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Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis

(repetitive epitope/motor protein/parasite/diagnosis)

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ABSTRACT **We report the cloning of a *Leishmania chagasi* antigen gene and an evaluation of leishmaniasis patient antibody responses to the recombinant protein, rK39. rK39 contains a 39-amino acid repeat that is part of a 230-kDa protein predominant in *L. chagasi* tissue amastigotes. Sequence analyses showed this protein, LcKin, to be related to the kinesin superfamily of motor proteins. Southern blot analyses demonstrated LcKin-related sequences in seven species of *Leishmania*, with conservation of the repeat between *L. chagasi* and *Leishmania donovani*. Serological evaluation revealed that 98% (56 of 57) of Brazilian and 100% (52 of 52) of Sudanese visceral leishmaniasis patients have high antibody levels to the rK39 repeat. Detectable anti-K39 antibody was virtually absent in cutaneous and mucosal leishmaniasis patients and in individuals infected with *Trypanosoma cruzi*. The data show that rK39 may replace crude parasite antigens as a basis for serological diagnosis of visceral leishmaniasis.**

Protozoan parasites of the genus *Leishmania* are widely distributed and transmitted by the bite of sandflies. In the vertebrate host, the infecting promastigotes differentiate into and replicate as amastigotes within macrophages. Symptoms range from self-healing skin lesions to diffuse cutaneous and mucosal manifestations, or severe visceral involvement of the spleen, liver, and lymph nodes. Visceral leishmaniasis (VL) is generally caused by *Leishmania donovani* in Africa, India, and southern Europe or *Leishmania chagasi* in Latin America. In VL, high antibody levels are observed prior to the detection of parasite-specific T-cell responses (1). This antibody response has been exploited for the diagnosis of *L. chagasi* and *L. donovani* infection (2–5). The current World Health Organization's estimate of 12 million cases of leishmaniasis and recent epidemics of VL in Sudan and India (6, 7) highlight the need for more effective control measures. Diagnostic tests use whole or lysed *Leishmania*, and a few studies have begun to examine patient antibody responses to specific antigens (2, 8–10). Such studies may improve diagnostic assays and help to evaluate B-cell responses during disease progression.

We report the cloning and expression of an *L. chagasi* gene sequence‖ and an evaluation of patient antibody responses to the recombinant product, rK39. The sequence encodes an immunodominant protein with a repetitive epitope closely conserved between *L. chagasi* and *L. donovani*. This repeat is part of a large kinesin-related protein expressed predominantly by amastigotes. More than 98% of Brazilian and 100% of Sudanese VL patients had readily detectable serum antibody responses to the K39 repeat.

MATERIALS AND METHODS

Parasites. *L. chagasi* (MHOM/BR/82/BA-2,C1), *L. chagasi* (MHOM/BR/84/Jonas), *Leishmania amazonensis* (IFLA/BR/67/PH8), *Leishmania brasiliensis* (MHOM/BR/75/M2903; obtained from Diane McMahon-Pratt, Yale University), *Leishmania guyanensis* (MHOM/BR/75/M4147), *L. donovani* (MHOM/Et/67/HU3), *Leishmania infantum* (IPT-1; obtained from Lee Schnur, The Hebrew University–Hadassah Medical School, Jerusalem), *Leishmania major* (LTM p-2, obtained from David Moser, Temple University, Philadelphia), and *Trypanosoma cruzi* (MHOM/CH/00/Tulahuen C2) were used. Promastigotes and epimastigotes were cultured in axenic media. *L. chagasi* and *L. amazonensis* amastigotes were obtained from spleens of Syrian hamsters and footpads of BALB/cByJ mice, respectively, and purified as described (11).

Patient Sera. Brazilian leishmaniasis sera and *T. cruzi* infection sera were from well-characterized patients in Bahia, Brazil; Sudanese leishmaniasis sera were from *Leishmania*-positive patients; normal sera were from healthy individuals in Sudan or the United States (2, 4, 12).

