were performed as described previously (36). Horseradish peroxidase-labeled antibodies were detected in the secondary system (Amersham Pharmacia Biotech) by using ECL detection kit. Molecular weight standards were run on the same gel.

The coverslips were mounted using Mowiol/Dabco and inspected by fluorescence microscopy. As duplicate infections were performed for each cell line, 300 macrophages were counted per coverslip, grouped according to parasite burden and the average taken.

RESULTS

Cloning of the L. mexicana ω1- and μ1-ADAPTIN Genes—For the cloning of the L. mexicana ω1-ADAPTIN gene, ω1-adaptin protein sequences from other organisms were used to find conserved blocks. A series of degenerate oligonucleotides were designed based on the peptide sequences QKGV/VFRL/TIQ/KRKY and VECDLIF and used in PCR with L. mexicana genomic DNA as the template. Sequencing of the amplified DNA identified a partial ORF with high homology to σ-adaptins. This PCR product was DIG-labeled and used to screen a λ-DashII L. mexicana genomic DNA library. Sequencing of a Lmx1l-ADAPTIN gene-containing subcloned DNA fragment showed the presence of an ORF of 495 base pairs (bp, Fig. 2C) encoding a protein of a predicted molecular mass of ~19.2 kDa (Figs. 1A and 3A) and a calculated isoelectric point of 7.9. Data base searches with the Lmx1l-ADAPTIN ORF displayed significant homology of this sequence with other AP complex σ-adaptins from various organisms. Phylogenetic analysis of these sequences showed that the Lmx1l-ADAPTIN ORF is grouped within the ω1 family of adaptins. The compiled sequences of this group are shown in Fig. 1A, where the sequence identities range between 44 and 52% from S. cerevisiae to H. sapiens. All of these sequences contain a segment that is considered a signature for the small chains (σ) of adaptor-like complexes, according to the PROSITE data base (indicated by a line in Fig. 1A).

A search of the Leishmania major sequencing project data base revealed a partial μ1-adaptin sequence that was used to synthesize degenerate primer pairs for the cloning of the Lmx3l-ADAPTIN gene. PCR was performed using these primers with L. mexicana genomic DNA as the template. Sequencing of the amplified DNA identified a partial ORF with high homology to other μ-adaptins. This PCR product was DIG-labeled and used to screen a λ-DashII L. mexicana genomic DNA library. An ORF of 1299 bp (Fig. 2D) was identified upon sequencing the Lmx3l-ADAPTIN gene-containing DNA fragment. This protein had a calculated molecular mass of ~49.1 kDa (Figs. 1B and 3B) and a pI of 6.9. Phylogenetic analysis grouped the Lmx3l-ADAPTIN ORF within the μ1-adaptin family with amino acid identities of 37–46% from A. thaliana to Drosophila melanogaster. Sequence alignments of this group are shown in Fig. 1B where the signature sequences for the medium chains (μ) of adaptor-like complexes are overlined (according to the PROSITE data base).

Targeted Gene Replacement of L. mexicana ω1- and μ1-ADAPTINS—Southern analysis with a range of restriction enzymes showed that the Lmx1l- and μ1-ADAPTIN genes are pres-
Adaptin Homologues of Leishmania mexicana

29403

sequences and those shown in asterisks represent spaces inserted for maximum alignment. Residues that are

Sequences were aligned using the PILEUP program, where

dashes denoted null mutant cell lines (Fig. 3, lane 2

otes (see Fig. 3, lane 2). All clones showed normal growth in standard culture medium compared with the parental WT strain or complemented null mutant cell lines (data not shown).

Biological Characterization of the L. mexicana α1- and μ1-ADAPTIN Gene Products—For the characterization of the Lmx1-1 and μ1-ADAPTIN gene products, rabbit polyclonal antibodies were raised against 15 residues corresponding to the

47 kDa was recognized in WT promastigotes, which was absent in Δα1 cells (see Fig. 3A, lanes 2 and 3). No protein was detected in Δμ1 parasites using anti-Lmx1-1-ADAPTIN antiserum, but a molecular mass species of ~47 kDa was recognized in WT promastigotes (see Fig. 3B, lanes 2 and 3). Both of these adaptins units were found to be of similar abundance and the same, respective molecular weight in both life cycle stages (Fig. 3, A and B, lanes 1 and 2). By comparison of the signal generated on immunoblot from a known number of parasites to that obtained for each recombinant protein as a standard, both Lmx1-1 and μ1-ADAPTIN were estimated to be present at ~1 × 10^4 copies per Leishmania cell (data not shown).

