The Immunologically Protective P-4 Antigen of Leishmania Amastigotes

A DEVELOPMENTALLY REGULATED SINGLE STRAND-SPECIFIC NUCLEASE ASSOCIATED WITH THE ENDOPLASMIC RETICULUM*

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The purified membrane-associated Leishmania pifanoi amastigote protein P-4 has been shown to induce protective immunity against infection and to elicit preferentially a T helper 1-like response in peripheral blood mononuclear cells of patients with American cutaneous leishmaniasis. As this molecule is potentially important for future vaccine studies, the L. pifanoi gene encoding the P-4 membrane protein was cloned and sequenced. Southern blot analyses indicate the presence of six tandemly arrayed copies of the P-4 gene in L. pifanoi; homologs of the P-4 gene are found in all other species of the genus Leishmania examined. DNA-derived protein sequence data indicated an identity to the P1 zinc-dependent nuclease of Penicillium citrinum (20.8%) and the C-terminal domain of the 3’ nucleotidase of Leishmania donovani (33.7%). Consistent with these sequence analyses, purified L. pifanoi P-4 protein possesses single strand nuclease (DNA and RNA) and phosphomonoesterase activity, with a preference for UMP > TMP > AMP >> CMP. Double-labeling immunofluorescence microscopic analyses employing anti-binding protein antibodies revealed that the P-4 protein is localized in the endoplasmic reticulum of the amastigote. Northern blot analyses indicated that the gene is selectively expressed in the intracellular amastigote stage (mammalian host) but not in the promastigote stage (insect of the parasite). Based upon its subcellular localization and single-stranded specific nuclease activity, possible roles of the P-4 nuclease in the amastigote in RNA stability (gene expression) or DNA repair are discussed.

Leishmania sp. are dimorphic intracellular parasites that cause a wide spectrum of human diseases, ranging from self-limited cutaneous to the more severe diffuse cutaneous and visceral forms. The parasite exists as a flagellated promastigote within the alimentary tract of its insect vector, the phlebotomine sand fly; within the mammalian host, the parasite transforms into the amastigote stage and resides in the phagolysosomal vacuole of the macrophage. Leishmania pifanoi, a member of the Leishmania mexicana complex, is associated with both simple and diffuse cutaneous leishmaniasis in the New World (1). The latter form of the disease is characterized by large histocytoma-like cutaneous nodules containing heavily parasitized macrophages and by a parasite-specific impairment of the cell-mediated immune response (2); patients with diffuse cutaneous leishmaniasis are generally resistant to current forms of chemotherapy (1). Over the past decade, leishmanial vaccine research has gained significant attention as clinical treatment failure is becoming increasingly common in many areas; furthermore, drugs used for therapy can be associated with significant adverse effects. However, problems exist with standard live vaccines employing virulent organisms (3, 4); consequently, a focus in leishmanial vaccine development is the identification of defined protective immunogens (5–9). Antigens specific for the amastigote (intracellular-mammalian host) stage of the parasite have been of interest in the construction of a leishmanial vaccine, as such developmentally regulated molecules may be biologically important for the intracellular survival of the parasite. Furthermore, the amastigote is the parasite stage responsible for the pathology associated with disease.

Relatively little is known about the mechanisms of amastigote adaptation and survival within the degradative milieu of the macrophage phagolysosome (10). Metabolic differences are known to exist between the promastigote and amastigote stages (11–14); in addition, several leishmanial molecules have been demonstrated to be up-regulated or specifically associated with the amastigote stage. These include specific glycosphin-golipids, parasite lysosomal enzymes (cysteine proteinase(s); arylsulfatase), the Leishmania donovani A2 gene, superoxide dismutase, and the proteophosphoglycan molecule(s) (15–19). The biological functions of these stage-specific molecules are of interest in terms of their potential role(s) in parasite virulence, pathogenicity, and intracellular survival. The leishmanial superoxide dismutase is considered to be involved in the detoxification of host cell radical oxygen intermediates known to be deleterious to the intracellular amastigote. The proteophosphoglycan molecule appears to have a role in parasite vacuole formation within the infected macrophage. Although not essential for survival, experimental studies of Leishmania genetically deficient in either the A2 or cysteine proteinase genes indicate that these molecules are important in parasite virulence.