Identification and Purification of rK39. A genomic library was constructed with sheared DNA of *L. chagasi* (MHOM/BR/82/BA-2,C1) in Lambda ZAPII (Stratagene) and screened with preadsorbed serum (13) of a *L. donovani* patient. The 39-kDa recombinant antigen of clone K39 (rK39) was purified from a 25–40% ammonium sulfate fraction of bacterial lysate by preparative isoelectric focusing (IEF) with the Rotofor IEF cell and 1% 3/10 ampholytes (pH range 3.5–9.5; Bio-Rad) in the presence of 8 M urea and 10 mM dithiothreitol. Peak fractions were concentrated by ammonium sulfate precipitation and dialyzed against 25 mM Tris·HCl, pH 8/150 mM NaCl (TBS). Protein concentrations were determined by using the Pierce BCA assay, and purity was assessed by Coomassie blue-staining following SDS/PAGE (14).

Immunoblot Analysis. Immunoblots of parasite lysates or purified rK39 were prepared (11). Filters were blocked with TBS containing 5% nonfat dried milk and probed with patient sera (1:250 dilution) or rabbit sera (1:400 dilution) diluted in TBS containing 0.1% Tween-20 and 1% bovine serum albu-

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Abbreviation: VL, visceral leishmaniasis.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L07879).

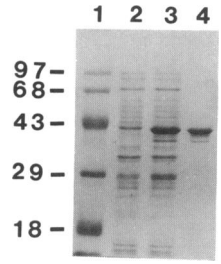

FIG. 1. Expression and purification of rK39. Coomassie blue-stained SDS/12% polyacrylamide gel of molecular mass markers (lane 1), *Escherichia coli* lysates from uninduced (lane 2) and induced (lane 3) cultures of clone K39, and purified rK39 (2 μg) (lane 4). Sizes are shown in kDa.

min. Bound antibody was detected with ^{125}I-labeled protein A (1×10^6 cpm per blot) followed by autoradiography.

Rabbit antiserum was raised by s.c. immunization of an adult New Zealand White rabbit (R & R Rabbitry, Stanwood, WA) with 200 μg of purified rK39 in Freund's incomplete adjuvant (IFA; GIBCO) together with 100 μg of muramyl dipeptide (Calbiochem) and a booster with 200 μg of rK39 in IFA alone. Three weeks later the rabbit received a booster i.v. with 25 μg of rK39, and serum was collected 6 days later.

K39 Gene Sequence Analysis. The radiolabeled insert of K39 was used to screen the *L. chagasi* genomic library to obtain clones flanking the K39 gene fragment. A set of overlapping deletions of clones K39 and LcKin were generated by exonuclease III digestion (15) to obtain the complete sequence of the coding and noncoding strands. Single-stranded template was prepared (16), and sequence was obtained by Sanger dideoxynucleotide chain-termination with [^{35}S]-labeled dATP (17) or by fluorescence-based sequencing on the Applied Biosystems Automated Sequencer model 373A. Sequence comparisons during GenPept (release 72.0) and Swiss-Prot (release 22.0) data bank searches were made with the Lipman/Pearson method (18), and final alignments were made according to the Needleman/Wunsch algorithm (19). Secondary structural predictions were according to Garnier–Robson (20) and Chou–Fasman (21).

Parasite DNAs were isolated, digested with *Pst* I, separated by agarose gel electrophoresis, and analyzed by Southern blotting (13). Blots were probed with a 2.4-kilobase (kb) *Hin*dIII fragment of LcKin derived from the 5′ end of the gene (probe A) or the 915-base-pair (bp) insert of clone K39 (probe B). Probes were radiolabeled with [α-^{32}P]dCTP to a specific activity of 9×10^8 cpm/μg of DNA by using random oligonucleotide primers (Boehringer Mannheim). Final washes were for 1 hr in 0.015 M NaCl/0.0015 M sodium citrate, pH 7/0.5% SDS at 68°C. Blots of *L. chagasi* DNA digested with *Bam*HI, *Hin*dIII, and *Pst* I were prepared and probed as above. Probe A contained one *Bam*HI, one *Pst* I, and no *Hin*dIII sites. Probe B did not contain sites for these enzymes.

