Molecular and Functional Analyses of a Novel Class I Secretory Nuclease from the Human Pathogen, *Leishmania donovani*.

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The primitive protozoan pathogen of humans, *Leishmania donovani*, resides and multiplies in highly restricted micro-environments within their hosts (i.e. as promastigotes in the gut lumen of their sandfly vectors and as amastigotes in the phagolysosomal compartments of infected mammalian macrophages). Like other trypanosomatid parasites, they are purine auxotrophs (i.e. lack the ability to synthesize purines de novo) and therefore are totally dependent upon salvaging these essential nutrients from their hosts. In that context, in this study we identified a unique 35-kDa, dithiothreitol-sensitive nuclease and showed that it was constitutively released/secreted by both promastigote and amastigote developmental forms of this parasite. By using several different molecular approaches, we identified and characterized the structure of *LdNuc*<sup>ε</sup>, a gene that encodes this new 35-kDa class 1 nuclease family member in these organisms. Homologous episcopal expression of an epitope-tagged *LdNuc*<sup>ε</sup> chimeric construct was used in conjunction with an anti-*LdNuc*<sup>ε</sup> peptide antibody to delineate the functional and biochemical properties of this unique 35-kDa parasite released/secreted enzyme. Results of coupled immunoprecipitation-enzyme activity analyses demonstrated that this "secretory" enzyme could hydrolyze a variety of synthetic polynucleotides as well as several natural nucleic acid substrates, including RNA and single- and double-stranded DNA. Based on these cumulative observations, we hypothesize that within the micro-environments of its host, this leishmanial "secretory" nuclease could function at a distance away from the parasite to harness (i.e. hydrolyze/access) host-derived nucleic acids to satisfy the essential purine requirements of these organisms. Thus, this enzyme might play an important role(s) in facilitating the survival, growth, and development of this important human pathogen.

*Leishmania* are a group of primitive pathogenic trypanosomatid protozoan parasites that cause over 2 million new cases of human cutaneous, mucocutaneous, and fatal visceral disease (i.e. leishmaniasis) per year worldwide (1). All *Leishmania* parasites undergo a digenetic life cycle, which includes differentiation, development, and transmission between both a sand fly vector and a mammalian host. Within their hosts, these parasites reside and multiply in highly restricted micro-environments (i.e. as extracellular, flagellated promastigote forms in the alimentary tract of their sandfly vectors and as obligate intracellular amastigote forms in the phagolysosomal compartments of infected mammalian macrophages) (2). It is important to point out that *Leishmania*, like other trypanosomatid parasites, are incapable of the de novo biosynthesis of purines (3, 4). Thus, it seems obvious that in order to survive, these purine auxotrophs must be capable of salvaging these essential nutrients from their host environments. One such purported purine salvage mechanism involves a unique cell surface membrane-anchored, bi-functional 3'-nucleotidase/nuclease that is present in several pathogenic *Leishmania* species and related trypanosomatids (5–9). To date, however, no report exists to indicate that these organisms might also be capable of producing and releasing/secretion analogous purine salvage enzymes (e.g. nucleotide hydrolases, nucleases, or other nucleic acid hydrolases) into their host environments. In light of the above, in this study a variety of biochemical and functional approaches were used for the following: 1) to identify a new and unique 35-kDa nuclease produced by *Leishmania donovani* parasites, and 2) to examine its constitutive release/secretion by both the promastigote and amastigote life cycle developmental forms of this organism. Based on those observations, we used several molecular approaches to identify and characterize the structure of *LdNuc*<sup>ε</sup>, a gene that encodes this new, "secretory" class 1 nuclease from these parasites. An anti-*LdNuc*<sup>ε</sup> peptide antibody was generated against the gene-deduced protein and shown to specifically recognize and immunoprecipitate the single 35-kDa native nuclease synthesized and released/secerted by both wild-type *L. donovani* promastigotes and amastigotes. Furthermore, an epitope-tagged *LdNuc*<sup>ε</sup> chimeric construct was used in a homologous leishmanial expression system to delineate the functional and biochemical properties of this unique 35-kDa parasite released/secreted enzyme.

**EXPERIMENTAL PROCEDURES**

Reagents—Unless specified, all chemicals and reagents were of analytical grade and were obtained from Sigma. Enzymes used for molecular studies were obtained from Roche Applied
amastigotes were also isolated from spleens of infected hamsters. Washed cell pellets were harvested by centrifugation at 80 °C until needed. For some experiments, culture supernatants from such serum-grown promastigotes were harvested and used in immunoprecipitation assays. To that end, when promastigote cultures reached a density of $\sim 1-2 \times 10^7$ cells ml$^{-1}$ (i.e. mid-log phase), they were centrifuged at $\sim 2100 \times g$ for 15 min at 4 °C. The supernatants from these were removed and refuged at $6000 \times g$ for 10 min at 4 °C. Following this, the cell-free supernatants were carefully removed, frozen, and stored at $\sim 80 ^\circ$C until used.

In addition to promastigotes, axenic amastigotes of this L. donovani cloned line were also generated and grown at 37 °C in RPMI 1640 medium, pH 5.5, containing 20% (v/v) FBS as described previously (11). Cell-free culture supernatants from such in vitro grown axenic amastigotes were prepared as indicated above for promastigotes and subsequently stored at $\sim 80 ^\circ$C until used. In vivo, the tissue-derived L. donovani amastigotes were also isolated from spleens of infected hamsters (Mesocricetus auratus, LVG strain; Charles River Breeding Laboratories, Inc., Wilmington, MA), as described previously (11-13), frozen, stored in liquid nitrogen, and used as needed for various experimental purposes.

For isolation of nucleic acids and proteins, parasite cell cultures were grown to about mid-log phase as above and harvested by centrifugation at $\sim 2100 \times g$ for 15 min at 4 °C (5). The resulting cell pellets were washed three times in ice-cold phosphate-buffered saline (PBS, 10 mM sodium phosphate, 145 mM NaCl, pH 7.4) by centrifugation as above and finally resuspended in buffers appropriate for the extraction of DNA, RNA, or proteins (see below).

**Generation of Culture Supernatants from Parasites Grown under Chemically Defined Conditions**—To analyze the nuclease activity released/secreted by both L. donovani wild-type (i.e. nontransfected cells) and episomally transfected promastigotes, these cells were grown in serum-free, chemically defined medium (M199 +) according to McCarthy-Burke et al. (14). When such cultures reached a density of $\sim 1-2 \times 10^7$ cells ml$^{-1}$ (i.e. mid-log phase), they were examined by phase contrast microscopy to ensure $>99.9\%$ cell viability and subsequently harvested by centrifugation at $\sim 2100 \times g$ for 15 min at 4 °C as described previously (15). Following an additional high speed recentrifugation step, to ensure the complete pelleting of cells (15), such culture supernatants were carefully removed and subsequently concentrated up to $\sim 100$-fold (100×) using Centricon™ Plus-20 centrifugal filtration devices according to the manufacturer’s instructions (Amicon Bioseparations, Millipore Corp.). These concentrated, cell-free culture supernatants were frozen and stored at $\sim 80 ^\circ$C until needed. For activity gel analyses, such samples were diluted with SDS-polyacrylamide sample buffer (16) either containing 50 mM DTT (final concentration, +DTT) or lacking any reducing agents (−DTT). Following heating in a boiling water bath for 5 min, these samples were assayed for their nuclease activity using polynucleotide-containing substrate gels as described below.