It appeared that with loss of the Lmx1-1-ADAPTIN gene, the stability of Lmx1-1-ADAPTIN was affected, as only low quantities of this protein were detected in Δμ1 parasites using anti-Lmx1-1-ADAPTIN antiserum, but expression was restored in the complemented null mutant cell line (Δμ1+cRIBμ1) (Fig. 3A, lanes 6 and 7). In contrast, Lmx1-1-ADAPTIN was detected in Δα1 cells at levels similar to WT and the Δα1+cRIBα1 complemented null mutant cell lines (Fig. 3B, lanes 2, 6, and 7).

To assess whether the Lmx1-1 and μ1-ADAPTINS were either

ent at one copy per haploid genome, as all the hybridizing fragments could be accounted for by the restriction maps of both loci (Fig. 2). This observation made it feasible to investigate the phenotypic effect of creating null mutants for these gene products. Two rounds of targeted gene replacement for the α1- and μ1-ADAPTIN genes were performed on wild-type (WT) L. mexicana, using the antibiotic resistance markers HYG and PHLEO (Fig. 2, C and D). A series of clones were isolated that lacked both alleles of the α1-ADAPTIN ORF (L. mexicana

Δα1:HYGΔα1::PHLEO, further on referred to as Δα1) (Figs. 2A, lane 13 and 3A, lane 3) or the μ1-ADAPTIN ORF (L. mexicana Δμ1:HYGΔμ1::PHLEO, hereafter referred to as Δμ1) (Figs. 2B, lane 9 and 3B, lane 3). All clones showed normal growth in standard culture medium compared with the parental WT strain or complemented null mutant cell lines (data not shown).
The sizes of DNA standards are indicated in kilobases. A, lanes 1–11: wild-type L. mexicana chromosomal DNA digested with the restrictions enzymes *Apa*I (1), *Ave*I (2), *Eco*RI (3), *Eco*RII (4), *Hin*dIII (5), *Not*I (6), *Pst*I (7), *Sac*I (8), *Sal*I (9), *Xba*I (10), *Xho*I (11). Lanes 12–15, genomic DNAs digested with *Apa*I derived from the cell lines: wild-type L. *mexicana* (12), Δσ1 (13), Δσ1 + pXμ1 (14), Δσ1 + pXμ1 (15). B, lanes 1–7 wild-type L. *mexicana* DNA digested with the restrictions enzymes *Hind*III/ *Eco*RI (1), *Eco*RI (2), *Eco*RII (3), *Hin*dIII (4), *Nru*I (5), *Pst*I (6), *Pvu*II (7). Lanes 8–11, genomic DNAs digested with *Pvu*II derived from the cell lines: wild-type L. *mexicana* (8), Δμ1 (9), Δμ1 + pRIBμ1 (10), Δμ1 (11). C and D, restriction maps of the *Lmxa*1- and Δμ1-ADAPTIN loci where the ORFs are highlighted in black, and restriction sites relevant for Southern blot analyses are indicated. The resistance genes HYG and PHLEO and the 5'- and 3'-flanking regions (marked by dashed lines) used for the construction of gene deletion cassettes are also shown.

cytosolic or membrane-associated, WT promastigotes were disrupted in a KCl-containing buffer and both of these proteins were found to partition into both fractions (Fig. 3C). In an attempt to study the nature of the association of these proteins with membranes, the total membrane fraction was incubated with different concentrations of salts and detergents, however the membrane forms of these proteins were resistant to extraction (data not shown). In contrast, lysis of the WT promastigotes in the presence of a KCl- and 0.8 M Tris-HCl (pH 7.5)-containing buffer released almost the total of the cellular pool into the soluble fraction (Fig. 3D).

The antisera failed in immunolocalization experiments, as staining of WT cells was not significantly different to that observed with the null mutant cell lines. This may be due to the low abundance of antigens or to a poor recognition of the native proteins by antibodies raised against the recombinant proteins.