K39 Antigen ELISA. rK39 or *L. chagasi* promastigote lysate was diluted in coating buffer (15 mM Na_2HCO_3/28 mM NaH_2CO_3, pH 9.6), and microassay plates (Probind; Falcon) were sensitized overnight at 4°C with rK39 (50 ng) or promastigote lysate (1 μg) followed by blocking with phosphate-buffered saline (PBS) containing 1% Tween-20 for 1 hr at room temperature. After five washes with PBS containing 0.1% Tween-20 (PBS-T), 50 μl per well of sera diluted 1:100 with PBS-T were incubated for 30 min at room temperature. Wells were washed five times with PBS-T, and bound antibody was detected by protein A-conjugated horseradish peroxidase (Zymed Laboratories) as described (2). Absorbance values are relative to the mean of five control sera assayed on each plate.

RESULTS

Identification of *L. chagasi* Antigen Genes. To characterize *Leishmania* antigens recognized by VL patients, a *L. chagasi* expression library was screened with sera from a *L. donovani* patient. From ≈32,000 recombinants, 7 clones were selected that contained inserts of 0.9–2.6 kb and produced immunoreactive recombinant proteins of 35–100 kDa. The recombinant antigen of clone K39 (rK39) was exceptionally reactive with test serum. rK39 migrated as a 39-kDa protein in induced bacterial extracts (Fig. 1, lane 3) and was purified (Fig. 1, lane 4) with a yield of 25–30 mg per liter.

Both *L. chagasi* and *L. donovani* VL Sera Recognize rK39. The reactivity of patient sera with rK39 was evaluated by immunoblot. Both rK39 and *L. chagasi* promastigote lysate were strongly recognized by *L. chagasi* (Fig. 2, lanes A–C) and *L. donovani* (Fig. 2, lanes D–F) infection sera but not by pools of sera from mucosal (Fig. 2, lanes G) or cutaneous (Fig. 2, lanes H) leishmaniasis patients or with Chagas disease patient sera (Fig. 2, lanes I). All sera reacted strongly with promastigote lysates. These results indicate that K39 may be specific to *L. chagasi* and *L. donovani* and/or K39 induces a strong antibody response only in VL patients.

Sequence Analysis of the K39 Gene. The DNA and deduced amino acid sequences of clone K39 were determined (Fig. 3*A*, nucleotides 2426–3319) revealing a single open reading frame encoding 298 amino acids with a predicted molecular mass of 32.7 kDa and pI of 4.4. rK39 contains an additional 6.2 kDa of plasmid fusion sequences. Of particular interest were 6.5 copies of a tandemly arrayed 39 amino acid repeat (Fig. 3*B*).

Clones containing sequences flanking the K39 gene fragment were isolated from the *L. chagasi* library. Sequence analysis of one overlapping clone, LcKin (Fig. 3*A*, nucleotides 1–3109), showed that the open reading frame extended 1971 bp in the 5′ direction, encoding 657 nonrepetitive amino acids; 5′ to the putative ATG initiation codon, 454 bp of sequence were obtained with multiple termination codons in each reading frame. Partial characterization of clones containing 3′ flanking sequences indicated that the repeat domain extended ≈3–4 kb.

Data bank searches revealed sequence similarity between LcKin and members of the superfamily of kinesin-related proteins, particularly in the N-terminal motor domain. Fig. 4