We have previously reported that three purified antigens (P-2, P-4, and P-8), up-regulated or selectively expressed in the...
amastigote stage, provide partial to complete protection in BALB/c mice against infection with *L. pifanoi* and *Leishmania amazonensis* (20). The enhanced resistance to infection in mice immunized with the P-4 antigen correlates with an increased interferon-γ (Th1-like) response. More recently, we have found that the P-4 antigen also can elicit a preferential Th1-like response in patients with American cutaneous leishmaniasis (21). For future vaccine studies of leishmaniasis and to understand better the potential biological function of the P-4 amastigote protein, we have cloned and sequenced the gene encoding the P-4 antigen from *L. pifanoi*. DNA-derived protein sequence data indicate that P-4 is a single strand-specific nuclease. Biochemical analyses have demonstrated that P-4 has both endo- and exonuclease activities and cleaves both RNA and single strand DNA substrates. The specific nuclease/ribonuclease activities, as well as developmental regulation of this molecule, suggest a potential role for P-4 in intracellular survival of these protozoan parasites.

**EXPERIMENTAL PROCEDURES**

**Parasite Strains and In Vitro Cultivation—** *L. pifanoi* (MHOM/VE/60/Ltr6) amastigotes were maintained at 31 °C in F-29 medium containing 20% heat-inactivated fetal bovine serum (FBS). *Life Technologies*, Inc., as previously reported (22). *L. amazonensis* (MHOM/BR/77/LTB0016), *Leishmania major* (MHOM/IS/79/LRCL251) strain WR309, *Leishmania braziliensis* (MHOM/BR/75/M2903), and *L. donovani* (MHOM/ET/67/LTL2) strain LV9 promastigotes were grown at 23 °C in Schneider's Drosophila medium supplemented with 20% FBS.

**Amino Acid Sequencing—** The P-4 antigen was purified from detergent-solubilized *L. pifanoi* amastigote membrane preparations by monoclonal antibody affinity chromatography as described previously (20). Isolated P-4 protein was then concentrated and further separated by SDS-PAGE. After staining of the proteins with Coomassie Blue, gel slices containing either the 33- or 35-kDa protein were excised and subjected to in-gel enzymatic digestion with either trypsin or chymotrypsin (Roche Molecular Biochemicals). Peptides were isolated by high pressure liquid chromatography on a Vydac C-18 column and subjected to amino acid sequence analysis at the Yale University School of Medicine Protein and Nucleic Acid Chemistry Facility. For N-terminal sequence analysis of the 33-kDa protein, proteins separated by SDS-PAGE were electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), stained with Coomassie Blue, and subjected to gas phase sequencer analysis.

**Amplification of cDNA by PCR and Genomic Library Screening—** *L. pifanoi* amastigote cDNA was generated from isolated total mRNA and was sequenced in both directions at least three times. Analyses of the resulting nucleotide and amino acid sequences were performed using the Swiss Institute of Bioinformatics ExPaSy Proteomics Server.

**Gel Electrophoresis and Molecular Karyotype Analysis—** Northern blot analysis was performed using total mRNA isolated from cultured parasites using a micro RNA isolation kit (Stratagene). The mRNA was fractionated electrophoretically in 1% agarose gels containing 2.2 M formaldehyde (23) and transferred to Nytran filters (Schleicher & Schuell). Blots were hybridized with the TA6.2 probe at 42 °C in 2× SSC, 50% formamide and washed at 42 °C with 2× SSC, 0.5% SDS. The filters were exposed to Kodak X-Omat AR film at −70 °C with a Cronex Lightning Plus intensifier screen.

**Phosphonoamosterase, Nuclease, and Endonuclease Assays—** The P-4 protein was isolated as indicated above and assessed for purity by SDS-PAGE analysis using Coomassie Blue staining as described previously (20). Phosphonoamosterase activity of purified P-4 protein was assessed by measuring the inorganic phosphate liberated following the hydrolysis of the indicated substrates. As described previously (26), the enz-...
MAI was used as a positive control. Incubation was carried out at 37 °C for 30 min and terminated by chilling and addition of 0.4 ml of ice-cold 10% trichloroacetic acid. The sample was clarified by centrifugation, and the absorbance at 260 nm was determined. Result are expressed as nanomoles of nucleotide released per 30 min.

