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Molecular cloning and characterization of the immunologically protective surface glycoprotein GP46/M-2 of *Leishmania amazonensis*

(repetitive sequences/protective immunity/gene family/parasitic protozoa)

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ABSTRACT **Immunization of mice with the GP46/M-2 membrane glycoprotein has been demonstrated to elicit protection against infection with the parasitic protozoan *Leishmania amazonensis*. As this molecule is important for future vaccine studies of leishmaniasis, the gene encoding the GP46/M-2 surface membrane glycoprotein of *Leishmania amazonensis* has been cloned and sequenced. The protein sequence derived from the DNA sequence data is consistent with the known biochemical and immunochemical properties of the protein and indicates a number of structural areas of interest. A repetitive sequence (24 amino acids repeated four times) occurs within the amino-terminal portion of the molecule and constitutes ≈22% of the total mature protein. The protease-resistant immunodominant carboxyl-terminal domain of the protein comprises approximately half of the molecule and consists of proline-rich and cysteine-rich areas of sequence; the distribution of cysteine residues is suggestive of metal binding motifs. The sequence predicts a hydrophobic leader peptide, and a putative attachment site for a glycosyl-phosphatidylinositol anchor is indicated at the carboxyl terminus, consistent with the membrane location of the protein. Southern blot analyses also indicate the presence of a GP46/M-2 gene family.**

Leishmania is a genus of parasitic protozoa capable of causing a spectrum of human disease (1, 2). Chemotherapeutic measures to control leishmanial infection can be difficult and are not always effective (3). However, vaccination, at least against the cutaneous forms of leishmaniasis, is possible (4). For centuries, vaccination with living organisms that produce a lesion has been practiced in the Middle East and has been the basis for vaccination programs in Israel and the U.S.S.R. However, complications that occur with the live vaccine (4) indicate the need for an attenuated or defined vaccine for cutaneous leishmaniasis. Studies of purified leishmanial antigenic components have resulted in significant protection using a murine model system (5–8).

Our laboratory has been focused on the identification of protective antigens for members of the *Leishmania mexicana* complex. Species of the *L. mexicana* complex have a wide geographical distribution, including South and Central America, the West Indies, and the southern United States (9–12). The parasites of this complex can cause cutaneous and diffuse cutaneous leishmaniasis. Diffuse cutaneous leishmaniasis, caused primarily by *Leishmania amazonensis* and *Leishmania pifanoi*, in a fraction of infected individuals (10), is characterized by disseminated large histocytoma-like nodules containing numerous parasites and by deficient cell-mediated immunity that may be associated with immunological suppression (12–14). In general, these cases are resistant to chemotherapy (12). Studies (8) in this laboratory using a murine model system indicate that a 46-kDa membrane glycoprotein of *L. amazonensis* (GP46/M-2) can be used to elicit a protective immune response against infection in susceptible mice. Using the amino-terminal sequence data derived from the purified protein, we have generated probes for, cloned, and sequenced the GP46/M-2 gene from *L. amazonensis*.¶

MATERIALS AND METHODS

***Leishmania*.** *L. amazonensis*, strain LTB0016 (MHOM/BR/77/LTB0016) was cultured in Schneider's *Drosophila* medium containing fetal bovine serum as described (15).

Protein Isolation and Amino-Terminal Sequence Analyses. The GP46/M-2 protein was isolated from membrane preparations of *L. amazonensis*, strain LTB0016 (MHOM/BR/77/LTB0016), as described (8, 16). The purification of GP46/M-2 was monitored by polyacrylamide gel electrophoresis (17) and visualized by Coomassie brilliant blue staining. The protein sequence of the M-2 protein was determined in the presence of Brij 97H detergent by M. Margolis (Massachusetts General Hospital, Boston).

Pulse–Chase Metabolic Labeling Experiments. Logarithmic-phase *L. amazonensis* promastigotes were metabolically labeled for 2 hr using [^{35}S]methionine (Amersham) and methionine-free Schneider's *Drosophila* medium as described (16). The organisms were washed and then placed in complete Schneider's medium for the various periods of chase. The mRNA, used for translation experiments, was isolated using the guanidinium isothiocyanate method (18) and translated using the rabbit reticulocyte system (19) (Amersham) and [^{35}S]methionine. Immunoprecipitation was performed as described (16) using either the M-2 monoclonal antibody, a negative control, *Leishmania panamensis*-specific monoclonal antibody (B-11) or, for the mRNA translation products, a rabbit heteroserum (16) raised to the purified GP46/M-2 protein.

