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 adaptor-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, *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), 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 \( \alpha \)) together with \( \beta_1 \) and \( \beta_2 \), respectively, \(-100 \text{ kDa}\), one medium-sized adaptin (\( \mu_1 \) or \( \mu_2 \), \(-50 \text{ kDa}\)) and one small adaptin (\( \sigma_1 \) or \( \sigma_2 \), \(-20 \text{ kDa}\)) (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 \( \alpha \)-adapts target the AP complexes to specific membranes (10, 11); \( \mu \)-adapts 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 *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 *Caenorhabditis elegans* and mice, and AP-2 is necessary for *C. elegans* embryonal development (14–17).

*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 *Leishmania* species studied, are vital for survival and virulence in the harsh environments that are encountered by the parasite (19–21).

*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 *Leishmania* and related organisms, such as *Trypanosoma brucei*, interesting models for protein trafficking...
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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 carboxyl 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 presence of these sorting motifs suggests that the molecular machinery for adaptin-mediated sorting may be 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 α1- and μ1-adaptins, due to their significant homology to other adaptins of these classes.

**EXPERIMENTAL PROCEDURES**

**Parasite Maintenance and Transfections—**L. mexicana promastigotes (MYNC/BZ/2/2/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- 

tron, Inc.) and 8 μg/ml hensin (Sigma). Transfections were performed as described previously (29), and recombinant clones were iso-

lated by limiting dilution on 96-well plates in SDM medium containing the appropriate drug for the selectable markers, used at the following concentrations of 32 μg/ml hygromycin (Sigma), 2.5 μg/ml phleomycin (Sigma), and 80 μM purines (Sigma).

**DNA Techniques—**Restriction enzyme digests, DNA ligations, trans-
formation 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). Plasmid DNA and DNA fragments from agarose gels were isolated using commercial kits according to the manufacturer’s instructions (Qiagen). Polymerase chain reactions (PCR) were performed using the Expand High Fidelity PCR system (Roche Applied Science). All PCR products were subcloned into the TA cloning vector (Invitrogen) and sequenced by the dyeoxy chain termination method using an ALFExpress automated sequencer (Amersham Biosciences). DNA probes were generated using a PCR-DIG labeling kit (Roche Applied Science). On nucleic acid blots the labeled DNA was detected using anti-DIG-Fab fragments coupled to alkaline phosphatase (Roche Applied Science) and CDP-Star as the chemiluminescent substrate according to the manu-

facturer’s instructions.

**Cloning of the L. mexicana 1-ADAPTIN Gene and Generation of Deletion and Addback Constructs—**The Block Maker program (www.

blocks.fhcrc.org) was used to align ungapped, highly conserved regions of the α1 subunit amino acid sequence from the organisms (SWISS- 

PROT accession codes given in parentheses): Homo sapiens (P56377), Mus musculus (Q00382), S. cerevisiae (P35181), and Arabidopsis thali-

ana (AAO86887). Peptide sequences from the resulting conserved blocks were used to design a series of degenerate primers: 5′-CA/GA/G-

GG/GA/CT/TA/A/A/I/GTI/TG/GA/G/C/T/G/T/T/G/A/I/G/C/G/A/I/G/A/G/TT/GA, CA/I/G/A/G/A/G/CT/TA/A/A/I/GTI/TG/GA/G/C/T/T/G/A/I/G/A/G/TT/GA, CA/I/G/A/G/A/G/CT/TA/A/A/I/GTI/TG/GA/G/C/T/T/G/A/I/G/A/G/TT/GA, and 3′-GA/G/TT/GA/CT/T/T/G/A/G/C/T/G/A/I/G/A/G/TT/GA. PCR was performed using this mixture of oligonucleotides with L. mexicana genomic DNA, and the derived 284-bp fragment was subcloned into the TA cloning vector (Invitrogen) and sequenced. This DIG-labeled PCR product was used to screen a λ-Dash II L. mexicana genomic DNA library (25), and a 3.3-kb NotI fragment from positive clones was subcloned into pBSK+ (Strat-

gen) and sequenced on both strands. Using data base searches the ORF corresponding to Lmx1-ADAPTIN was identified by homology to other known σ-adaptins. The sequence data for the Lmx1-ADAPTIN-containing genomic DNA fragment has been submitted to the DDBJ/ 