**Parasite Cell Lysates**—Washed cell pellets of L. donovani wild-type promastigotes, axenic amastigotes, and in vivo-derived amastigotes as well as episomally transfected promastigotes were solubilized in SDS-polyacrylamide sample buffer (16) lacking any reducing agents and heated in a boiling water bath for 5 min. Following this, the solubilized samples were cooled to room temperature, frozen, and stored at $\sim 80 ^\circ$C until analyzed. The protein concentration in these cell lysates was determined using the bicinchoninic acid method according to the manufacturer’s instructions (Micro BCA, Pierce). Subsequently, equivalent amounts of protein from such lysates were separated in poly(A)-containing SDS-polyacrylamide gels under nonreducing conditions and stained in situ for their nuclease activity (17). In preliminary experiments to test the effects of reducing agents on nuclease activity, cell lysates were also prepared in SDS-polyacrylamide sample buffer containing 50 mM DTT (final concentration).

**In Situ Nuclease Activity/Zymogram Gels**—Parasite cell lysates, aliquots of concentrated serum-free culture supernatants from promastigotes (above) and samples from immunoprecipitation and affinity binding assays were separated in SDS-PAGE mini-gels (8 × 8 cm) and processed for in situ staining of nuclease activity essentially according to Bates (17) as modified from Zlotnick et al. (18). Briefly, samples were separated by SDS-PAGE using a 10% resolving gel (1 mm thick) containing poly(A) (300 µg ml$^{-1}$, final concentration) and a discontinuous buffer system (19). Following separation, gels were washed at room temperature with four changes ($\sim 100$ ml for 15 min each) of buffer containing 100 mM HEPES, 0.1% (v/v) Triton X-100 (Protein Grade™ Detergent, Calbiochem), pH 8.5, on an orbital shaker. This protocol was adopted to remove SDS from the gels and facilitate renaturation of nuclease activity. Subsequent to washing, these gels were incubated in the same buffer for 2 h at 37 °C with gentle agitation in a rocking hybridization oven (Shake ‘n Bake™, Boekel Scientific, Feasterville, PA). Following incubation, gels were rinsed briefly with deionized water and fixed for 10 min in 7.5% (v/v) aqueous acetic acid. Subsequently, these gels were washed in three changes of deionized water ($\sim 200$ ml, 10 min each), stained with toluidine blue O (0.2% (w/v) in 10 mM HEPES, pH 8.5) for 15 min, and destained using multiple changes of deionized water (20) all at room temperature using an orbital shaker. The nuclease activity in these gels was readily apparent as distinct, clear/colorless bands of substrate hydrolysis in an otherwise uniformly stained dark blue background of unhydrolyzed polynucleotide substrate.

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3 The abbreviations used are: FBS, fetal bovine serum; aa, amino acid; DIG, digoxigenin; DTT, dithiothreitol; gDNA, genomic DNA; HA, hemagglutinin; LdNuc, gene encoding the released/secretory nuclease of L. donovani; mt, nucleotide; ORF, open reading frame; PBS, phosphate-buffered saline; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; KLH, keyhole limpet hemocyanin; IFA, immunofluorescence; NRS, normal rabbit serum; FITC, fluorescein isothiocyanate; ER, endoplasmic reticulum; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid).
For some experiments, zymogram gels were prepared as above except that various other individual synthetic polydeoxyribonucleotides (i.e. poly(C), poly(G), poly(I), or poly(U), all from Sigma) were substituted for the poly(A) substrate.

Nomenclature—The designations used in this report for genes, proteins, and plasmids follow the nomenclature for Trypanosoma and Leishmania as outlined by Clayton et al. (21).

Oligonucleotide Primers, PCR, and Cloning—In preliminary experiments, we found that during their growth in vitro, L. donovani wild-type promastigotes released/secreted an ~35-kDa nuclease activity into their culture medium. Based on these observations, we searched the Leishmania major (Friedlin) GeneDB data base for putative nucleases. Among the 41 sequences present in the data base, only two possessed a putative signal peptide, had an open reading frame (ORF) encoding an ~35-kDa deduced protein, and lacked any apparent membrane anchor motifs. These two sequences were annotated as follows: P1/S1 nucleases (GeneDB Data Bank identifier, LmjF30.1500 and LmjF30.1510) and located on chromosome 30 of L. major. In addition to the latter annotations, an analogous nuclease gene sequence was reported from an Iranian rodent isolate of L. major (22).

Comparisons of these three L. major sequences were done using the Gap-Global alignment program of Genetic Computer Group (GCG) via NIH helix (molbio.info.nih.gov/molbio/gcglib/compare.html). Such analyses showed that these sequences had ~96% identity with each other. Based on these observations, primers were designed against one of these L. major sequences (22), toward amplifying the P1/S1 nuclease homolog from L. donovani. To that end, a sense primer (5’-ATGCGTCCGTTTGTCCGGCTCCGTG-3’) with a methionine initiation codon (boldface type) corresponding to aa 1–9 and a reverse/antisense primer (5’-GGCCGCCACTTCCAACGTGCAGCGCG-3’) corresponding to aa 308–316 of the above L. major sequence (22) were synthesized by β-cyanoethylphosphoramidite chemistry using an Expedite™ nucleic acid synthesis system (Applied Biosystems). For PCR amplifications, these primers were used with 50 ng of L. donovani genomic (g) DNA as a template and a high fidelity polymerase mix (High Fidelity PCR Master Mix, Roche Applied Science). The latter was possible because trypanosomatid protozoans generally do not possess introns within the coding regions of their ORFs (23, 24). After an initial “hot start” at 94°C for 2 min, the conditions used for amplification were as follows: 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (35 cycles), and a final step at 72°C for 5 min. The resulting ~950-bp amplified product was cloned into the pCR®2.1-TOPO vector (Invitrogen). This plasmid was subjected to nucleotide sequencing using vector-encoded M13-forward and -reverse primers. Results of those analyses showed that this PCR clone had a high level of identity with the L. major putative “P1/S1 nuclease” sequences above. Subsequently, this PCR clone was labeled with digoxigenin-DUTP using a PCR-Dig labeling kit (Roche Applied Science) according to the manufacturer’s instructions. The resulting digoxigenin-labeled probe (LdNuc*-DIG950) was used to screen an L. donovani cosmid library to obtain a complete genomic copy of the LdNuc* nuclease gene.