EMBL3 Library Construction and Screening. *L. amazonensis* high molecular weight genomic DNA was isolated as described (20) from a clone (LTB0016 C1S1) of strain LTB0016 (P.J.L., unpublished results). *Leishmania* genomic DNA was partially digested with the restriction enzyme *Sau*3A. DNA fragments in the size range of 15–20 kilobases (kb) were isolated and ligated to *Bam*HI-digested λEMBL3 arms. The resultant λ library was plated, and plaque-lift

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38368).

filters were hybridized with a 62-base oligonucleotide that was synthesized, at the Yale Oligonucleotide Synthesis Center, under the direction of D. Crothers, on the basis of the amino-terminal amino acid sequence (21, 22) of the purified GP46/M-2 protein (see *Results and Discussion*). Hybridizations with the ^{32}P-labeled 62-mer were performed using 6× SSC/0.5% SDS/5× Denhardt's solution/salmon sperm DNA (100 μg/ml)/10 mM EDTA at 45°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). Washes were done in 2× SSC/0.5% SDS at 60°C. A strongly hybridizing clone (7A12) was isolated and the DNA insert was mapped with restriction enzymes. A 2.7-kb *Bam*HI fragment containing the entire coding region of GP46/M-2 and some flanking sequence was subcloned into pUC19 (p3BF16).

Southern and Northern Blot Analyses. For Southern blot hybridizations, DNA was subjected to electrophoresis in 0.8% agarose gels, blotted onto Nytran filters (Schleicher & Schuell), and analyzed as described (23, 24). Hybridization of Southern blots was performed either as described above for the 62-base oligonucleotide or using a nick-translated ^{32}P-labeled *Bam*HI–*Sph* I internal DNA fragment from the cloned gene at 62°C using 2× SSC/0.5% SDS, as described (22, 24). The filters were air-dried and exposed to Kodak X-Omat AR film at −70°C with Cronex Lightning Plus intensifier screens.

Northern blot analysis was performed using total mRNA isolated using the phenol/guanidinium isothiocyanate method (18). mRNA was fractionated electrophoretically on 1.2% agarose gels containing 2.2 M formaldehyde (23) using RNA molecular markers (Bethesda Research Laboratories) as standards. Blots were probed with the ^{32}P-labeled plasmid p3BF16 at 50°C in 2× SSC/0.5% SDS, washed at 65°C using 2× SSC/0.5% SDS, and processed for radioautography as described above.

DNA Sequencing and Analysis. The 2.7-kb *Bam*HI fragment from p3BF16 was subcloned in both orientations into M13mp19 or into pBluescript (Stratagene). A set of overlapping deletions of plasmids containing inserts in either orientation for both strands of the fragment were generated by either the method of Dale *et al.* (25) using the Cyclone cloning system (International Biotechnologies) or the method of Henikoff (26) using the Erase-a-Base system (Promega Biotec) and sequenced by a modified Sanger dideoxynucleotide chain-termination method (27) using either T7 DNA polymerase (Bethesda Research Laboratories) or the *Thermus aquaticus* DNA polymerase (Promega Biotec).

DNA and amino acid sequence analyses were done using the University of Wisconsin Genetics Computer Group programs (28) and accessing the GenBank and EMBL data bases of protein and DNA sequences (July, 1990).

RESULTS AND DISCUSSION

Pulse–Chase Experiments. The molecular processing of the *L. amazonensis* GP46/M-2 protein was investigated by examining metabolically labeled promastigotes and mRNA *in vitro* translation products. Monoclonal antibodies and rabbit heteroserum raised to the M-2 protein were used in immunoprecipitations. The results from these experiments are shown in Fig. 1. Over the 2-hr chase, a 42-kDa molecule is progressively replaced by a 46-kDa protein; a single-step processing event appears to be involved in this transition. The mRNA translation product migrates at 44 kDa, suggesting that the protein is first rapidly processed to a 42-kDa form and subsequently glycosylated, producing the mature 46-kDa molecule. These results are consistent with endoglycosidase experiments that indicate that the GP46/M-2 protein contains a single carbohydrate side chain (16).