EMBL/GenBank™ data base under accession code AF514805. For the derivation of double targeting gene replacement cassettes, PCR was carried out for the 5′-upstream region of Lmx1-ADAPTIN using the primers GTCGACGGCAGCCGGCGTCGCGCG and CCATGGCATGGCCACCGCCGCTGAC (where a Nco restriction site was intro-

duced at the translation initiation codon of Lmx1-ADAPTIN ORF), and for the 3′-downstream region of Lmx1-ADAPTIN using the primers TCGAGATCTCATGAGTTCGATC and TCTTAGATCATACACCTTGGATGC and a Lmx1-ADAPTIN gene containing DNA fragment, to introduce ClaI and XbaI restriction en-

zymes sites for subcloning of the ORF into the pXS6PAC episomal vector (34).

For protein expression under the control of the RNA polymer, PCR was performed with the following primers: ATCGATATGATCATCCGTG and TCTTAGATCATACACCTTGGATGC and a Lmx1-ADAPTIN gene containing DNA fragment, to introduce ClaI and XbaI restriction en-

zymes sites for subcloning of the ORF into the pXS6PAC episomal vector (34). For protein expression under the control of the RNA promoter, PCR was performed with the following primers: ATCGATATGATCATCCGTG and TCTTAGATCATACACCTTGGATGC and a Lmx1-ADAPTIN gene containing DNA fragment, to introduce ClaI and XbaI restriction en-

zymes sites for subcloning of the ORF into the pXS6PAC episomal vector (34). The integration cassette was excised by digestion with PacI and PmeI for transfection of L. mexicana promastigotes containing the HYG- and PHLEO- Lmx1-ADAPTIN gene replacement cassettes were excised by NcoI digestion. For episomal and chromosomal protein expression in the gene deletion mutant background, vectors containing the ORF were con- 

structed. The Lmx1-ADAPTIN ORF obtained from the PCR (see “Materials and Methods” below), was excised from the TA cloning vector by BamHI digestion and subcloned into the pXS6PAC episomal vector (34). For protein expression under the control of the RNA polymer, PCR was performed with the following primers: ATCGATATGATCATCCGTG and TCTTAGATCATACACCTTGGATGC and a Lmx1-ADAPTIN gene containing DNA fragment, to introduce ClaI and XbaI restriction en-

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zymes sites for subcloning of the ORF into the pXS6PAC episomal vector (34).
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the control of the rRNA promoter this blunt-ended LmxL1-ADAPTIN ORF-containing DNA fragment was subcloned into Cia/IIdigested ends-filled in psSsu-int plasmid (35). The integration cassette was excised with digestion with PcaI and PmeI for transfection of L. mexicana promastigotes.

Antibodies—For high level expression and purification of L. mexicana orL and or1-ADAPTIN recombinant proteins the pQE-8 vector (Qiagen) was used. In order to subclone the full-length ORFs into the BumIII/HindIII sites of this plasmid, PCR was carried out to introduce these restriction sites using the primer pairs: CCGGGATCCGATTT-CAGTTTGGTGC and AAGGTTTCAACCTTTGGATGCGGTT for LmxL1-ADAPTIN and GGATCCGGCGTCTGCTGAGTAA and AAGGTTTCAACCTTTGGATGCGGTT for LmxL1-ADAPTIN. To avoid sequencing the entire amplified ORF recombinant ORF and the 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 μg/ml) by Ni-nitrilotriacetic acid-agarose chromatography were performed according to the manufacturer’s instructions (Qiagen). Rabbits 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 LmxL1-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-LmxL1-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 orL- and or1-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. The protein was then resuspended at the equivalent of 5 × 105 cells/ml in 50 mM Tris-HCl pH 8.0 containing 5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 5 μM o-phenanthroline, and 100 units/ml benzamidine (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 polyvinylidene 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 using ECL system (Amersham Biosciences) and the number of bands for re-probing were both carried out according to the manufacturer’s instructions.