The endonuclease activity of the P-4 protein was assessed using covalently closed single-stranded M13mp DNA, a “bubble” DNA substrate, and single strand oligonucleotides. Briefly, covalently closed single-stranded M13mp DNA (0.5 μg) was incubated with different concentrations of P-4 protein at 25 °C in 30 μl of buffer containing 50 mM sodium acetate, 30 mM NaCl, and 1 mM ZnSO4 for 2 h. The reaction was stopped, followed by electrophoresis in 1% agarose gel. DNA was stained with ethidium bromide and then photographed using Polaroid type 55 film. The P-4 protein was tested for its ability to cleave bubble-structured DNA consisting of a central unpaired region of 29 nucleotides in one strand and 30 nucleotides in the other strand flanked by 30 base pairs on both sides. Substrate was formed by annealing one 90-mer oligonucleotide and one 99-mer oligonucleotide (as indicated below); one of the strands (89- or 99-mer) was labeled with [γ-32P]ATP at the 5′-end. The bubble substrate gel was purified after annealing the oligonucleotide 5′-CCAGTATCATACAGCTTGTGAGATCCGCCCGGTAGCAGT- GACCG-3′ to the oligonucleotide 5′-CGTTCAACGTGCGCCATACACGAGCCGGCTGACACCGGT- TGGCAAG-3′. The bubble-structured DNA (2 ng) was incubated with different concentrations of P-4 at 25 °C for different times. Reactions were stopped by adding an equal volume of denaturing solution (95% (v/v) formamide, 10 mM EDTA, pH 8.0, 0.1% bromphenol blue, and 0.1% xylene cyanol), and samples were heated to 95 °C for 3 min. Products were separated on a denaturing 12% polyacrylamide gel and visualized by autoradiography and photographed using a DC 220 ZOOM camera. Size markers were made by labeling of the 10-bp DNA ladder with [γ-32P]ATP.

To determine the nucleotide preference of the P-4 nuclease, 5′-end-labeled oligonucleotide 5′-CGTTCAACGTGCGCCATACACGAGCCGGCTGACACCGGTCAGTGGCAAG-3′ or 5′-CCAGTATCATACAGCTTGTGAGATCCGCCCGGTAGCAGTGACCGGT-3′ was used as substrate. P-4 digestion was conducted as indicated above, and the products were visualized by autoradiography after resolution in denaturing 12% polyacrylamide gel.

Subcellular Localization of the P-4 Single Strand-specific Nuclease—All incubations were carried out on ice. After washing three times in PBS, L. pifanoi amastigotes were incubated in PBS containing 4% paraformaldehyde for 15 min and then washed once in PBS. Fixed cells were then permeabilized by incubation for 5 min, in PBS containing 0.05% Triton X-100, washed once in PBS, and then incubated in PBS containing 5% FBS and 5% normal goat serum. After washing twice with PBS, amastigotes were then either incubated for 45 min with either normal rabbit serum or anti-P-4 monoclonal antibody and rabbit anti-guinea pig IgG, generously provided by Dr. J. Bangs, University of Wisconsin, Wisconsin, diluted in PBS, 5% FBS. The parasites were then washed three times with PBS containing 5% FBS and 0.05% Tween 20. Washed amastigotes were then incubated for 45 min with fluorescein-conjugated goat antirabbit IgG (1:100; Molecular Probes), rhodamine-conjugated goat anti-mouse IgG (1:100; Jackson Laboratories, Inc.), and DAPI (1:1000; Sigma). Organisms were then washed, air-dried onto poly-L-lysine-coated slides, and mounted in aqueous mounting medium (Biomedia Corp., Foster City, CA). Fluorescence was visualized using either a Nikon Microphot-FXA microscope; images were digitalized with a film scanner equipped with the microscope. Alternately, the localization of P-4 and/or BiP proteins were examined using confocal microscopy employing a Zeiss axiovert 100 and LSM 510 software.