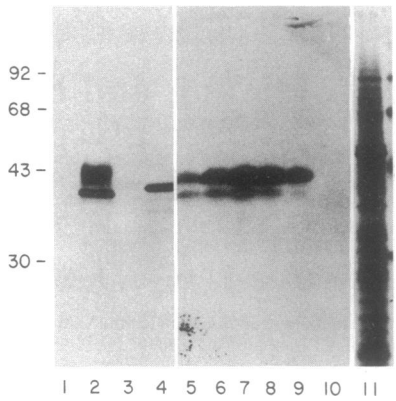


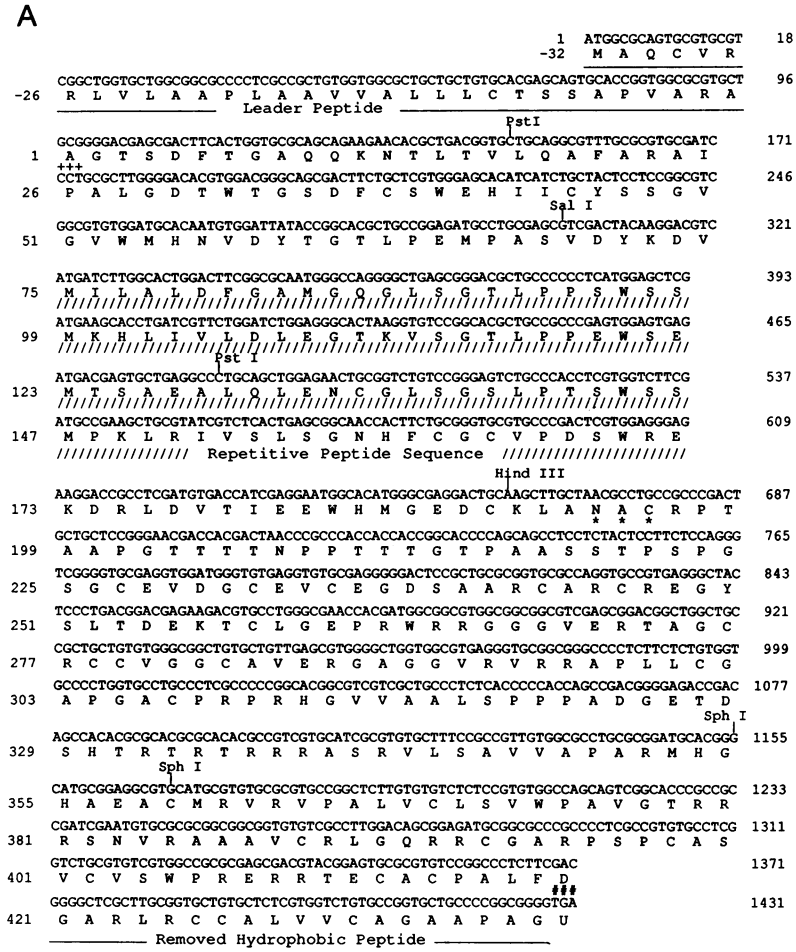
FIG. 1. Autoradiographic results of pulse–chase experiments. Metabolically labeled logarithmic phase *L. amazonensis* promastigotes were incubated in medium without radioisotope for chase periods of 0, 10, 20, 40, 75 (lanes 2 and 5–8, respectively), and 120 min (lanes 9 and 10). Either [^{35}S]methionine-labeled *L. amazonensis* promastigote mRNA translation products (lanes 3 and 4) or metabolically labeled organisms (lanes 1, 2, and 5–10) were employed and immunoprecipitated with normal rabbit serum (lanes 1 and 3), rabbit anti-GP46/M-2 serum (lanes 2 and 4), monoclonal antibody M-2 (specific for the GP46/M-2 protein of *L. amazonensis*; lanes 5–9), or a negative control monoclonal antibody (B-11; lane 10). Lane 11 shows total labeled *in vitro* translation products. Molecular mass in kDa are shown to the left.