Attempts to Demonstrate *LmxMBAP* Adaptor Complex Interaction—With the finding that *LmxMBAP* contains putative sorting signals in its carboxyl terminus, two alternative approaches for the detection of adaptor complex subunits were taken. First, a biochemical affinity purification strategy was used. Using a synthetic peptide corresponding to the last 20 amino acids of the *LmxMBAP*, which contains the sorting signals YMKF and II, coupled to an affinity matrix via an inserted amino-terminal cysteine residue, following binding of *Leishmania* promastigote cytosolic extracts, eluted proteins profiles were assessed for potential binding proteins. However, by comparison to eluates from a glutathione-Sepharose column, used as a negative control, no novel bands were detected which could be used for further analysis. Second, the yeast two-hybrid system was used with the cytoplasmic domain of *LmxMBAP* as bait to screen both promastigote and amastigote cDNA libraries. These experiments proved to be unsuccessful as many false-positives were identified with sequencing of isolated colonies. Once the deletion mutants became available, it could be demonstrated by immunofluorescence studies that the absence of the *Lmxa*1- and Δμ1-ADAPTINS did not change the endosomal/lysosomal localization of *LmxMBAP* (data not shown and see Ref. 25). It may be added, that characterization of the Δσ1 and Δμ1 cell lines for the presence of the GPI-anchored surface molecules, which have been implicated as being important for virulence, LPG, GP63, and PPGs (19, 21, 37), was performed using immunofluorescence with a panel of monoclonal antibodies directed against epitopes of these molecules (described in Ref. 38). By comparison to WT cells, the null mutants also displayed these molecules on their surface (data not shown).

Null Mutant Δσ1 and Δμ1 *L. mexicana* Parasites Are Not Infectious for Macrophages or Mice—By comparison to WT *L. mexicana* parasites, Δσ1 and Δμ1 promastigotes were unable to establish infection of *in vitro* cultured macrophages (Fig. 4, A and B). This effect was directly correlated with the loss of the *L. mexicana* σ1- and μ1-ADAPTIN gene products as shown by the ability of the complemented null mutant cell lines to infect the host cells. In the case of the complemented Δσ1 mutants, expression under the control of the ribosomal promoter (Δσ1 + pRIBr1) showed that 98% of the macrophages were parasitized, whereas only 62% of the macrophages were infected with the episomally complemented Δσ1 mutant.
Adaptin Homologues of Leishmania mexicana

The results obtained in this study show the identification and initial characterization of two adaptin homologues of the pathogenic, protozoan L. mexicana. The significant sequence identities of these two ORFs to α1- and μ1-adaptins from other organisms (Fig. 1, A and B) and phylogenetic analyses which group these proteins within these classes of adaptins suggest that these subunits are potential components of a L. mexicana AP-1 complex homologue that by analogy to other heterotetrameric AP-1 complexes would be involved in the formation of clathrin-coated vesicles at the TGN.

The high degree of α1-adaptin sequence conservation implicates an important functional role of this subunit within the AP complexes. Although it has been proposed that α1-adaptins may be involved in targeting of the complexes to appropriate membranes (11), a definitive role has yet to be determined. The ORFs of the α1-adaptins contain several conserved blocks, which are also present in the L. mexicana α1-adaptin primary sequence. One of these segments conform to the consensus, which is considered a signature for the α1-adaptins according to the PROSITE data base (Fig. 1A).

The μ-adaptins have been implicated in cargo selection by recognition of distinct sorting signals found within the cytoplasmic tails of certain transmembrane proteins (12). Resolution of the crystal structure of the μ2-adaptin complexed to the tyrosine motif, YXXΦ, has identified the domains involved in signal recognition that form a hydrophobic pocket into which the Y and Φ residues fit (42). These amino acid segments are