### RESULTS

Cloning of the P-4 Gene—We have previously shown that on SDS-PAGE, affinity purified P-4 appears as a doublet of proteins with estimated molecular masses of 33 and 35 kDa (20). Pulse-chase labeled procyclic flagellates, examined in vivo biochemical processing of these proteins, indicated that the 35-kDa protein is the precursor of the 33-kDa protein (65). In agreement with this, the alignment of high pressure liquid chromatography elution profiles of trypsin-digested 33- and 35-kDa proteins indicated that these two proteins were closely related, if not identical (data not shown).

### Table I

| Peptides | Sequence | Designed primers* |
|----------|----------|-------------------|
| N-terminal | XGVXGHMLLAIA | |
| I | AQLDLNEEEIKQR | A1 |
| II | XRHOLDLENEEQKQ | A5 |
| III | MLENKWTQHMAVW | |
| IV | HTISRY | |
| V | XVXTSPGVTGGLTL | |
| VI | YTDLFL | |
| VIII | XLSATADKLVETY | A4 |
| VII | FSELETLVDMAHEE(S) | |

* Degenerate oligonucleotide primers were synthesized based on underlined amino acids.

X designates an undetermined residue.

The amino acid sequences of the N terminus and eight internal tryptic and/or chymotryptic peptides (Table I) from the 33-kDa P-4 protein were obtained (see “Experimental Procedures”). Based on amino acid sequences of peptides II, III, and VII, degenerate oligonucleotides A1, A4, and A5, respectively, were synthesized (see “Experimental Procedures”) using a mixed primer strategy (32, 33). Reverse transcriptase-PCR amplification of *L. pifanoi* amastigote RNA employing either A1/A4 or A5/A4 primer sets each yielded a single fragment of approximately 540 or 520 bp, respectively. These PCR products were cloned into pCRTM II vectors; the DNA sequences obtained encoded a polypeptide that included five peptides of trypsin/chymotrypsin-digested P-4 (peptides I–IV, VI, and VII), clearly indicating that these PCR products represented a segment of a cDNA encoding the P-4 protein. To obtain a complete copy of the P-4 gene, a *L. pifanoi* EMBL3cos genomic library was screened using a radiolabeled TA6.2 cDNA clone as probe. Two separate phage clones containing genomic P-4 gene copies were isolated; subfragments containing the P-4 genes were subcloned and sequenced from each phage clone. The two cloned copies of the P-4 gene were found to have identical sequences. The sequence of the cDNA clone TA6.2 was contained within the genomic clones; some differences in the derived protein sequence appeared to exist between the TA6.2 cDNA clone and genomic sequences. These differences did not involve residues involved in either zinc-binding sites nor hypothesized to be involved in the active site of the enzyme. Furthermore, the complete derived P-4 protein sequence included the chymotryptic peptides V and VIII (Table I), not found within derived protein sequence of the TA6.2 cDNA clone.

The final DNA sequence encoding the P-4 protein is shown in Fig. 1A. The gene encoding P-4 is 948 nucleotides in length (Fig. 1A); the open reading frame encodes a polypeptide of 316 amino acids with a predicted mass of 35.1 kDa and predicted pI of 8.9. Based upon the structural features signal peptide, a putative signal peptide recognition site (34) is predicted between Gly-30 (amino acid residue 30) and Trp-31 (Fig. 1A, marked with ▼). This is consistent with the known N-terminal sequence of the 33-kDa protein (Table I). The deduced protein sequence contains two putative N-linked glycosylation sites (NFT and NTS, Fig. 1B) at amino acid residues 108–110 and 251–253, as well as potential casein kinase II phosphorylation sites and protein kinase C phosphorylation sites. The existence of such post-translational modifications, however, needs to be confirmed experimentally. The P-4 proteins have been demonstrated to be membrane-associated (20). Based on the prediction of Gerber et al. (35), it is not likely that P-4 is a glycosylphosphatidylinositol-anchored protein. Sequence analyses, however, predicted three putative transmembrane domains from residues 1–38, 134–151, and 286–299. However, based upon the enzymatic activity (see below) of the protein, which is
Fig. 1. A, the complete genomic DNA sequence and derived protein sequence for the P-4 protein are shown. Nucleotides are numbered on the left. The deduced amino acid sequence is displayed above the DNA sequence. The putative cleavage site for the signal peptide is indicated (\(\nabla\)); the termination codon is indicated by a †. The sequence data are available from GenBank\textsuperscript{TM} under the accession number AF057351. B, comparative analysis of the sequences of the P-4 single strand-specific nuclease and the \textit{P. citrinum} P1 nuclease and the 3'-nucleotidease of \textit{L. donovani}. The potential glycosylation sites of the P-4 nuclease are double underlined. Conserved areas of sequence among these three proteins are indicated by using \textit{boldface} type; residues implicated from crystallographic analyses (36) in either the zinc-binding site and/or the active site of the \textit{P. citrinum} P1 nuclease are indicated with an *. The amino acid residues that match with the N-terminal and chymotryptic/tryptic peptides of the purified 33-kDa protein are \textit{underlined}.