Amino-Terminal Protein Sequence. The amino-terminal sequence of the isolated and purified GP46/M-2 protein was determined. The protein sequence derived was Ala-Gly-Thr-Ser-Asp-Phe-Thr-Glu-Ala-Gln-Gln-Thr-Asn-Thr-Leu-Thr-Val-Leu-Gln-Ala-Phe-Ala-Arg-Ala-Ile-Pro-Ala-Leu-Gly-. The sequence from Phe-6 through Pro-26 was used to design a 62-base oligonucleotide probe, based on the considerations of Lathe (21) and the codon usage and G/C bias in the third codon position reported for other *Leishmania* genes (22). The sequence chosen for the oligonucleotide was 5′-TTCACG-GAGGCGCAGCAGACGAACACGCTGACGGTGCTG-CAGGCGTTCGCGCGCGCGATCCC-3′.

DNA Sequence Analysis of the GP46/M-2 Gene. The gene encoding the GP46/M-2 protein was cloned. Fig. 2*B* shows the restriction map of a 20-kb insert in EMBL3 clone 7A12 that contains two copies of the gene encoding the GP46/M-2 protein. A 2.7-kb *Bam*HI fragment containing GP46/M-2 gene A was subcloned into pUC19 (p3BF16); a partial restriction enzyme digest map of p3BF16 is shown (Fig. 2*B*). The coding region (1431 base pairs) is represented by the solid area with the ATG start codon beginning 70 base pairs from the *Bam*HI site on the 5′ end of the fragment.

The codon usage in the third position was 77% G/C, consistent with that found for other sequenced *Leishmania* genes (22). No significant protein sequence identity/similarity was found between GP46/M-2 and other sequenced molecules using the GenBank and EMBL data bases accessed through the University of Wisconsin Genetics Computer Group programs (28).

Fig. 2*A* displays the entire nucleotide and amino acid sequences of GP46/M-2A and also indicates some of the coding region restriction enzyme sites, as illustrated for p3BF16 (Fig. 2*B*). The sequence derived from direct protein sequencing (data presented above) and that predicted from the genomic DNA sequence are in agreement for 27 out of 29 residues. The exceptions were the prediction from the DNA-derived sequence of glycine in place of glutamic acid at residue 8 of the mature protein and lysine for threonine at residue 12 (see above and Fig. 2*B*). These data, cDNA sequence data obtained from the LTB0016 clone C1S1 (P.J.L. and J. Bock, unpublished data) and vaccination



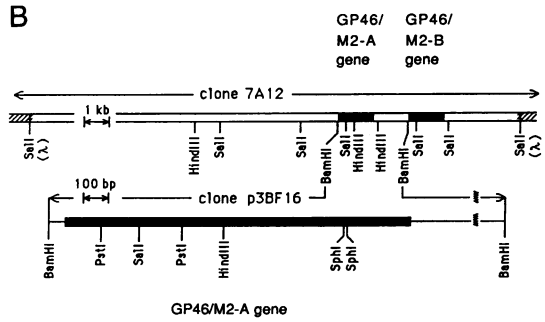


FIG. 2. Restriction maps and sequence of the GP46/M-2 clones. (*B*) Partial restriction maps for EMBL3 clone 7A12 and pUC19 clone p3BF16 are illustrated. For the 20-kb genomic clone (7A12), all *Hin*dIII, *Sal* I, and *Bam*HI sites are indicated. For the 2.7-kb insert in p3BF16, restriction enzyme sites are indicated only in the coding region. (*A*) The complete nucleotide sequence for the gene encoding for the GP46/M-2 protein and the corresponding amino acid sequence. Some predicted restriction endonuclease sites are indicated above the nucleotide sequence; the corresponding amino acid and nucleotide residue numbers are given in the columns at the left and right hand sides, respectively, of the sequences. Indicated are the amino-terminal residue alanine (+++), the single predicted N-linked glycosylation site [NAC (Asn-Ala-Cys); ***], and the potential site for the addition of the glycosyl-phosphatidyl-inositol anchor (###). The 24-amino acid repetitive sequence of the GP46/M-2 protein is aligned and indicated by hatched lines underneath the sequence.