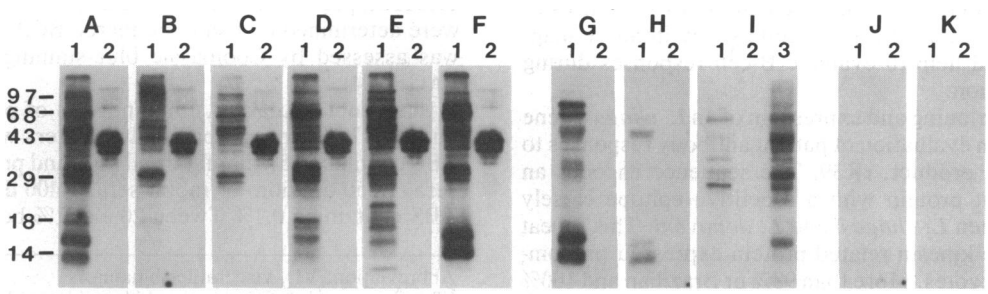

FIG. 2. Reactivity of patient sera with rK39. Blots containing 10 μg of *L. chagasi* promastigote lysate (lanes 1), 50 ng of purified rK39 (lanes 2), and 10 μg of *T. cruzi* epimastigote lysate (lane 3) were probed with individual *L. chagasi* (lanes A–C) or *L. donovani* (lanes D–F) VL sera, pooled mucosal (lane G, $n = 4$) or cutaneous (lanes H, $n = 4$) leishmaniasis sera, or *T. cruzi* infection (lane I, $n = 5$) sera. Pooled normal human sera ($n = 3$) and no primary antibody controls are shown in lanes J and K, respectively. Sizes are shown in kDa.