Infections of Mice and Peritoneal Macrophages—For mouse infection studies, groups of four Balb/c mice were used for each cell line. These experiments were performed in duplicate. The left hind footpad was infected with 107 promastigotes per day. Macrophage infection experiments, peritoneal cells were isolated from Balb/c mice by peritoneal lavage and seeded onto coverslips (13-mm diameter) at 2.5 × 107 to 107 cells per coverslip. DMEM, Dulbecco’s modified Eagle’s medium (DMEM) or a medium (50:50), which contained 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin/streptomycin, and 2 mM glutamine. After overnight incubation at 37 °C with 5% CO2 in air, non-adherent cells were removed by three washes of the coverslips with pre-warmed complete DMEM. Approximately 50% of the cells, which had adhered to the coverslips, judged as peritoneal macrophages by their morphology, were infected with stationary phase promastigotes, which had been washed and resuspended in complete DMEM, at a parasite to macrophage ratio of 2:1. Two coverslips were used per cell line. Following incubation overnight at 33 °C with 5% CO2 in air, residual-free promastigotes were removed by three washes using pre-warmed complete DMEM. Incubation at 33 °C with 5% CO2 in air was continued and coverslips removed at the required time points, washed with pre-warmed PBS, and fixed and stained with DAPI (see below). The number of parasitized macrophages and the number of L. mexicana amastigotes per host cell were counted by inspection with a fluorescence microscope.

Immunofluorescence Microscopy— Coverslips containing parasitised peritoneal macrophages were washed three times with pre-warmed PBS, fixed by submerging in PBS containing 2% formaldehyde and 0.05% glutaraldehyde for 30 min, washed twice with room temperature PBS, incubated at room temperature for 30 min with DAPI (10 μg/ml) in blocking solution of PBS containing 2% bovine serum albumin and 0.05% NH4Cl, followed by three washes with room temperature PBS. The coverslips were mounted using Mowiol/Dabco and inspected by fluorescent 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 orL- and or1-ADAPTIN Genes—For the cloning of the L. mexicana orL-ADAPTIN gene, orL-adaptin protein sequences from other organisms were used to find conserved blocks. A series of degenerate oligonucleotides were designed based on the peptide sequences QGK/V/F/R/L/I/I/K/R/W 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 L. mexicana genomic DNA library. Sequencing of a LmxL1-ADAPTIN gene-containing subcloned DNA fragment showed the presence of an ORF of 495 base pairs (bp, Fig. 2C) coding 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 LmxL1-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 LmxL1-ADAPTIN ORF is grouped within the or1 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 or1-adaptin sequence that was used to synthesize degenerate primer pairs for the cloning of the LmxL1-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 L. mexicana genomic DNA library. An ORF of 1299 bp (Fig. 2D) was identified upon sequencing the LmxL1-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 LmxL1-ADAPTIN ORF within the μ-adaptin family with amino acid identities of 37–46% from A. thaliana to Dro sophila 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 orL- and or1-ADAPTINS—Southern analysis with a range of restriction enzymes showed that the LmxL1- and or1-ADAPTIN genes are pres-
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...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).

Biochemical Characterization of the L. mexicana α1- and µ1-ADAPTIN Gene Products—For the characterization of the Lmxα1- and Lmxµ1-ADAPTIN gene products, rabbit polyclonal antibodies were raised against 15 residues corresponding to the carboxyl terminus of Lmxα1-ADAPTIN, which should have no correlate to other L. mexicana α-ADAPTIN sequences (Fig. 1A), or full-length, His-tagged recombinant Lmxα1-ADAPTIN. Irrelevant antibodies were removed by absorption of the antisera against each respective null mutant cell line, Δα1 and Δμ1. The specificity of each antibody is shown by immunoblotting in Fig. 3. The anti-Lmxα1-ADAPTIN antibody detected one protein species of ~19 kDa 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-Lmxα1-ADAPTIN antisera, but a molecular mass species of ~47 kDa was recognized in WT promastigotes (see Fig. 3B, lanes 2 and 3). Both of these adaptin subunits 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 Lmxα1- and Lmxµ1-ADAPTIN were estimated to be present at ~1 × 10^4 copies per Leishmania cell (data not shown).