Screening of an L. donovani Cosmid Library—An L. donovani cosmid library (a generous gift from by Dr. Buddy Ullman, Oregon Health Sciences University, Portland) was screened using the LdNuc*-DIG950 probe under high stringency hybridization and washing conditions (i.e. 0.1× SSC; 0.1% SDS at 65°C). Following three rounds of isolation and hybridization, several positive clones were identified. One of these (LdNuc*-FRED/II) was chosen for further analyses. DNA was isolated from this cosmid clone using a plasmid purification kit (Qiagen) and subjected to nucleotide sequencing.

Nucleotide Sequencing and Analyses—DNA was sequenced using the fluorescent dyeoxy chain terminator cycle sequencing method (25) at The Johns Hopkins University DNA Analysis Facility (Baltimore, MD). Sequence data obtained from both strands were analyzed using the GCG software package (26) running on a National Institutes of Health Unix System and Sequencer™3.0 software (Gene Codes Corp., Ann Arbor, MI). Furthermore, such sequences were also subjected to BLAST-N and BLAST-P analyses using the NCBI BLAST-link (www.ncbi.nlm.nih.gov/BLAST/). Signal peptide sequence and protease cleavage sites were predicted using the SignalP link available at the worldwide ExPASy proteomics server available on-line. Protein domain analysis was done using the web-based Simple Modular Architecture Research Tool (available on-line). Protein multiple sequence alignments were done using the ClustalW program (27) using a MacVector® 7.0 software package (Accelrys).

Isolation of Genomic DNA and Southern Blot Analysis—Total gDNA was isolated from mid-log phase L. donovani promastigotes using the Gnome DNA Isolation Kit (Bio101, Carlsbad, CA) as described by Joshi et al. (28). Aliquots of this DNA were digested with the following individual restriction endonucleases BamHI, Clal, EcoRI, HindIII, Ncol, PvuII, SphI, and Xhol, separated on 1% agarose gels, transferred to positively charged nylon membranes (Roche Applied Science), and cross-linked to the membranes by UV irradiation using a Stratalinker® 2400 (Strategene). Subsequently, such blots were hybridized under high stringency conditions with the digoxigenin-labeled LdNuc*-DIG950 probe according to manufacturer’s recommendations (Roche Applied Science). Following washing at high stringency (i.e. 0.1× SSC, 0.1% SDS at 65°C), the hybridized fragments were visualized using an anti-digoxigenin, alkaline phosphatase-conjugated antibody in conjunction with a chemiluminescent substrate (CSPD) according to the manufacturer’s recommendations (Roche Applied Science). Images from such blots were captured using BIOMAX™-MR x-ray film (Eastman Kodak Co.).

Isolation of RNA and Northern Blot Analysis—Total RNA was isolated from promastigotes and axenic amastigotes of L. donovani using RNA STAT-60 according to the manufacturer’s recommendations (Tel-Test, Inc., Friendswood, TX). Total RNA (10 μg) was separated in 1.2% formaldehyde-agarose gels (29) and subjected to Northern blot analysis with the LdNuc*-DIG950 probe as per Joshi et al. (28).

Generation of a Rabbit Anti-LdNuc* Antibody—Based upon analyses of the antigenic indices of the LdNuc*-deduced protein, a single immunogenic peptide sequence was identified. A synthetic peptide, corresponding to this sequence (i.e. aa resi-
L. donovani Secretory Nuclease
dues 157–172, Leu-His-Thr-Ile-Ser-Arg-Tyr-Ser-Ser-Glu-Tyr-Pro-His-Gly-Asp-Lys) was synthesized using 9-fluoromethylxoycarbonyl chemistry (Commonwealth Biotechnologies, Inc., Richmond, VA). An N-terminal cysteine residue was incorporated into this peptide to facilitate its conjugation to keyhole limpet hemocyanin (KLH). Subsequently, the peptide was purified by high pressure liquid chromatography and conjugated to KLH using an Imject® maleimide-activated mCKLH kit according to the manufacturer’s instructions (Pierce). This conjugate was used to generate our anti-LdNuc antibody in a New Zealand White rabbit according to the provider’s (Spring Valley Laboratories, Woodbine, MD) standard immunization protocol (i.e. primary immunization followed by three boosts, using 250 μg of conjugated peptide per injection). The resulting anti-LdNuc antibody was used in subsequent immunoprecipitation, indirect immunofluorescence (IFA), and Western blot analyses as indicated below. Preimmune serum from this rabbit (NRS) served as control in all experiments.

Generation of the pKSNEO::LdNuc-HA Epitope-tagged Expression Construct—A homologous episomal system was used to express an epitope-tagged LdNuc-HA chimeric protein in L. donovani promastigotes. To that end, a construct was designed that contained the complete open reading frame of the L. donovani Nuc gene (including its 5′-end encoding the putative signal peptide) joined, at its 3′-end, with a nine amino acid sequence encoding the influenza virus hemagglutinin (HA) epitope (Roche Applied Science). This construct was generated by PCR using the LdNuc-c′-FRED/II cosmid clone as template with a forward primer 5′-CATACTAGATGCCCAGCTTTGTCGCGCTCT-3′ (containing an SpeI restriction site shown in boldface) and a reverse primer 5′-CGTACTAGTTTACCGCTAGTCCGCACGTTCAGGTACGGGTACCC-3′ (containing an SpeI restriction site shown in boldface; stop codon in boldface with underlines; and the HA epitope sequence underlined). The PCR conditions used were as follows: a hot start at 94 °C for 2 min, followed by 35 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final step at 72 °C for 5 min. The resulting ~1000-bp amplified product (LdNuc-HA) was gel-purified and cloned into the pCR2.1-TOPO vector (Invitrogen) to generate the pCR21::LdNuc-HA plasmid. The insert was excised from the latter using SpeI. Subsequently, the excised fragment was ligated into the SpeI site of the pKSNEO expression vector (30) to generate the pKSNEO::LdNuc-HA plasmid construct. The orientation of LdNuc-HA in pKSNEO was examined by restriction enzyme analysis, and the nt sequence of this construct was confirmed by DNA sequence analysis.

Homologous Episomal Expression of LdNuc—Both the pKSNEO::LdNuc-HA construct and the [pKSNEO] control plasmid were transfected into L. donovani promastigotes using electroporation conditions essentially as described by Shakarian et al. (10). Following overnight recovery in medium M199+ with 10% FBS (at 26 °C), these cells were selected for their growth in the same medium containing 15 μg ml⁻¹ Genetin® (G418, Invitrogen). Once such drug-resistant parasites emerged, they were further selected in increasing concentrations of G418 up to 100 μg ml⁻¹. For routine purposes, these transfectants were maintained and grown at 26 °C in complete growth medium containing 100 μg ml⁻¹ of G418. For some experiments, these transfectants were also grown under serum-free conditions, in chemically defined (M199+) medium (14) supplemented with 100 μg ml⁻¹ of G418. Both cell lysates and cell-free culture supernatants were prepared from these transfectants as described above.