studies employing vaccinia–GP46/M-2A recombinant expression vectors (D. Rodriquez, J. Rodriguez, L. Rivas, K.L.L., M. Esteban, and D.M.-P., unpublished data) are consistent with the cloned genomic gene encoding the GP46/M-2 molecule. The differences found between the protein sequence derived from the genomic DNA sequence and that of the isolated protein may be due to potential errors in the protein sequence data or because the GP46/M-2 is encoded by a gene family (see below). In addition, the original protein sequence data were obtained from populations of late logarithmic phase organisms of the *L. amazonensis* strain LTB0016, whereas the sequenced genomic clone has been derived from promastigotes of a clone (C1S1). Consequently, further analyses will be required to determine the basis for the variation between the genomic DNA-derived sequence and original protein sequence data.

Structural Analysis of GP46. Analyses of the primary protein sequence were performed to reveal biochemically important features and to suggest possible protein secondary structures. Three specific areas of amino acid sequence were identified within the GP46/M-2 molecule (Fig. 3); these are discussed below.

A Kyte–Doolittle hydropathicity plot (29) of the protein revealed two hydrophobic regions, at the amino- and carboxyl-terminal portions of the molecule. Consensus sequences specific for the cleavage and removal of hydrophobic leader peptides from membrane-associated proteins (for review, see ref. 30) predict a leader peptide for GP46/M-2 that extends for 32 amino acid residues from a methionine (amino acid residue −32) through an alanine (amino acid residue −1, Fig. 4) and predicts that the amino terminus of the protein is an alanine, consistent with protein sequence data.

The attachment of glycoproteins to the outer membrane surface through a specific glycolipid linkage has been described for a variety of organisms (31–33). Preliminary data (L. Rivas and D.M.-P., unpublished results) have indicated that the mature form of GP46/M-2 contains a carboxyl-terminal-linked glycolipid that acts to anchor the protein to the parasite surface membrane. Carboxyl-terminal protein sequence data derived from glycolipid-anchor-containing proteins (33) indicates that, after synthesis of the protein is complete, relatively short hydrophobic peptides are removed prior to the addition of a glycolipid tail. Commonly, the two amino acids found immediately after the cleavage site include combinations of alanine, asparagine, aspartic acid, glycine, and serine residues and a hydrophobic sequence of variable length (7–12 amino acids) is found on the carboxyl-terminal side of the cleavage site. In GP46/M-2, the region from

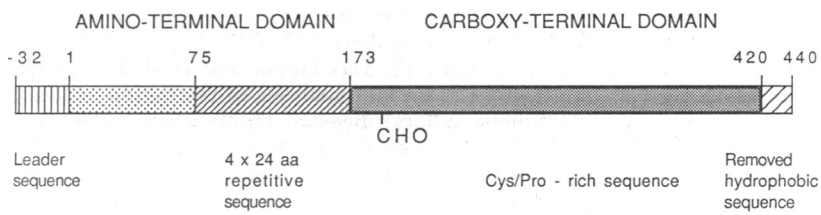


FIG. 3. Diagrammatic representation of the structural features of the gene encoding the GP46/M-2 protein of *L. amazonensis*.

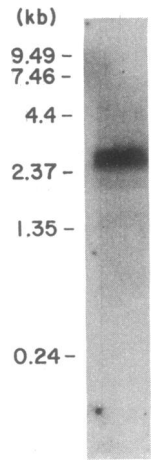


FIG. 4. Autoradiographic results from Northern blot experiments employing 10 μg of isolated total *L. amazonensis* promastigote mRNA and ^{32}P-labeled plasmid p3BF16.

Cys-426 to the end of the protein fits the basic criterion for a hydrophobic peptide and a Asp-Gly-Ala amino acid sequence is found upstream at residues 420–422 that could be cleaved between Asp-420 and Gly-421 with the subsequent attachment of glycosyl-phosphatidylinositol anchor to Asp-420.