It appeared that with loss of the Lmxα1-ADAPTIN gene, the stability of Lmxα1-ADAPTIN was affected, as only low quantities of this protein were detected in Δα1 parasites using anti-Lmxα1-ADAPTIN antisemur, but expression was restored in the complemented null mutant cell line (Δα1::cRBlμ1) (Fig. 3A, lanes 6 and 7). In contrast, Lmxµ1-ADAPTIN was detected in Δμ1 cells at levels similar to WT and the Δμ1::cRBlμ1 complemented null mutant cell lines (Fig. 3B, lanes 2, 6, and 7).

To assess whether the Lmxα1- and Lmxµ1-ADAPTINS were either...

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**FIG. 1.** Comparison of the primary structures of L. mexicana α1 and µ1 with those from various organisms of the adaptor small chain (α) and medium chain (μ) family, respectively. Sequences were aligned using the PILEUP program, where dashes represent spaces inserted for maximum alignment. Residues that are conserved across all five species are indicated by asterisks. The numbering corresponds to the number of amino acids of the L. mexicana sequences and those shown in parentheses indicate those from the other species. A, multiple sequence alignment of the L. mexicana α1-ADAPTIN with H. sapiens, M. musculus (Mus mus.), A. thaliana (A. thal.), and S. cerevisiae (S. cerei.) with respective GenBank™ accession codes: AB521978, M62419, U89209,5, and Z26014. The overlined region corresponds to the adaptor complex small chain signature (PROSITE accession code PS00989). B, multiple sequence alignment of the L. mexicana µ1-ADAPTIN with D. melanogaster (D. mel.), M. musculus (Mus mus.), C. elegans (C. elegan.), A. thaliana (A. thal.) with respective GenBank™ Accession Codes: AJ06219, M62419, L62691, and AF008631. The overlined regions correspond to the adaptor complex medium chain signatures (PROSITE accession codes PS00990 and PS00991), and the amino acids involved in the binding of the tyrosine motif is highlighted in bold lettering.
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 MYKF 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 clones. Once the deletion mutants became available, it could be demonstrated by immunofluorescence studies that the absence of the Lmxα1- and μ1-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 Δα1 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 Δα1 and Δμ1 L. mexicana Parasites Are Not Infectious for Macrophages or Mice—By comparison to WT L. mexicana parasites, Δα1 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 α1- 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 Δα1 mutants, expression under the control of the ribosomal promoter (Δα1+cRIB1) showed that ~86% of the macrophages were parasitized, whereas only ~62% of the macrophages were infected with the episonally complemented Δα1 mutant
fixed macrophages stained for nucleic acid taken at various times. After 1 day, all cell lines had invaded macrophages; however, the percentage of infected macrophages was lower for the null mutants compared with the WT (Fig. 4, C and D). By day 3, macrophages harboring WT cells showed the formation of the parasitophorous vacuole (PV); however, at this time point the number of macrophages infected with the Δσ1 and Δμ1 mutant cell lines had significantly decreased and those parasites seen in macrophages appeared to be in very small vacuoles (Figs. 4, C and D and 5). Subsequent time points showed colonized macrophages, infected with WT and the complemented null mutant (Δσ1+pCRibI1 and Δμ1+pXμ1) cells lines, contained mature parasite-harbouring compartments, where the ovoid-shaped parasites were close to the membrane of the organelle, a characteristic feature for *Leishmania mexicana* PVs. In contrast, by day 4 and day 5 most of the Δσ1 and Δμ1 mutant parasites had been cleared, respectively.

Several previous studies have shown that in vitro macrophage infection experiments correlate with *in vivo* mouse infectivity (20, 39–41). In this study this correlation holds true, as the Δσ1 and Δμ1 *L. mexicana* parasites proved to be avirulent to Balb/c mice even at the high parasite dose (10^7/mouse) used, as no significant swelling of the inoculated footpads was observed (Fig. 4, E and F). In both cases virulence could be restored by the complemented null mutant cell lines. The rate of onset and progression of disease where the Δσ1+pCRibI1 cell line was used for infection was comparable to that of the WT parental strain, and lesion development was slower in mice infected with the Δσ1+pXμ1 promastigotes (Fig. 4E). Although some of the mice infected with the complemented null mutant cell lines, Δμ1+pCRibμ1 and Δμ1+pXμ1, failed to develop lesions (50% in both cases), for the rest the progression of disease was still much slower than the control WT promastigotes (shown in Fig. 4F). Unsuccessful attempts to re-isolate parasites from the injected foot-pad, lymph nodes, and spleen from mice infected with the Δσ1- or Δμ1-null mutant cells, indicated that there were no persistent parasites in these animals.