Growth Kinetics of Transfected Parasites—Both pKSNEO and pKSNEO::LdNuc-HA transfected parasites were monitored at regular intervals during the course of their growth in vitro. For experiments, triplicate cultures of both transfectants were initiated at 1–2 × 10⁶ cells ml⁻¹ (i.e. from stock cultures in their exponential phase of growth). Aliquots from such cultures were taken at regular intervals, diluted appropriately, and counted using a Coulter® counter (Model Z1, Beckman-Coulter) as described previously (11).

Western Blots—Lysates of both L. donovani wild-type and episomally transfected parasites as well as aliquots of their concentrated cell-free (chemically defined) culture supernatants (all in 1× SDS-polyacrylamide sample buffer as above) were separated in SDS-polyacrylamide gels (10%, pre-cast, Tris-glycine polyacrylamide, Novex® gels; Invitrogen). These gels were trans-blotted onto polyvinylidene difluoride membranes (Invitrogen), and the membranes were blocked and washed as described previously (6). Such blots were probed with either our rabbit anti-LdNuc peptide antibody and its NRS control or with a mouse anti-HA monoclonal antibody (clone, HA.11, Covance Research Products, Berkeley, CA) or an appropriately matched purified mouse IgG1, κ control immunoglobulin (Sigma). Subsequent to incubation and washing, the blots were reacted with either a donkey anti-rabbit or a sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare) as described previously (6). Immunodetection was carried out using ECL Western blot kit reagents according to the manufacturer’s recommendations (GE Healthcare), and images were captured using BIOMAX™-MR x-ray film (Kodak).

Indirect Immunofluorescence Analyses—The intracellular distribution of LdNuc was investigated using indirect immunofluorescence microscopy. For such assays, both wild-type promastigotes and axenic amastigotes as well as episomally transfected promastigotes were prepared essentially as described previously (31). Briefly, prior to use, the wells (5 mm diameter) printed, Teflon-masked, glass microscope slides (Cel-Line/Erie Scientific Co. Portsmouth, NH) were treated for 30 min at room temperature with an aqueous solution (10 mg ml⁻¹, w/v) of poly-l-lysine hydrobromide (Sigma), rinsed with de-ionized water, air-dried, and stored at room temperature. For experiments, mid-log phase cultures of parasites were harvested, washed three times by centrifugation as above, and resuspended in PBS. Aliquots of such cell suspensions were placed onto the poly-l-lysine-coated wells of glass slides (above) and mixed immediately with an equal volume of freshly prepared 4% (w/v) para-formaldehyde (Polysciences, Inc., Warrington, PA) in 10 mM PIPES (U. S. Biochemical Corp.), pH 6.5. After 20 min of incubation at room temperature in a humid chamber, nonadherent cells were removed by aspiration. Subsequently, adherent cells were treated for 5 min at ~20 °C with absolute methanol, washed three times in PBS (5 min each) at
room temperature, and incubated in “blocking buffer” (0.5% (w/v) bovine serum albumin (United States Biochemical Corp.), 0.045% (v/v) fish gelatin (Sigma) in PBS) for 1 h at room temperature.

A set of these fixed and blocked cells was reacted with our rabbit anti-\(Ld\)Nuc\(^c\) peptide antiserum or NRS appropriately diluted in the blocking buffer as above. Following washing with PBS, these cells were reacted for 1 h at room temperature with a Texas Red-conjugated goat anti-rabbit IgG (H + L) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted in blocking buffer. Subsequently, cells were washed three times with PBS and mounted in Vectashield\(^R\) mounting medium (Vector Laboratories, Inc., Burlingame, CA) and examined using epi-fluorescence microscopy. In addition, one set of the fixed and blocked transfected promastigotes were also reacted with a mouse anti-HA monoclonal antibody (Covance) or its isotype-matched control mouse immunoglobulin (Sigma), appropriately diluted in blocking buffer as above for 1 h at room temperature. After three washes in PBS as above, these cells were treated for 1 h at room temperature with a fluorescein isothiocyanate (FITC)-conjugated, goat anti-mouse IgG (H + L) secondary antibody (Sigma) appropriately diluted in blocking buffer. Subsequently, cells were washed with PBS and mounted in Vectashield\(^R\) mounting medium (Vector Laboratories) and examined using epi-fluorescence microscopy, as above. All images were acquired using a Leica DMIRBE inverted fluorescence microscope (Leica Microsystems) and appropriate Texas Red or FITC excitation/barrier filters. Such images were processed using Adobe Photoshop 5.5 (Adobe Systems Inc.).

Immunoprecipitation and Detection of Parasite Secreted/Released Nuclease Activity—In preliminary studies we found that \(L.\) donovani wild-type promastigotes grown under chemically defined conditions constitutively released/secreted an ∼35-kDa nuclease activity into their culture supernatants, and this activity was specifically recognized by our rabbit anti-\(Ld\)Nuc\(^c\) peptide antibody. Although promastigotes can be grown under serum-free conditions, axenic amastigotes of this parasite can only be grown in serum-containing medium (11). Therefore, to ascertain whether \(L.\) donovani amastigotes also produced and released/secreted this ∼35-kDa nuclease, it was necessary to test their culture supernatants with the anti-\(Ld\)Nuc\(^c\) peptide antibody. For comparative purposes, both wild-type promastigotes and axenic amastigotes were grown in serum-containing media, and their cell-free culture supernatants were harvested, neutralized with 1 M Tris, pH 8.5 (as needed), and subsequently used in immunoprecipitation experiments. For these assays, aliquots of such culture supernatants were reacted in immunoprecipitation (IP) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% v/v Nonidet P-40 (Sigma), pH 7.4) with the rabbit anti-\(Ld\)Nuc\(^c\) peptide antibody or NRS in combination with protein A/G-agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Following incubation at 4 °C for 4 h on a platform rocker, beads were pelleted by microcentrifugation and washed three times with IP buffer and twice with 50 mM Tris-HCl, pH 7.4. Subsequently, the bead-bound immunoprecipitates were eluted by boiling in SDS-polyacrylamide sample buffer lacking reducing agent (−DTT), and a parallel set was eluted with sample buffer containing reducing agent (+DTT). The resulting samples were analyzed for their nuclease activity using SDS-polyacrylamide/poly(A) zymogram gels as described above.