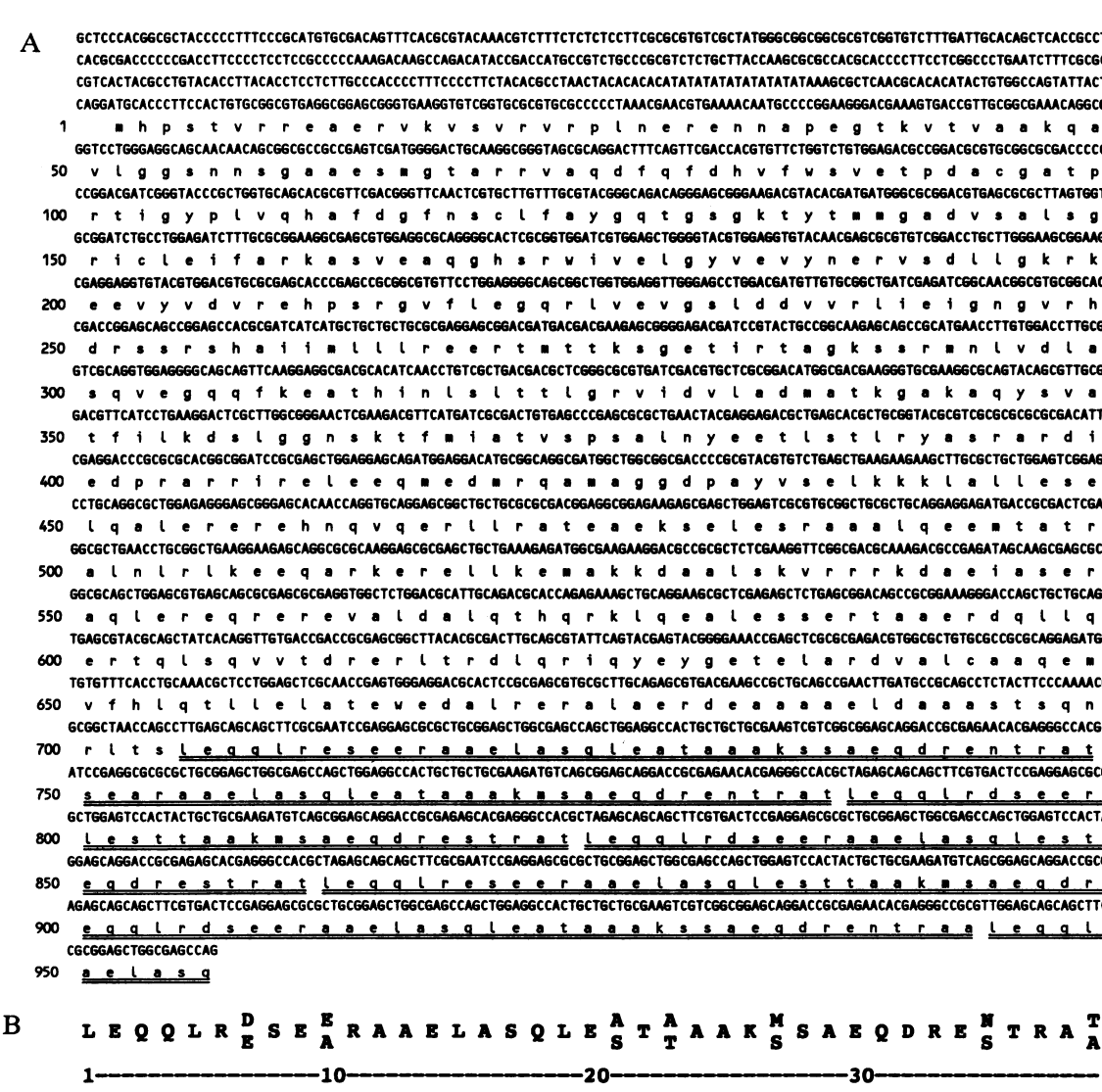

FIG. 3. Sequence analysis of clones LcKin and K39. (*A*) DNA and deduced amino acid sequence in single-letter code of overlapping clones LcKin (nucleotides 1–3109) and K39 (nucleotides 2426–3319) with residues doubly underlined to indicate each repetitive segment. (*B*) Consensus sequence of the 39-amino acid repeat of K39 with degeneracies indicated.

shows a comparison between the N-terminal domain of LcKin and the motor domain of the *Caenorhabditis elegans* kinesin (22), with 43.8% identity over 349 amino acids. Strong sequence conservation was observed in the putative ATP and microtubule binding domains (23). The remaining 500 residues showed limited similarity to the tail regions of kinesin and myosin. Secondary structure analyses predicted this portion of LcKin (amino acids 426–955) to contain >90% helical structure characteristic of the coiled-coil tail regions of several motor proteins. Thus, the repetitive rK39 epitope appears to present in *L. chagasi* as part of the tail region of a *Leishmania* kinesin-related protein.

Southern Blot Analysis of LcKin Gene Sequences. Genomic DNAs from several *Leishmania* species were analyzed by Southern blot by using probes of LcKin derived from the kinesin homology domain (probe A, Fig. 5*A*) or from the K39 repetitive domain (probe B, Fig. 5*B*). Probe A hybridized strongly to multiple *Pst* I restriction fragments of all *Leishmania spp.* tested (Fig. 5*A*, lanes 3–9), indicating conservation in the kinesin homology domain. Polymorphisms in the size and number of hybridizing fragments were noted. Less conservation in the repetitive domain of the LcKin gene was observed as probe B hybridized with varying intensity to *Pst* I restriction fragments of *L. chagasi, L. amazonensis, L. brasiliensis, L. donovani, L. infantum*, and *L. major* but not *L. guyanensis* (Fig. 5*B*, lanes 3–9). The K39 repeat appeared most closely related between *L. chagasi* and *L. donovani* (Fig. 5*B*, lanes 3 and 7). No hybridization with either probe was observed with *T. cruzi* DNA (Fig. 5, lane 10).

When using *L. chagasi* digested DNA, two *Pst* I fragments were detected, with probe B indicating the presence of a second copy of the LcKin gene or polymorphism in restriction sites present in the 3′ repetitive sequences (Fig. 5*B*, lane 3). Probe A hybridized to three fragments in each of the *Bam*HI, *Hin*dIII, and *Pst* I digests of *L. chagasi* DNA (Fig. 5*A*, lanes 1–3). One *Bam*HI, one *Pst* I, and no *Hin*dIII sites are present within the probe A sequence. Taken together, the Southern blot data show that the LcKin gene is present in a minimum of two or three copies in the *L. chagasi* genome and that related sequences are present in seven species of *Leishmania* examined.

Identification of the Native LcKin Antigen. Rabbit anti-rK39 serum was used to probe SDS/PAGE blots of *L. chagasi* promastigote and tissue amastigote lysates. The antiserum bound specifically to purified rK39 (Fig. 6*A*, lane 2) and to an ≈230-kDa *L. chagasi* antigen present in amastigotes (Fig. 6*B*, lane 6) and to a lesser degree in promastigotes (Fig. 6*B*, lane 5). No reactivity with this serum was detected in promastigote and amastigote lysates of *L. amazonensis* (Fig. 6*B*, lanes 7–8). Comparable amounts of lysate were loaded in all lanes