dependent upon zinc-binding site included in residues 138–151, it is unlikely that these residues represent a membrane-spanning region of the protein.

To define the biological function of the P-4 protein(s), the nucleotide and amino acid sequences of P-4 were compared with those in the data banks. These analyses indicated similarities in amino acid sequences among the mature P-4 protein (amino acid residues 31–316), and the C-terminal region of \textit{L. donovani} 3'-nucleotidase/nuclease (3'-NT/Nu; GenBank\textsuperscript{TM} accession number L35078, with an open reading frame of a 477 amino acids), and the zinc-dependent \textit{P. citrinum} nuclease P1 (GenBank\textsuperscript{TM} accession number P24289, with an open reading frame of a 270 amino acids). Within these regions, P-4 shared a 33.7% identity with 3'-NT/Nu and a 20.8% identity with nuclease P1. Furthermore, it was evident that the residues implicated in zinc binding and/or the enzyme active site (Fig. 1B, marked with *) were conserved among these three proteins (36), suggesting that the levels of identity found were significant.
...the sequence divergence, these results never-though it is still unclear whether the weaker hybridization at least 10-fold weaker than that observed for 1400–1500 kb; the hybridization signals for these species were gene was identified on one chromosome of approximately Bam Eco I, and using enzymes that cut once (RV, nuclease and probed with the labeled cDNA clone, TA6.2. By existence of homologous chromosomes differing in size have separated by CHEF electrophoresis. Southern blots of zonensis, L. braziliensis, and L. donovani, were sep-rated by CHEF electrophoresis and then hybridized with a labeled TA6.2 probe. The experimental conditions are as indicated under “Experimental Proce-sis of the P-4 genes among the Leish-mania. The experimental conditions are as indicated under “Experimental Proce-dures.” Left panel, the radioautographic results from Southern blot analyses fol-low ing CHEF electrophoresis indicating the chromosome location for the P-4 genes in L. pifanoi (6-h exposure) and other spe-cies of Leishmania (120-h exposure). Right panel, the ethidium bromide-stained agarose gel. The yeast chromo-some M markers (Amersham Pharmacia Biotech) are as indicated (kb).

FIG. 2. Molecular karyotype analy-sis of the P-4 genes among the Leish-mania. The experimental conditions are as indicated under “Experimental Proce-dures.” Left panel, the radioautographic results from Southern blot analyses fol-low ing CHEF electrophoresis indicating the chromosome location for the P-4 genes in L. pifanoi (6-h exposure) and other spe-cies of Leishmania (120-h exposure). Right panel, the ethidium bromide-stained agarose gel. The yeast chromo-some M markers (Amersham Pharmacia Biotech) are as indicated (kb).

Fig. 2. Molecular karyotype analysis of the P-4 genes among the Leishmania. The experimental conditions are as indicated under “Experimental Procedures.” Left panel, the radioautographic results from Southern blot analyses following CHEF electrophoresis indicating the chromosome location for the P-4 genes in L. pifanoi (6-h exposure) and other species of Leishmania (120-h exposure). Right panel, the ethidium bromide-stained agarose gel. The yeast chromosome M, markers (Amersham Pharmacia Biotech) are as indicated (kb).