Immunoprecipitation experiments similar to those described above were also performed using cell-free culture supernatants obtained from episomally transfected \(L.\) donovani promastigotes grown in chemically defined medium. To that end, such supernatants were harvested, concentrated up to ∼100-fold, and duplicate aliquots subjected to immunoprecipitations with either our rabbit anti-\(Ld\)Nuc\(^c\) peptide antibody in combination with protein A/G-agarose beads (Santa Cruz Biotechnology) or a mouse monoclonal anti-HA immunoglobulin matrix (Covance). Controls in these assays consisted of samples reacted with either NRS or a purified normal mouse IgG1 immunoglobulin, as appropriate. Following incubation and washing as above, one set of bead-bound immunoprecipitates was eluted by boiling in SDS-polyacrylamide sample buffer lacking DTT, and a parallel set was eluted with sample buffer containing this reducing agent. The resulting samples were analyzed for their nuclease activity using zymogram gels as described above.

Nuclease Assays Using Nucleic Acid Substrates—In vitro transcribed RNA was used as substrate to evaluate the ribonuclease activity of the \(L.\) donovani \(Ld\)Nuc\(^c\) nuclease. For these experiments, an RNA transcript was generated by \textit{in vitro} transcription from a cloned fragment of the \textit{LmexCht1} gene (28) in the PCR-T7 plasmid (Invitrogen) using a T7 polymerase and the Megascript T7 transcription kit (Ambion Inc., Austin, TX) as per the manufacturer’s instructions. Prior to use, this RNA was ethanol-precipitated, washed with 70% ethanol, and resuspended in diethyl pyrocarbonate-treated water. For some assays, aliquots of this \textit{in vitro} transcribed RNA were incubated directly with 100× concentrated cell-free culture supernatants from \(L.\) donovani promastigotes transfected with either the \textit{pKSneo} control or the \textit{pKSneo::LdNuc\(^c\)-HA} plasmid grown in chemically defined medium. In other assays, such culture supernatants were subjected to immunoprecipitation with either a mouse monoclonal anti-HA affinity bead matrix or with normal mouse immunoglobulin controls, as described above. Subsequent to washing, bead-bound immune complexes were incubated with the \textit{in vitro}-transcribed RNA substrate above. Such reactions were carried out in parallel at both pH 5.0 (25 mM sodium acetate buffer) and at pH 8.5 (25 mM HEPES buffer) in a total assay volume of 20 μL. Following incubation at 37 °C for 30 min, samples were mixed with 5 μL of Novex\textsuperscript{®} Hi-Density TBE (5×) sample buffer (Invitrogen), heated at 65 °C for 5 min and the hydrolysis products separated/resolved in 6% TBE-polyacrylamide gels (Novex\textsuperscript{®}, Invitrogen). Subsequently gels were stained with SYBR Green II (Invitrogen) as per the manufacturer’s instructions, visualized using a 300 nm ultraviolet trans-illuminator, and digital images captured with a Nikon CoolPix 4500 camera. Images were processed using Adobe Photoshop 5.5.

Both an ∼7,249-nt single-stranded (ss-) DNA (M13mp18 phage, Sigma) and a circular 7,249-bp double-stranded (ds) DNA (M13mp18 RF1, Sigma) were also used as substrates to test the nuclease activity of the released/secreted \(Ld\)Nuc\(^c\)
L. donovani Secretory Nuclease

enzyme. For experiments, such ssDNA (0.3 μg) or dsDNA (0.2 μg) was incubated with either aliquots of 100× concentrated cell-free parasite culture supernatants or their anti-HA bead-bound immunoprecipitates (as above) in a total reaction volume of 10 μl containing a final concentration of either 25 mM sodium acetate, pH 5.0, or 25 mM HEPES buffer, pH 8.5. Following incubation (30 min at 37 °C), samples were mixed with 1 μl of 10× gel loading buffer (BlueJuice™, Invitrogen), and the hydrolysis products were resolved in 0.8% E-Gels® (Invitrogen) as per the manufacturer’s instructions. Subsequently, images were captured using an ultraviolet trans-illuminator and a UVP-Biochemi Imaging System (UVP-Bioimaging Systems, UVP, Inc., Upland, CA) and processed using Adobe Photoshop 5.5, as above.

RESULTS

Identification of the L. donovani Secreted Nuclease Activity—Previously, we have shown that promastigotes of all pathogenic Leishmania species possess a unique, ≥40-kDa, bi-functional 3’-nucleotidase/nuclease enzyme anchored in their cell surface membrane (5, 7, 9, 17). Furthermore, it was suggested that this unique parasite, class I nuclease, must play important roles in the salvage of host-derived purines (5, 9). In light of those observations, it was of interest to determine whether this or related nuclease activities might also be released/secreted by Leishmania parasites. Thus in preliminary experiments, both cell lysates and 100-fold concentrated cell-free culture supernatants from L. donovani promastigotes grown in chemically defined medium were analyzed for their nuclease activities in SDS-polyacrylamide/poly(A)-containing substrate zymogram gels. For such assays, samples were prepared either in the presence of DTT (50 mM final concentration (6, 7)) or absence of this reducing agent (17).

Results obtained from these in situ activity gel assays showed that parasite lysates prepared with DTT possessed only a single ~43-kDa band of nuclease activity (Fig. 1, panel A, lane 1). The latter represents the endogenous “nuclease” activity of the bi-functional L. donovani 3’-nucleotidase/nuclease (6, 7). However, no nuclease activity was detected in the cell-free culture supernatants of promastigotes in the presence of this reducing agent (Fig. 1, panel A, lane 2). These observations are consistent with previous reports (6, 7) that showed that the L. donovani 3’-nucleotidase/nuclease was a DTT-resistant, surface membrane-anchored enzyme. In contrast to the latter, cell lysates of promastigotes prepared in the absence of DTT showed two prominent bands of nuclease activity, i.e. one of ~43 kDa and a second of ~35 kDa (Fig. 1, panel B, lane 1). Furthermore, under these nonreducing conditions, a single, distinct ~35-kDa band of nuclease activity was also present in the concentrated cell-free culture supernatants of these parasites (Fig. 1, panel B, lane 2). Taken together, results of these activity gel assays demonstrated that L. donovani promastigotes possess a novel, to date unidentified, ~35 kDa, DTT-sensitive nuclease in addition to their previously described 43-kDa, bi-functional L. donovani 3’-nucleotidase/nuclease activity. Furthermore, this ~35-kDa nuclease appeared to be constitutively released/secreted by these parasites into their culture medium during their growth in vitro.