**DISCUSSION**

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 σ1- 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 heterotrimeric AP-1 complexes would be involved in the formation of clathrin-coated vesicles at the TGN.

The high degree of σ-adaptin sequence conservation implicates an important functional role of this subunit within the AP complexes. Although it has been proposed that σ-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 σ1-adaptins contain several conserved blocks, which are also present in the *L. mexicana* σ1-adaptin primary sequence. One of these segments conform to the consensus, which is considered a signature for the σ-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...
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. C, 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 10⁷ 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 \( \mu_1 \)-adaptins 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 \( L. \) \textit{mexicana} \( \mu \)-ADAPTIN primary sequence shows the presence of many highly conserved sequence blocks found in \( \mu \)-adaptins of other organisms, including the PROSITE signature sequence, and amino acids involved in sorting motif interaction are identical to the class of \( \mu_1 \)-adaptins from other organisms. Based on these criteria, the \( L. \) \textit{mexicana} \( \mu_1 \)- and \( \mu_2 \)-ADAPTINs identified in this study have been assigned \( L.mx \) \( \mu_1 \)- and \( \mu_2 \)-ADAPTIN (according to the genetic nomenclature for \textit{Leishmania} stipulated by Ref. 43). The number of molecules per \textit{Leishmania} promastigote for each adaptin was estimated at \( \sim 1 \times 10^4 \), 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 \( Lmx \) \( \mu_1 \)- and \( \mu_1 \)-ADAPTINS were found to be partially associated with membranes. However unlike the adaptins studied in other organisms, the \( L. \) \textit{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 \( L. \) \textit{mexicana} adaptins from the membrane.

Studies performed by disrupting the \( S. \) \textit{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 \( Lmx \) \( \mu_1 \)- and \( \mu_1 \)-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 \( \mu_1 \)-adaptn gene resulted in embryonic lethality (45). It was
found that no free γ-, β1-, or α1-subunits were present in fibroblasts and γ-β1-α1 subcomplexes were unable to associate with membranes, which has been suggested to cause the re-routing of mannose 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 *Lmx*1-ADAPTIN gene resulted in the down-regulation of *Lmx*1-ADAPTIN protein, as only minute amounts of this adaptin were detected in the Δα1 cell line. Re-introduction of the *Lmx*1-ADAPTIN gene into Δα1 cells restored the expression of the *Lmx*1-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 *Lmx*1- 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 *Lmx*1- 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, Δα1 and Δμ1, were unable to establish infection when introduced into macrophages or mice (Fig. 4). That the Δα1 and Δμ1 parasites retained the ability to invade macrophages is shown by the kinetic experiments performed, however, with time the invading Δα1 and Δμ1 parasites were killed and cleared by the host cells, whereas WT and complemented null mutant (Δα1+cRIBα1 and Δμ1+pXM1) 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, Δα1 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 GPIs do not render these parasites avirulent for mice (20, 38, 40, 41).

Infection of mice with the Δα1 and Δμ1 cell lines showed that in comparison to WT and the respective complemented cell lines, the null mutants were impaired in lesion formation (Fig. 4, E and F). For the Δα1+cRIBα1- and Δμ1+pXM1-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 heterotetrameric AP-1 complexes in other organisms, it is most likely that the *Lmx*1- 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 prolif-

eration of amastigotes. Initially, it was assumed that these adaptin subunits may be involved in the sorting of the *Lmx*MBAP 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 Δα1 and Δμ1 cell lines, as in WT parasites. Although the *Lmx*MBAP 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 *Lmx*1- 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 *Lmx*1- and μ1-ADAPTINS may require the simultaneous overexpression of all four subunits either in *Leishmania* or a heterologous system.

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

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