Results suggested the possibility of tandemly repeated copies of P-4 gene. To verify this possibility, partial digestions of L. pifanoi DNAs with EcoRV or PstI were performed. A clear repetition of five copies of a band of 2.4 kb is evident after digestion with both enzymes (Fig. 3B). These Southern blot analyses indicate that at least six copies of the P-4 gene, arranged as a tandem repeat, are present in the L. pifanoi genome. These results indicate that P-4 is a member of a gene family of proteins; these findings are of importance for further genetic studies examining the function of the P-4 genes.

Functional Analysis of the P-4 Molecule: Phosphomonoesterase, Exonuclease, and Endonuclease Activities—As sequence data indicated that the P-4 protein was potentially related to single strand-specific nucleases, the enzymatic activities of purified P-4 was studied with respect to the specificity of hydrolyzing ribo- and deoxyribonucleotide substrates. It was evident that P-4 protein(s) displayed phosphomonoesterase activities, with the following substrate preference: 3'-UMP > 3'-TMP > 3'-AMP >> 3'-CMP (Table II); no activity was detected with 5'-AMP (data not shown). In addition, the P-4 protein contained nuclease activities, with the following substrate preference: ssDNA > RNA > dsDNA. These data clearly indicate that P-4 is preferentially a single strand nuclease, with exonuclease activity. In comparison to mung bean nuclease, P-4 has comparable activity toward ssDNA; however, P-4 nuclease appears to be relatively more active toward RNA.

The endonuclease activity of the P-4 nuclease was examined using single-stranded circular M13 DNA. As seen in Fig. 4, when single-stranded circular (SSC) DNA M13mp was incubated with purified P-4 protein, the DNA was degraded; the level of degradation (partial to complete) correlated with the concentration of the P-4 protein. These results reveal that the P-4 protein has an associated endonuclease as well as exonuclease function because it acts on covalently closed SSC DNA. The apparent reaction of the P-4 nuclease with dsDNA and ssDNA was examined further using a bubble DNA substrate (Figs. 5 and 6). The bubble DNA substrate was preferentially cleaved within the single-stranded areas when incubated with P-4 (Fig. 6A, lane 2); these results further confirm the endonucleolytic activity of the protein and suggest that P-4 is a single strand-specific nuclease. In addition, it was evident that the P-4 nuclease preferentially digested the poly(T) (90 nucleotides; Fig. 6A) strand of the bubble substrate and not the poly(C) (89 nucleotides) strand (Fig. 6B). These data are consistent with the phosphomonoesterase specificities found for the P-4 nuclease. Further analyses of the digestion (Fig. 6C) of either the monomeric 89- or 90-mer oligonucleotides indicated a strong preference of the P-4 nuclease for thymidine. The vari-
ous fragments generated were consistently found to represent selectively cleavages at thymidine residues. This is of interest and undoubtedly reflects the lower level of P-4 employed for these digestion (10 ng). Activity toward the phosphomonoesterase substrates suggested that the specificity for thymidine monophosphate > adenosine monophosphate at low P-4 levels, whereas the P-4 nuclease had relatively comparable activities toward both substrates at higher enzyme concentrations.

Developmental Expression of the P-4 Gene—We have previously shown that the P-4 monoclonal antibody recognizes antigenic components selectively expressed by axenic and macrophage-derived amastigotes of *L. pifanoi* and *L. amazonensis* but not by the respective promastigote forms (40, 41). To establish that P-4, in fact, was developmentally regulated, we isolated total RNAs from amastigotes and promastigotes of *L. pifanoi* and performed Northern blot analyses using labeled TA6.2 as a probe. Results of these analyses indicated that *L. pifanoi* amastigotes displayed high levels of P-4 RNA and that at least four transcripts, 2.48, 4.96, 7.44, and 9.1 kb, could be identified (Fig. 7A, lane a). Weaker hybridization signals, only evident upon longer exposure, were detected in *L. pifanoi* promastigotes (Fig. 7A, lane p). These results are consistent with Northern blot analysis of *L. amazonensis* organisms, in which specific P-4 mRNAs of 2.48, 4.96, and 7.4 kb were expressed only in the amastigote but not in promastigote developmental stage (35) (Fig. 7B). Reprobing of the filter with a labeled probe for the *Ldp23* gene (23), which is expressed in both the promastigote and amastigote stage of the parasite, indicated that equal amounts of *L. amazonensis* promastigote and amastigote RNA were present (Fig. 7C). Consequently, P-4 mRNA does not appear to be expressed by the promastigote stage of *L. amazonensis*. The weak signal in the case of *L. pifanoi* promastigotes is likely due to low number of axenic amastigotes that are generally present in the promastigote cultures (65). Together, these studies indicate that the P-4 nuclease is predominantly, if not exclusively, expressed by the amastigote stage of the parasite.