Nuclease Activity in Various L. donovani Developmental Forms—SDS-polyacrylamide/poly(A)-containing substrate gels were also used to determine whether the ~35-kDa nuclease was present in the amastigote developmental form of L. donovani. For these assays, whole cell lysates of promastigotes, axenic amastigotes, and in vivo tissue-derived amastigotes (i.e. isolated from infected hamster spleens) were prepared in the absence of DTT and subjected to in situ zymogram gel analyses. Results of such assays showed that both promastigotes and axenic amastigotes possessed a discrete ~43-kDa band of L. donovani 3’-nucleotidase/nuclease activity and a second very distinct ~35-kDa band of nuclease activity (Fig. 2, lanes I and 2, respectively). The latter activity appeared to be more prominently expressed in axenic amastigotes than in promastigotes. Interestingly, in these activity gel assays in vivo, tissue-derived amastigotes showed only a single, prominent ~35-kDa band of nuclease activity (Fig. 2, lane 3). Our results showing the presence of the ~43-kDa, L. donovani 3’-nucleotidase/nuclease activity in both promastigotes and axenic amastigotes and its down-expression/apparent absence from in vivo-derived amastigotes are in agreement with previously published observations concerning this enzyme (11). More importantly, results of these assays demonstrated that the unique ~35-kDa nuclease activity was constitutively expressed by both life cycle developmental forms of this human pathogen. Furthermore, in contrast to the L. donovani 3’-nucleotidase/nuclease, the activity of this ~35-kDa nuclease was completely abrogated in all samples treated with DTT (data not shown) indicating its sensitivity to


denatured and 100-fold concentrated cell-free culture supernatants from L. donovani promastigotes grown in chemically defined medium, prepared in SDS-polyacrylamide sample buffer containing reducing agent (+ DTT), and separated in a poly(A)-containing SDS-polyacrylamide substrate gel. Following renaturation and staining with toluidine blue O, nuclease activity was visualized in these gels as a clear/colorless zone of substrate hydrolysis in an otherwise uniformly stained, dark blue background of unhydrolyzed poly(A) substrate. Arrow indicates the ~43-kDa band of nuclease activity of the bi-functional, 3’-nucleotidase/nuclease present in the promastigote cell lysate (Lys.). Note the absence of such activity in the culture supernatant (Cult. Sup) from these cells. Panel B, samples as in panel A (lanes 1 and 2) prepared in SDS-polyacrylamide sample buffer lacking reducing agent (i.e. minus DTT) and analyzed as above. The asterisk denotes the nuclease activity of the ~43-kDa 3’-nucleotidase/nuclease present in the lysate (Lys.) of promastigotes but absent from their culture supernatant (Cult. Sup). The arrow indicates the ~35-kDa band of nuclease activity present in both the cell lysate and culture supernatant from promastigotes. Protein molecular mass standards in kDa are shown on the left of each panel.
this reducing agent. It is of interest to note that a second, slightly smaller (<35 kDa), and more diffuse band of nuclease activity was also observed in some preparations of axenic amastigotes and in vivo amastigotes, and presumably this represents a proteolytic degradation product of the endogenous 35-kDa enzyme.

Identification and Characterization of the LdNuc Gene—It is important to point out that the functional activity of the 35-kDa promastigote released/secreted nuclease could be readily detected in zymogram gels using ~100× concentrated cell-free culture supernatants; the actual amount of protein present in such samples, however, was far below the level of detection. These observations suggested that the parasite released/secreted nuclease might have high specific activity. Thus, in preliminary experiments we used a variety of different affinity-based bead matrices (e.g. binding to concanavalin A and the nucleotide-dye mimetics: Cibacron Blue F3GA and Reactive Red) in attempts to isolate/enrich sufficient quantities of this nuclease for its functional characterization, including direct amino acid sequencing (data not shown). In that regard, despite repeated attempts, we were unable to obtain sufficient quantities of this protein for such purposes. However, we adopted a molecular approach to successfully identify the gene (i.e. LdNuc) encoding this released/secreted nuclease and used it to examine/elucidate some of biochemical and functional properties of this unique parasite enzyme. To that end, an annotated protein data base of a closely related leishmanial species (i.e. Leishmania major (Friedlin) GeneDB data base) was searched for putative nuclease homologs. Our search criteria included deduced proteins that had the following: 1) a calculated molecular mass of ~35 kDa, 2) possessed a putative signal peptide, and 3) lacked any apparent membrane-anchor motif(s). Among the 41 putative nuclease in the L. major (Friedlin) data base, only two met our search criteria. These two deduced proteins were annotated as LmjF30.1500 and LmjF30.1510 and both were designated as putative (i.e. no demonstrated functional activities) P1/S1 nuclease. In addition to the latter, a very similar putative nuclease gene sequence was also reported in the literature from a separate (Iranian) isolate of L. major (22). Sequence comparisons of these three L. major putative nuclease were done using the Gap-Global Alignment program of the Genetic Computer Group (GCC). Such analysis showed that these three sequences had ~96% identity with each other. Based on these observations, oligonucleotide primers were synthesized against one of these L. major sequences (22) and used in PCR with Ld-gDNA as template, to amplify a gene encoding the ~35-kDa L. donovani nuclease. A single ~950-bp product obtained from these amplification reactions was gel-purified, cloned, and subjected to nucleotide sequencing. The resulting sequences were subjected to both BLAST-N and BLAST-P analyses. Those analyses showed that the PCR clone contained an ORF, which showed both high nt and deduced aa sequence identity (90 and 84%, respectively) with the putative L. major P1/S1 nuclease above.

The PCR fragment above was labeled with digoxigenin-dUTP, and the resulting probe (LdNuc*-DIG950) was used to screen an L. donovani gDNA cosmid library by hybridization. Following three rounds of screening with this probe, a positive cosmid clone (LdNuc*-FRED/II) was selected for further analysis. Results of nt sequence analyses revealed that the LdNuc*-FRED/II clone contained a complete ORF of 951 bp, which was designated as LdNuc~. Sequence analyses showed that the composition of the LdNuc~ ORF was GC-rich (~59%), which is consistent with the overall GC content of the Leishmania genome (32). Furthermore, such analyses showed that the LdNuc~ ORF encodes a polypeptide of 316 amino acids with a calculated molecular mass of 35,003 Da and a pI of 6.59 (Fig. 3, panel A).

Comparison of the deduced amino acid sequence encoded by the LdNuc~ ORF to all available and nonredundant data bases using BLAST-P showed that it has homologies to purported (33–37) and functional (7–9, 38–42) nuclease from a variety of diverse sources. Furthermore, analyses of the LdNuc~-deduced protein using the Pfam data base indicated that it belongs to the P1/S1 family of class I nuclease. The prototypical members of this family include the P1 and S1 nuclease (EC 3.1.30.1) of Penicillium citrinum and Aspergillus oryzae, respectively (40, 41), which typically cleave RNA and single-stranded DNA with no apparent base specificity.