Pulse–chase and endoglycosidase experiments characterizing the GP46/M-2 protein (16) suggested the presence of a single asparagine-linked glycosylation site. Nonetheless, a consensus amino acid sequence [Asn-Xaa-Thr or Asn-Xaa-Ser (34)] was not found in the GP46/M-2 protein sequence. However, an Asn-Ala-Cys (34, 35) sequence is present at position Asn-193 (Fig. 2*B*); this exact sequence has been described (34, 35) as a glycosylation site for bovine protein C. The location of the putative GP46/M-2 glycosylation site agrees with data that localizes the carbohydrate to within the 27-kDa carboxyl-terminal segment of the protein (16).

GP46/M-2 Repetitive Sequence. The amino-terminal half of the GP46/M-2 molecule contains a sequence of 24 amino acids that is repeated four times and constitutes nearly a quarter of the total protein sequence. The aligned repeated sequences are indicated in Fig. 2*B*. The carboxyl-terminal segment of the repetitive sequence appears to be relatively conserved, while there appears to be more variation in the amino-terminal portion. In addition, the type of amino acid (hydrophobic versus hydrophilic) appears to be conserved at certain repeat residue positions. For example, in positions 7 and 14 of the repetitive sequence, the amino acids used are Phe/Leu/Val, all of which are hydrophobic; in other positions (repeat residues 10, 12, and 17), the amino acids are all capable of hydrogen bonding. These patterns may reflect the action of selective constraints on the molecule.

Repetitive amino acid sequences have been reported for *Leishmania major* and *Leishmania donovani* (36) as well as *Trypanosoma cruzi* (37) and have been found to exist in numerous surface protein antigens of malarial parasites (38–41). The ability of the malarial parasites to vary these immunodominant segments of repetitive protein sequence has been implicated in mechanisms for evasion of the host immune response; successful evasion may occur by a genetically restricted immune response to a specific repetitive motif or by the rapid variation of immunodominant repetitive sequences. Genetic polymorphism as reflected by a number of molecular and biochemical techniques (42–50) occurs within and between the species of the genus *Leishmania*. However, antigenic variation similar to that found for malarial parasites has not been documented as yet for *Leishmania*; in fact, those repetitive sequences studied to date appear to be present in all species of *Leishmania* examined (36).

For the GP46/M-2 protein, whether variation occurs within the repetitive sequence between various strains of *L. amazonensis* is currently unclear. However, this is unlikely to create a problem as evidence suggests that conserved segments of the molecule exist and that these may be responsible for the protective immune response elicited *in vivo*. First, in protective immunity studies using the murine model system (8), genetic restriction of the immune response to the GP46/M-2 molecule is not evident. In addition, the immunodominant segment of the molecule appears to involve the carboxyl-terminal rather than the amino-terminal domain of the molecule containing the repetitive sequence (16). Furthermore, monoclonal antibodies directed to different epitopes of the GP46/M-2 molecule consistently recognize the *L. amazonensis* species, regardless of the biological source of the strain or its geographical origin (11).

Cysteine/Proline-Rich Protease-Resistance Immunodominant Carboxyl-Terminal Domain. A Chou–Fasman (51) analysis for the prediction of secondary protein structure predicts the presence of segments of α-helix with a predominance of "turn" structure, especially in the carboxyl-terminal portion of the protein. The carboxyl-terminal half of the GP46/M-2 protein is rich in proline and cysteine residues (Fig. 3) that account for ≈19% of the carboxyl-terminal sequence and for ≈75% of the total proline/cysteine residues of the mature protein. The cysteine/proline-rich composition of the carboxyl-terminal segment of the protein is consistent with a stable folded structure and the general refractoriness to protease digestion observed for this portion of the molecule (16). The proteolytic stability may be responsible, in part, for the apparent immunodominant response to this segment of the protein (16). In addition, such refractoriness may be essential for the maintenance of the biological function of this molecule for the promastigote as it develops within the hydrolytic environment found in the midgut of the sand fly.