Indirect Immunofluorescent Microscopic Studies—Preliminary immunofluorescence studies suggested that, although occasional nuclear staining was observed, the P-4 nuclease was predominantly located internally in the perinuclear area of the *Leishmania* amastigotes. To determine if the P-4 protein was associated with the endoplasmic reticulum of the parasite, studies examined the co-localization of P-4 with the binding protein (BiP), a major peptide-binding chaperone found in the...
endoplasmic reticulum. Co-localization experiments employing an anti-P-4 monoclonal antibody, a polyclonal anti-binding protein (BiP) antibody (against *Trypanosoma brucei* BiP, provided by Dr. J. Bangs (42)), and DAPI (DNA staining) were performed. As shown in Fig. 8, the P-4 molecule is predominantly found perinuclearly in the amastigote; the staining pattern for P-4 consistently overlapped with that found for BiP. It was noticeable that the localization of the BiP protein within the amastigote appeared more diffuse than that observed for P-4; this localization, however, appears to be characteristic of the BiP protein in kinetoplastids (42). Therefore, confocal microscopic analyses were performed to evaluate further the co-localization of BiP and P-4 (Fig. 8, D and E). The graphical representation of the subcellular localization indicates that P-4 (Fig. 8E, red line) was consistently found to co-localize with that of the BiP protein (Fig. 8E, green line). These results suggest that the P-4 single strand-specific nuclease mainly resides and potentially may function within the ER of the *Leishmania* amastigote stage.

**DISCUSSION**

The leishmanial P-4 protein antigen has been demonstrated to be a single strand-specific nuclease associated with the endoplasmic reticulum of the amastigote (mammalian host) stage of the parasite. Although the phosphomonoesterase specificity found for the P-4 nuclease is similar (preferring 3'-substrates) to that reported for the *L. donovani* 3'-NT/Nu (26, 43) and the two proteins share some homology, P-4 is biochemically distinct from 3'-NT/Nu in several aspects. The 3'-NT/Nu is an external surface membrane protein of *Leishmania* expressed by the promastigote stage and is encoded by a single copy gene. The P-4 nuclease is encoded by gene family with at least six copies/haploid genome and appears to be selectively expressed by the amastigote stage. The 3'-NT/Nu is thought to be involved in purine salvage; the level of 3'-NT/Nu expression is up-regulated under conditions of purine deprivation. These organisms are incapable of *de novo* purine synthesis; thus, enzymes devoted to the transport and metabolism of purines are critical for intracellular survival of the parasites (44). The difference in subcellular localization of the two enzymes undoubtedly reflects the distinction in the function of these two distantly related molecules/genes.