**DISCUSSION**

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FIG. 4. Analysis of macrophage and mouse infections by promastigotes of *L. mexicana* WT, Δσ1, and Δμ1 and the respective complemented null mutant cell lines. Peritoneal macrophages were infected at a ratio of two stationary phase promastigotes per cell. The percentage of parasitised host cells (sample size 300) was counted 6-days postinfection. The bars represent the average of duplicate determinations, and S.E. are indicated. A, infection of peritoneal macrophages by *L. mexicana* WT, Δσ1, Δσ1+cRIBσ1 and Δσ1+pXσ1. B, infection of peritoneal macrophages by *L. mexicana* WT, Δμ1, Δμ1+cRIBμ1, and Δμ1+pXμ1. Time course of infection of peritoneal macrophages after challenge by *L. mexicana* WT, Δσ1, Δσ1+cRIBσ1 (C) and WT, Δμ1, and Δμ1+pXμ1 (D). The ratio of infected to uninfected macrophages was determined at days 1, 2, 3, 4, 5, and 7 postinfection. For mouse infections, Balb/c mice were challenged with $10^7$ promastigotes in the left hind footpad. The swellings caused by *L. mexicana* WT, Δσ1, Δσ1+cRIBσ1, and Δσ1+pXσ1 (E) and *L. mexicana* WT, Δμ1, Δμ1+cRIBμ1, and Δμ1+pXμ1 (F) were measured relative to the uninfected right hind footpad. These experiments were performed in duplicate, using four mice in each group, and S.E. are shown.
conserved in the $\mu_2$ subunits from all species. The binding domains present in the $\mu_1$-adapts are very similar, therefore it has been postulated that one amino acid change may alter the binding affinity and specificity for the X-residue. Examination of the $L.\text{mexicana}$ $\mu$-ADAPTIN primary sequence shows the presence of many highly conserved sequence blocks found in $\mu$-adapts of other organisms, including the PROSITE signature sequence, and amino acids involved in sorting motif interaction are identical to the class of $\mu_1$-adapts from other organisms. Based on these criteria, the $L.\text{mexicana}$ $\sigma$- and $\mu$-ADAPTINS identified in this study have been assigned $Lm\sigma_1$- and $Lm\mu_1$-ADAPTINS (according to the genetic nomenclature for Leishmania stipulated by Ref. 43). The number of molecules per $Leishmania$ promastigote for each adaptin was estimated at $\approx 1 \times 10^4$, and 50% of this amount was found to be in the membrane-associated pool. Therefore this amount appeared to be too low for detection by the antisera used in localization studies.

One feature of coat proteins is their ability to cycle between cytosolic and membrane-bound pools (1). The nature of association of AP complexes with membranes has been investigated by extracting membrane fractions with varying concentrations of different salts and detergents, where the results obtained indicated that the complexes behave as peripheral membrane proteins (44). The findings reported here showed that both $Lm\sigma_1$- and $Lm\mu_1$-ADAPTINS were found to be partially associated with membranes. However unlike the adaptins studied in other organisms, the $L.\text{mexicana}$ adaptins were not susceptible to dissociation from membranes after treatment with moderate concentrations of salt or detergent (Fig. 3, C and D and data not shown). Most of the total cellular pool could be solubilized in the presence of 0.8 M Tris-HCl (pH 7.5), and this must be a specific Tris effect rather than that of an elevated ionic strength, as NaCl had no effect in releasing these $L.\text{mexicana}$ adaptins from the membrane.

Studies performed by disrupting the $S.\text{cerevisiae}$ AP subunits genes, of which there are 13 genes encoding homologues of the heterotetrameric complexes to assemble three AP complexes with one extra $\mu$ chain, showed that the deletion strains did not display any discernible phenotype (14). It was therefore proposed that, for these mutant unicellular eukaryotes, alternative mechanisms are used for sorting and coated vesicle formation. It appears also that this is the case for both $Lm\sigma_1$- and $Lm\mu_1$-ADAPTINS, as it was possible to generate null mutants of both of these genetic loci and obtain viable promastigotes.

In contrast, studies on disrupting AP complex genes in higher eukaryotes has led to the conclusion that these heterotetramers are essential for the development of multicellular organisms (13). In particular, targeted disruption of the mouse $\mu_1A$-adaptin gene resulted in embryonic lethality (45). It was
found that no free γ-, β-, or α1-subunits were present in fibroblasts and γβ1-α1 subcomplexes were unable to associate with membranes, which has been suggested to cause the rerouting of mannosese 6-phosphate receptors. Interestingly, these μ1A-adaptin cells showed reduced expression levels of α1-adaptin, which indicate reduced stability of free adaptins. The results obtained in this study showed that disruption of the \( Lmx1-\)ADAPTIN gene resulted in the down-regulation of \( Lmx1-\)ADAPTIN protein, as only minute amounts of this adaptin were detected in the Δμ1 cell line. Re-introduction of the \( Lmx1-\)ADAPTIN gene into Δμ1 cells restored the expression of the \( Lmx1-\)ADAPTIN protein to WT levels (Fig. 3, A and B). This observation suggests that both of these \( L. \) mexicana adaptins are components of the same AP complex. Overexpression of both \( Lmx1-\) and μ1-ADAPTIN genes, from either an episome or under the control of the ribosomal promoter, did not result in large quantities of protein detected, but rather WT levels were present in these cell lines (Fig. 3, A and B), which suggests that the excess proteins not incorporated into an AP complex are degraded.