Cysteine residues are generally involved in disulfide bond formation; however, the presence of sequences within this region with the motifs Cys-Xaa$_{-2-4}$-Cys/His-Xaa$_n$-Cys-Xaa$_{2-4}$-Cys (residues 232–246) and His-Xaa$_4$-Cys (residues 184–189) are suggestive of metal binding proteins (52). The stability of this segment/domain of the GP46/M-2 molecule, as observed for other molecules, could be due in part to metal ion binding (53). Alternatively, the cysteine residues in the carboxyl-terminal segment may contribute to stabilization through formation of disulfide bonds (54). The structural features maintaining the integrity of this immunodominant proline/cysteine-rich region of the GP46/M-2 molecule is of potential interest to vaccine studies.

Northern and Southern Blot Analyses. Northern blot analysis of promastigote mRNA revealed a single 2.3-kb RNA (Fig. 4) sufficiently large enough to encode the GP46/M-2 protein.

The results from Southern blot analyses of nuclear DNA of *L. amazonensis* digested with various restriction endonucleases and probed with a ^{32}P-labeled 62-base oligonucleotide probe are shown in Fig. 5*A*. Both weakly and strongly hybridizing bands were observed for the restriction endonucleases *Hin*dIII and *Bam*HI, although no restriction sites for these enzymes are present within the area of the GP46/M-2 gene sequence corresponding to the oligonucleotide probe. As shown in Fig. 5*B*, *L. amazonensis* DNA, digested with *Hin*dIII or *Pst* I and probed with the *Bam*HI–*Sph* I fragment of p3BF16, generated more fragments than predicted on a basis of the map shown in Fig. 2*A* and known DNA sequence. These data indicate that there may be multiple copies or a gene family of GP46/M-2 in *L. amazonensis*. Recent estimates suggest that there are 10–12 copies of the gene per haploid genome (S. Das Gupta and D.M.-P., unpublished results). Southern blot analyses of restriction endonuclease (infrequent cutters) digested chromosomes of *Leishmania* (D.M.-P., Y. Traub-Csekö, K.L.L., and S. Beverley, unpublished data) confirm these data, indicating the presence of GP46/M-2 families in *L. major* and *L. donovani*. The pres-

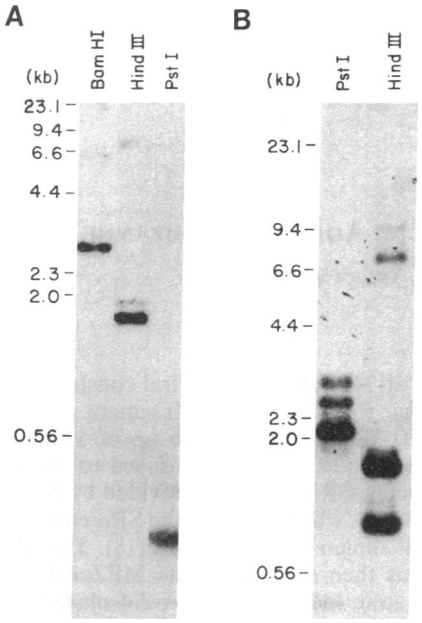


FIG. 5. (*A*) Autoradiographic results from Southern blot experiments employing *L. amazonensis* genomic DNA digested with various restriction endonucleases, as indicated, and ^{32}P-labeled 62-base oligonucleotide probe from the amino-terminal sequence of the GP46/M-2 protein. (*B*) Autoradiographic results from Southern blot analyses employing genomic DNA digested with various restriction endonucleases and a ^{32}P-labeled *Bam*HI–*Sph* I fragment from the coding region of the GP46/M-2 protein derived from the p3BF16 clone.

ence of conserved gene families suggests that this is an important class of parasite molecules and these proteins should serve as vaccine candidates.

CONCLUSIONS

The gene encoding the immunologically protective GP46/M-2 protein of *L. amazonensis* has been cloned and sequenced. The DNA sequence data agree with the known biochemical and immunochemical features of the molecule. Southern blot analyses suggest multiple copies or a family of GP46/M-2 genes in *L. amazonensis*. The amino acid sequence of the protein is distinct from those reported for *Leishmania* membrane proteins and appears to represent a unique class/family of antigenic surface molecules. The GP46/M-2 molecule has been demonstrated in a murine model system to elicit a protective immune response against infection with *L. amazonensis*; consequently the ability of the related molecules of other species to elicit protection will be of interest in terms of a future vaccine against leishmaniasis.

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