The P-4 single strand-specific nuclease was found to localize perinuclearly and to co-localize with the BiP protein, a chaperone molecule, and marker for the endoplasmic reticulum (42). Although P-4 sequence has a signal sequence for import into the endoplasmic reticulum, the protein is lacking a known/obvious ER retention sequence. ER retention sequences appear to be conserved (45, 46), even among the kinetoplastid protozoa (42). However, recent evidence suggests that targeting/import/retention in the ER can be complex (47–50) and may not be identical among various genera/species (51). Consequently, it will be of interest to determine the signals/areas of protein sequence in-
Nuclease activity has been reported in several systems to be associated with the endoplasmic reticulum; such enzymes appear to be important in RNA stability/expression during development or stress. The mRNA stability/half-life within a cell can change dramatically in response to environment, e.g. cytokines, starvation, and hormonal stimulation (52, 53). An estrogen-regulated Xenopus liver polysome nuclease has been shown to be involved in the selective destabilization of albumin mRNA (54); the enzyme has been demonstrated to recognize selectively two sites approximately 311 nucleotides from the 5’-end of the albumin coding area. An endoribonuclease that degrades polysome-associated myc mRNA is tightly bound to polysomes; the mRNA is first deadenylated and then degraded 3’ to 5’ (55). Furthermore, in the unfolded protein response, the Saccharomyces cerevisiae endoplasmic reticulum-associated Ire1p endonuclease has been shown to be involved in the excision of the HAC1 mRNA intron (56, 57); this specialized RNA splicing allows the translation of the Hac1p transcription factor responsible for the increased expression of ER resident proteins, the chaperon BiP, and protein disulfide isomerase.

In the case of Leishmania, studies indicate that RNA stability contributes to gene regulation of both the promastigote and amastigote stages. Studies of the gp63 and GP46 gene families, as well as the amastigote specifically expressed A2 gene, indicate that RNA stability contributes to the preferential gene expression (58–61). In the case of the gp63/GP46 gene families, this is observed as the specific expression of various gene family members during log and stationary growth phases (58–60); these studies clearly indicate the existence of RNase activity in gene regulation in the promastigote stage of the parasite. Studies of mRNA regulation/stability indicate the importance of 3’ area of non-coding sequence in conferring RNA stability (58, 61). It is possible that the ER-associated nuclease P-4 may play a role in gene regulation/expression in the amastigote stage. 

Alternately, it is possible that the P-4 nuclease is involved in nucleotide excision and repair (62, 63) in the amastigote. The specificity of the P-4 enzyme is not restricted to RNA; single strand DNA substrates are readily digested. The endonuclease activity of the P-4 nuclease might allow it to participate in the excision process preceding repair (63). The amastigote resides within the phagolysosome of the macrophage; within this milieu, the parasite is subjected to the oxidative (superoxide anion, H2O2, NO) onslaught of the cell. Consequently, DNA dam-

**Fig. 7.** Autoradiographic results from Northern blot experiments employing 10 μg of isolated total RNA from either Leishmania pifanoi or L. amazonensis organisms. Radiolabeled TA6.2 (540 bp) was used as a probe. A, L. pifanoi. Lane a shows axenically cultured amastigotes (31 °C). Lane p indicates late-log promastigotes derived from amastigotes after transformation at 22 °C. B, L. amazonensis. Lane a shows tissue-derived amastigotes; lane p, cultured late-log phase promastigotes. C, L. amazonensis. The same samples as in B but hybridized with a probe for Ldp23 (22). M, markers (RNA ladder, Life Technologies, Inc.) are as indicated (kb).

**Fig. 8.** Evidence for the co-localization of BiP and P-4 protein. A, L. pifanoi amastigotes stained with DAPI which stains the nuclei only. B, localization of P-4 protein in the same cells as in A. C, localization of BiP in the same cells as in A. D, confocal microscopic analyses of the localization of BiP protein and the P-4 nuclease. E, a graphical representation of the level of BiP and P-4 proteins across an arbitrary linear area (D, red arrowhead); the intensities of the BiP and P-4 proteins are indicated by the green and red lines, respectively.
age and repair are essential to the continued survival of the organism. The association of the ER with the nucleus could potentially allow for the transport of the nucleases as required. It may be that P-4 nuclease activity is only required in cases of parasite stress and DNA damage (e.g., oxidative metabolites of the macrophage); however, the enzyme resides proximal to the required site of action, hypothetically ready to be mobilized as required. At present the precise physiological role of the P-4 gene product in parasite differentiation and in the host-parasite interface is unknown but potentially may be involved in RNA stability and/or DNA excision/repair. Further investigation, involving genetic (64) and/or biochemical approaches, should prove useful to distinguish among these possibilities.

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The Immunologically Protective P-4 Antigen of *Leishmania* Amastigotes: A Developmentally Regulated Single Strand-Specific Nuclease Associated with the Endoplasmic Reticulum

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