The \( Lmx1-\) and μ1-ADAPTINS were dispensable for growth of promastigote stage parasites; however, the function(s) of these proteins were clearly essential for transformation to amastigotes or proliferation of these mammalian stage cells, as the null mutant promastigote cell lines, Δμ1 and Δμ1, were unable to establish infection when introduced into macrophages or mice (Fig. 4). That the Δμ1 and Δμ1 parasites retained the ability to invade macrophages is shown by the kinetic experiments performed, however, with time the invading Δμ1 and Δμ1 parasites were killed and cleared by the host cells, whereas WT and complemented null mutant (Δμ1+μ1RIB1 and Δμ1+μ1RIB1) cells survived and proliferated (Figs. 4, C and D). The start of vacuole formation is observed in macrophages colonized by WT and complemented null mutant cells on day 3. In contrast, Δμ1 and Δμ1 parasites appear unable to induce PV formation and were rapidly cleared from macrophages. In comparison to WT cells, both these null mutant cell lines also displayed the GPI-anchored molecules on their surface, which have been proposed to be required for infection, and it is unlikely that these molecules would require adaptin-mediated sorting. It must be noted that for \( L. \) mexicana, absence of LPG, GP63, PPGs, and GIPs do not render these parasites avirulent for mice (20, 38, 40, 41).

Infection of mice with the Δμ1 and Δμ1 cell lines showed that in comparison to WT and the respective complemented cell lines, the null mutants were impaired in lesion formation (Figs. 4, E and F). For the Δμ1+μ1RIB1- and Δμ1+μ1RIB1-complemented cell lines although the mice showed lesion formation, the onset and rate of disease progression was lower in comparison to the WT control. In vivo, expression of the μ1-ADAPTIN may be lower without selection pressure, which would result in reduced amounts of α1-ADAPTIN, as Western blot analysis showed that lower levels of α1-ADAPTIN were detected in the Δμ1 cell line (Fig. 3A). Therefore in the complemented Δμ1 cell lines, the slower progression of disease may be due to the overall effect of reduced amounts of α1- and μ1-ADAPTINS. In addition, several reports have shown that, for reasons unknown, complementation systems only give qualitative information when compared to infection with wild-type parasites (39, 46–48).

By analogy to heterotrimeric AP-1 complexes in other organisms, it is most likely that the \( Lmx1-\) and μ1-ADAPTINS would play a functional role for sorting of proteins at the TGN for delivery to the endosomal/lysosomal system. However, it is difficult to speculate which parasites proteins need to be accurately sorted that are required for transformation and proliferation of amastigotes. Initially, it was assumed that these adaptin subunits may be involved in the sorting of the \( Lmx\)BAP due to potential sorting signals contained within its cytoplasmic domain (25), but localization studies have shown that this protein is delivered to the endosomal/lysosomal network in both the Δμ1 and Δμ1 cell lines, as in WT parasites. Although the \( Lmx\)BAP is expressed in both life cycles stages of \( L. \) mexicana, parasites that lack this protein still retain the ability to cause disease in mice (49). It is also unlikely that the \( Lmx1-\) and μ1-ADAPTINS would play an essential role in the sorting of cysteine proteases to megasomes, which have been characterized in \( L. \) mexicana, as parasites lacking these proteins are able to establish infection in mice (46).

The results obtained in this study lead to the conclusion that like yeast, \( L. \) mexicana promastigotes maintained under laboratory conditions do not require α1- and μ1-ADAPTIN function for viability. However, with similarity to multicellular organisms where it is essential that multiple transport and sorting events are highly accurate for development, introduction of these parasites into the mammalian host leads to a stringent requirement for the α1- and μ1-ADAPTINS during differentiation or proliferation or both, which is required for infectivity of these unicellular, pathogenic organisms. A better biochemical and functional characterization of the AP-1 complex containing the \( Lmx1-\) and μ1-ADAPTINS may require the simultaneous overexpression of all four subunits either in \( L. \) mexicana or a heterologous system.

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Adaptin Homologues of Leishmania mexicana

σ1- and μ1-Adaptin Homologues of *Leishmania mexicana* Are Required for Parasite Survival in the Infected Host

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