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LcKIN    MHPSTVRRREAERVKVSVRVRPLNEREMNAPEGTKVTVAAKQAAAVVTVKVLGGENNSGAAESMGTARRVAQDFQFDHVFWSVETPDACGATPATQADVFRTIGYPLVQHAF 111
                                  :: . :   . : :Q .   T K :   N.:.:: :   : .: . .F:FDH :WS .  D:       TQ :V: .:G  :::HAF
CELKIN                            MSSVKVAVRVRPFNQREISNTSKCVLQVNGNTTTINGHSINKENFSFMFDHSIWSFARNDP---HFITQKQVYEELGVEMLEHAF  82
                                  ATP BINDING
LcKIN    DGFNSCLFAYGQTGSGKTYTMMGADVSALSGEGNGVTPRICLEIFARKASVEAQGHSRWIVELGYVEVYMERVSDLLGKRKKGVKQGGEEVYVDVREHPSRGVFLEGQRLV 222
         :G:N C:FAYGQTGSGK:YTMMG   .A . :. G:.PR:C ::FAR .: :.: : :. VE::Y:E:Y ERV.DLL        . ...  : VREHP  G :::  :
CELKIN   EGYNVCIFAYGQTGSGKSYTMMG---KANDPDEMGIIPRLCNDLFARIDN-MNDKDVQYSVEVSYMEIYCERVKDLL--------NPMSGGMLRVREHPLLGPYVDDLTKM 181

LcKIN    EVGSLDDVVRLIEIGNGVRHTASTKMNDRSSRSHAIIMLLLREERTMTTKSGETIRTAGKSSRMNLVDLAGSERVAQSQVEGQQFKEATHINLSLTTLGRVIDVLADMATK 333
         .V S :D: .L:: GN .R .A:T:MN. SSRSHA:: ::L :.R    ..::: .:.K S:::LVDLAGSER.. : .EGQ::KE:::IN SLTTLG VI. LA: :TK
CELKIN   AVCSYHDICMLMDEGNKARTVAATNMNSTSSRSHAVFTIVLTQKR----HCADSNLDTEKHSKISLVDLAGSERANSTGAEGQRLKEGANINKSLTTLGLVISKLAEESTK 288
                                                  MICROTUBULE BINDING
LcKIN    GAKAQYSVAPFRDSKLTFILKDSLGGNSKTFMIATVSPSALNYEETLSTLRYASRARDIVNVAQVNEDPRARRIRELEEQNEDMRQAMAGGDP                  426
           K:: :V P:RDS LT.:L:::LGGNSKT M:A::SP:.:N::ETLSTLRYA.RA::IV  A VNEDP.A: IREL:E:: .:R: : : :
CELKIN   KKKSNKGVIPYRDSVLTWLLRENLGGNSKTAMLAALSPADINFDETLSTLRYADRAKQIVCQAVVNEDPNAKLIRELMEEVIKLRHILKDKGI                  381

43.8% identity in 349 aa overlap
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FIG. 4. Protein sequence comparison between LcKin (amino acids 1–426) and the motor domain of the kinesin related protein of *C. elegans* (22) in single-letter code. Identical residues are indicated by letter; colons and periods represent conservative and neutral amino acid substitutions, respectively. Putative ATP and microtubule binding domains are overlined (23).

as shown by the reactivity of a rabbit antiserum raised against a constitutively expressed *L. chagasi* ribosomal phosphoprotein, LcP0 (Fig. 6*C*) (24).

Reactivity of Patient Sera with rK39. The reactivity of patient sera with rK39 was evaluated by ELISA. Among VL patients, 98.2% of Brazilian sera (56 of 57) and 100% of Sudanese sera (52 of 52) were positive on rK39 (absorbance values were >3 standard deviations above the mean of normal controls). Values ranged from 0.05 to >2.0 (mean = 1.38) among Brazilian VL sera and from 0.094 to >2.0 (mean = 1.60) among Sudanese VL sera (Fig. 7*B*). Detectable antibody to rK39 was restricted to VL patients, as little or no anti-rK39 response was observed in mucosal or cutaneous leishmaniasis sera or *T. cruzi* infection sera, despite reactivity with crude *L. chagasi* lysate (Fig. 7*A*).