Based on the von Heijne algorithm (43, 44), the hydrophobic, N-terminal, 24 amino acids of the LdNuc~-deduced protein constitutes a putative signal peptide (Fig. 3, panel A). Cleavage at this site, presumably in the endoplasmic reticulum of the...
parasite, would result in a mature protein with Val25 as its N-terminal amino acid residue. Such cleavage would result in a mature protein consisting of 291 amino acids with a calculated molecular mass of 32,525 Da and a theoretical pI of 6.43. The \( \text{LdNuc} \)'-deduced protein was also analyzed using various other structural algorithms. Those analyses indicated that this parasite enzyme lacked any apparent hydrophobic transmembrane domains or glycosylsinositol phosphate anchor signature sequences (45). Similarly, no KDEL or analogous endoplasmic reticulum (ER) retention sequences (46) or any other intracellular organelle specific-targeting sequences were identified in the \( \text{LdNuc} \)'-deduced protein. Based on its overall hydrophilicity, the presence of an N-terminal signal peptide and the absence of both membrane anchors and ER retention motifs suggest that the \( \text{LdNuc} \) represents a soluble/released protein.

These deduced structural features are in good agreement with our experimental observations, which demonstrated that the endogenous wild-type nuclease was constitutively released/secreted by \( L. \ donovani \) parasites during their growth in vitro. Furthermore, it is of interest to point out that many microbial class I nucleases have also been shown to be soluble/secretory enzymes (47–51).

The \( \text{LdNuc} \)'-deduced protein was also analyzed for potential \( N \) and \( O \)-linked glycosylation and phosphorylation sites using NetOGlyc, NetNGlyc, and NetPhos web-based tools. Results of such analyses indicated that the \( \text{LdNuc} \) possessed two potential \( N \)-linked glycosylation sites at Asn\(^{108} \) and Asn\(^{251} \) (Fig. 3, panel A). The latter is consistent with our preliminary observations, which showed that the native, wild-type parasite (promastigote) released/secreted nuclease was a mannose-containing glycoprotein (i.e. was bound to concanavalin A beads, and such binding was inhibited with \( \alpha \)-methylmannoside; data not shown). Results of NetOGlyc analyses failed to identify any potential \( O \)-linked glycosylation sites in the \( \text{LdNuc} \)'-deduced protein. In contrast, results of NetPhos analyses showed that \( \text{LdNuc} \) contained at least 14 potential sites for phosphorylation by several different mechanisms (e.g. casein kinase II, protein kinase C, etc.). These include seven potential phosphorylation sites on serines (i.e. Ser\(^{70} \), Ser\(^{128} \), Ser\(^{161} \), Ser\(^{164} \), Ser\(^{165} \), Ser\(^{185} \), and Ser\(^{289} \)), six on threonines (i.e. Thr\(^{115} \), Thr\(^{234} \), Thr\(^{252} \), Thr\(^{256} \), Thr\(^{262} \), and Thr\(^{280} \)), and a single site on tyrosine (i.e. Tyr\(^{203} \)) (Fig. 3, panel A).

Previously it was shown that many class I nucleases possess five conserved blocks of aa residues designated as domains I–V (8). Furthermore, it was suggested in that report that some of these conserved domains may contain one or more histidine residues that could be involved in the binding of divalent metal cofactors. In that regard, domains I–IV of the P1 nuclease of \( P. \ citrinum \) contain nine conserved aa residues, which have been implicated in the coordinate binding of essential zinc ions (52). Analysis of the \( \text{LdNuc} \)'-deduced protein showed that it possesses all nine of these conserved aa residues (Fig. 3, panel A, double underlined aa), lending further support that this parasite enzyme is a member of P1/S1 family of nucleases. Moreover, some members of the class I nuclease family have been shown to possess intra-chain disulfide bridges (e.g. the Cys\(^{80} \)-Cys\(^{85} \) and Cys\(^{72} \)-Cys\(^{216} \) bonds in both the P1 nuclease of \( P. \ citrinum \) and S1 nuclease of \( A. \ oryzae \), which are essential for their enzymatic functions (40, 41). In that context, our analyses showed that the mature \( \text{LdNuc} \)'-deduced protein possesses four cysteine residues (Fig. 3, panel A, Cys\(^{35} \), Cys\(^{79} \), Cys\(^{196} \), and Cys\(^{271} \)), which might be involved in forming disulfide bridges. Such disulfide bonds appear to be essential for the function of the endogenous \( L. \ donovani \) wild-type enzyme, as treatment with reducing agents (e.g. DTT) abolished its nuclease activity (cf. Fig. 1, panels A and B). In addition to the above, the \( \text{LdNuc} \)'-deduced protein was also analyzed for its putative antigenic epitopes using \( B \)-turn probability, antigenic index, and surface probability (i.e. hydrophilicity) using a variety of web-based tools and algorithms, including the Plotstructure, the GCG-Lite Protein Sequence Analysis tool available via NIH Helix (at molbio.info.nih.gov/molbio/molbio_docs/ggc/plotstructure.html).

Among the epitopes identified, one corresponding to aa residues 157–172 was chosen to generate a rabbit anti-\( \text{LdNuc} \)' peptide antibody (Fig. 3, panel A). Subsequently this anti-\( \text{LdNuc} \)' peptide antibody was used in a variety of experiments.
onto nylon membranes. Such membranes were subjected to SphI, or XhoI), separated by gel electrophoresis and blotted. In light of our Western blot results above, the rabbit anti-

L. donovani Secretory Nuclease

~6.0 kb in both the promastigote and axenic amastigote developmental forms of the parasite (Fig. 5, panel B). In that regard, the ~3-kb message is sufficiently large enough to encode the entire ~35 kDa, LdNuc\(^{c}\) protein, and the 6-kb message could represent its precursor. Alternatively, the 6-kb mRNA might reflect hybridization of the LdNuc\(^{c}\)-DIG950 probe with some other structurally related gene sequence(s) in the parasite genome. Interestingly, the overall hybridization signals obtained in these blots appeared to be significantly more intense with RNA isolated from axenic amastigotes (i.e. the developmental stage that produces disease in mammals) than from promastigotes (i.e. the insect vector stage of the parasite). Results of these Northern blot analyses demonstrated that the LdNuc\(^{c}\) gene(s) appeared to be constitutively transcribed throughout the developmental life cycle of this parasite. Furthermore, these results also suggested that LdNuc\(^{c}\) mRNA is differentially up-regulated by amastigote forms of this human pathogen.

Reactivity of the Rabbit Anti-LdNuc\(^{c}\) Peptide Antibody with the Wild-type L. donovani Released/Secreted Nuclease—Western blot analyses were done to determine whether the anti-peptide antibody generated against the deduced amino acid sequence from the LdNuc\(^{c}\) gene would recognize the endogenous wild-type L. donovani ~35-kDa released/secreted nuclease. To that end, lysates of both wild-type promastigotes and axenic amastigotes were separated in SDS-polyacrylamide gels, trans-blotted onto polyvinylidene difluoride membranes, and reacted in Western blots with our rabbit anti-LdNuc\(^{c}\) peptide antibody or with its control NRS. In such blots, the anti-LdNuc\(^{c}\) antibody reacted with only a single ~35-kDa band of endogenous nuclease activity immunoprecipitated by this antibody (IP: \(\alpha\)-Nuc) from culture supernatants of both promastigotes and axenic amastigotes (lanes 1 and 2, respectively). Protein molecular mass standards in kDa are shown at the left.