DISCUSSION

We have identified a 230-kDa antigen of *L. chagasi*, LcKin, with homology to the kinesin superfamily of motor proteins. LcKin is predominant in amastigotes, is present in diverse species of *Leishmania*, and contains an extensive repetitive domain. Southern analyses showed the repeat of LcKin to be variable among species, but closely related in *L. chagasi* and *L. donovani*. We demonstrated high antibody titers in Brazilian and Sudanese VL patients to rK39, which contains several 39-amino acid repeats, indicative of the conservation of the repeat between *L. chagasi* and *L. donovani*.

Characterization of a gene encoding a protozoan motor protein, represented here by the cloning of LcKin, has been previously unreported to our knowledge. Members of the kinesin superfamily share on average 40% sequence identity in the N-terminal 350–400 amino acids composing the motor domain (25). These microtubule-based motors are involved in such varied intracellular processes as organelle and synaptic vescicle transport, chromosome segregation, and spindle pole body separation, nuclear fusion, protein sorting, and flagellar beating (25, 26). Although they share little sequence similarity outside of the motor domain (26), the nonmotor tail domain is predominantly α-helical in structure and likely forms a coiled-coil interacting with different intracellular ligands. The LcKin gene product is similar to members of this family in primary sequence, particularly in the putative ATP and microtubule binding domains, as well as in predicted secondary structure, although the specific cellular processes involving LcKin are not yet known.

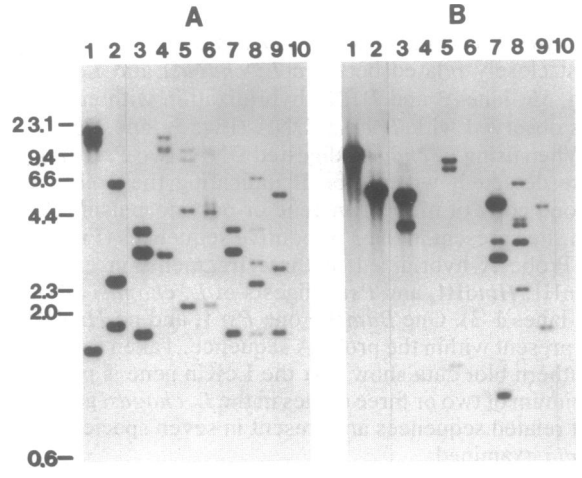

FIG. 5. Southern blot analysis of LcKin gene sequences. Genomic DNA (2.5 μg per lane) from *L. chagasi* digested with *Bam*HI (lane 1), *Hin*dIII (lane 2), and *Pst* I (lane 3) or Pst I-digested DNA from *L. amazonensis* (lane 4), *L. braziliensis* (lane 5), *L. guyanensis* (lane 6), *L. donovani* (lane 7), *L. infantum* (lane 8), *L. major* (lane 9), or *T. cruzi* (lane 10) were analyzed by Southern blotting. Blots were probed with a 2.4-kb *Hin*dIII fragment from the LcKin kinesin homology domain (*A*) or with the 915-bp repetitive insert of K39 (*B*). Sizes are shown in kb.

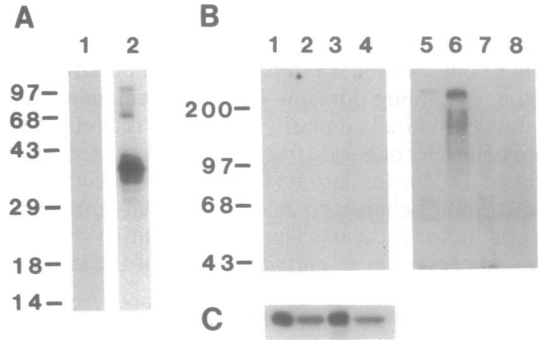

FIG. 6. Reactivity of rabbit anti-rK39 antiserum on rK39 and *Leishmania* lysates. (*A*) Immunoblot of purified rK39 (50 ng) transferred from SDS/12% polyacrylamide gels and probed with preimmune rabbit serum (lane 1) or rabbit anti-rK39 (lane 2). (*B*) Immunoblot of 10 μg of *L. chagasi* promastigote (lanes 1 and 5) and amastigote (lanes 2 and 6) lysates or 10 μg of *L. amazonensis* promastigote (lanes 3 and 7) and amastigote (lanes 4 and 8) lysates transferred from SDS/7.5% polyacrylamide gels and probed with preimmune rabbit serum (lanes 1–4) or rabbit anti-rK39 (lanes 5–8). (*C*) Reactivity of rabbit antisera raised against *L. chagasi* ribosomal protein PO (24) with material in lanes 1–4 of *B*. Sizes are shown in kDa.

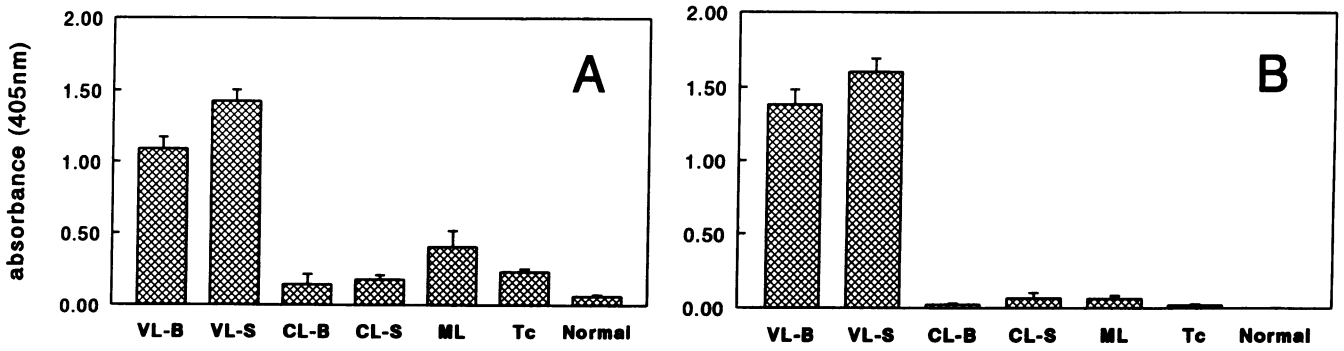

FIG. 7. ELISA evaluation of patient seroreactivity on *L. chagasi* promastigote lysate (*A*) or purified rK39 (*B*). Absorbance values (mean + SEM) of Brazilian VL (VL-B, $n = 57$), Sudanese VL (VL-S, $n = 52$), *T. cruzi* infection (Tc, $n = 35$), Brazilian cutaneous leishmaniasis (CL-B, $n = 13$), Sudanese cutaneous leishmaniasis (CL-S, $n = 13$), mucosal leishmaniasis (ML, $n = 15$), and normal ($n = 15$) sera.

A striking feature of LcKin was the high prevalence of antibody to the K39 repeat in VL patients from geographically distinct regions. This response was restricted to VL patients, reflecting the relatedness among members of the *L. donovani* complex (27–29). The characterization of patient B-cell responses to defined *Leishmania* antigens has been minimal (2, 8–10). Our studies are unique in showing VL-restricted antibody responses to a recombinant antigen of *L. chagasi* and showing a marked restriction of this response to *L. chagasi*- and *L. donovani*-infected patients.

Repetitive amino acid domains have been observed in many parasitic protozoan antigens, often being immunodominant B-cell epitopes that detect high levels of antibody in infected patients (16, 30–34). It is unclear if such responses contribute to the development of protective immunity or are immunological "smokescreens" limiting the development of protective responses (30, 35). In preliminary studies, 10 of 25 asymptomatic or subclinical patients infected with *L. chagasi* had elevated levels of anti-K39 antibody (unpublished data). As only 30–35% of such individuals develop acute VL (36), the fate of those with a positive anti-K39 titer may be informative. Such prospective studies should help to determine whether the anti-K39 response is associated with the progression or resolution of VL. Nevertheless, from a practical standpoint, the data presented here as well as data from field studies in epidemic regions of VL in Sudan (H.W.G., W.G.S., D.R.B., J.M.B., S.G.R., unpublished data) show that rK39 may be useful in the serological diagnosis of acute VL.

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