Expression of the LdNuc\(^{c}\) Message during the Developmental Life Cycle of L. donovani—Northern blot analyses were performed to evaluate the expression of LdNuc\(^{c}\) mRNA during the parasite developmental life cycle. To that end, total RNA from both promastigotes and axenic amastigotes was separated in agarose/formaldehyde gels, blotted onto nylon membranes, and hybridized with the LdNuc\(^{c}\)-DIG950 probe. Results obtained from such blots showed that the LdNuc\(^{c}\) probe specifically hybridized with two distinct messages of ~3.0 and ~6.0 kb in both the promastigote and axenic amastigote developmental forms of the parasite (Fig. 3, lane 2). Interestingly, the overall hybridization signals obtained in these blots appeared to be significantly more intense with RNA isolated from axenic amastigotes (i.e. the developmental stage that produces disease in mammals) than from promastigotes (i.e. the insect vector stage of the parasite). Results of these Northern blot analyses demonstrated that the LdNuc\(^{c}\) gene(s) appeared to be constitutively transcribed throughout the developmental life cycle of this parasite. Furthermore, these results also suggested that LdNuc\(^{c}\) mRNA is differentially up-regulated by amastigote forms of this human pathogen.

including Western blot analyses, indirect IFA, and immunoprecipitation assays to demonstrate its specific reactivity with the wild-type L. donovani released/secreted nuclease.

Southern Blot Analysis of the LdNuc\(^{c}\)—To investigate the genomic organization and copy number of the LdNuc\(^{c}\) gene, L. donovani gDNA was digested with various restriction endonucleases (i.e. BamHI, Clal, EcoRI, HindIII, Ncol, PvuII, Spel, Sphl, or Xhol), separated by gel electrophoresis and blotted onto nylon membranes. Such membranes were subjected to Southern hybridization using the LdNuc\(^{c}\)-DIG950 probe (i.e. corresponding to the complete L. donovani LdNuc\(^{c}\) ORF) under high stringent conditions. Based on nt sequence analyses, the LdNuc\(^{c}\) ORF did not contain any predicted cleavage sites for BamHI, EcoRI, HindIII, Spel, or Xhol but had single predicted cleavage sites for Ncol, Clal, and PvuII, and two predicted cleavage sites for Sphl. Cumulative results obtained from Southern blot hybridization experiments with these restriction endonucleases (data not shown) indicated that there were two copies of the LdNuc\(^{c}\) gene within the diploid genome of this parasite.

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\[\text{FIGURE 4. Reactivity of the anti-LdNuc}^{c}\text{ peptide antibody with the native parasite released/secreted nuclease. Panel A, Western blot detection of the ~35-kDa native parasite nuclease. Whole cell lysates (25 \(\mu\)g of total protein) of L. donovani promastigotes (Pro) and axenic amastigotes (AxAm) were subjected to SDS-PAGE and Western blotting (WB) with a rabbit anti-LdNuc}^{c}\text{ peptide antibody. This antibody reacted with only a single ~35-kDa protein (arrow) in both promastigotes and axenic amastigotes. Protein molecular mass standards in kDa are shown at the left. Panel B, indirect immunofluorescence analyses. Log phase promastigotes (Pro) and axenic amastigotes (AxAm) were reacted with a rabbit anti-LdNuc}^{c}\text{ peptide (primary) antibody followed by a Texas Red-conjugated goat anti-rabbit secondary antibody. Differential interference contrast images showing the elongated pyriform shape of promastigotes (panel A1) and the rounded/spheroid morphology of axenic amastigotes (panel B1) and their fluorescence images acquired in the Texas Red channel (IFA, \(\alpha\)-Nuc) are shown in panels A2 and B2, respectively. Bar = 2 \(\mu\)m. Panel C, immunoprecipitation (IP) of native wild-type released/secreted nuclease activity. Aliquots of cell-free culture supernatants (Cult. Sup.) from mid log phase (i.e. ~1.5 \(\times\) 10\(^{5}\) cells ml\(^{-1}\)) promastigotes and axenic amastigotes were reacted with the rabbit anti-LdNuc\(^{c}\) peptide antibody, and the resulting immunoprecipitates were solubilized in sample buffer lacking DTT and subsequently analyzed for their nuclease activity in SDS-polyacrylamide zymogram gels containing poly(A) as in Fig. 1, above. Arrow denotes the single ~35-kDa band of endogenous nuclease activity immunoprecipitated by this antibody (IP: \(\alpha\)-Nuc) from culture supernatants of both promastigotes and axenic amastigotes (lanes 1 and 2, respectively). Protein molecular mass standards in kDa are shown at the left. \]
the rabbit anti-LdNuc\(^c\) peptide antibody or its control NRS. Subsequently, cells were reacted with a Texas Red-conjugated secondary antibody and examined by epi-fluorescence microscopy. In these assays, the anti-LdNuc\(^c\) antibody reacted with the endogenous parasite nuclease and showed a dispersed intracellular pattern of staining within both promastigotes and axenic amastigotes (Fig. 4B, panels A2 and B2, respectively). Such staining could reflect the processing of this parasite nuclease through the ER in these organisms. In support of this concept, very similar IFA-staining patterns have also been reported for a variety of other secretory proteins that are processed through the ER in these parasites (10, 28, 53). No fluorescence was detected in any parasite sample treated with the control NRS (data not shown).

To further investigate the foregoing observations, experiments were also carried out to ascertain whether the rabbit anti-LdNuc\(^c\) peptide antibody would, in fact, recognize and immunoprecipitate the \(~35\)-kDa, DTT-sensitive nuclease activity released/secreted by wild-type parasites during their growth in vitro. For such experiments, cell-free culture supernatants from both promastigotes and axenic amastigotes were reacted with the anti-LdNuc\(^c\) peptide antibody or control NRS in a protein A/G-agarose bead-based assay as described above. The resulting immunoprecipitates were solubilized in sample buffer (\(−/+\) DTT) and subsequently analyzed for their nuclease activity in SDS-polyacrylamide zymogram gels containing poly(A) as substrate. Results of these assays showed that the anti-LdNuc\(^c\) antibody specifically immunoprecipitated the single \(~35\)-kDa nuclease activity present in culture supernatants of promastigotes (Fig. 4, panel C, lane 1). Of equal significance was the observation that this antibody also immunoprecipitated a single \(~35\)-kDa nuclease activity from the culture supernatants of axenic amastigotes (Fig. 4, panel C, lane 2). The latter results demonstrated, for the first time, that wild-type axenic amastigotes also produced and released/secreted a \(35\)-kDa nuclease activity into their culture medium during growth in vitro. It is also relevant to point out that the nuclease activity present in the immunoprecipitates obtained from these parasite culture supernatants was completely abrogated following treatment with DTT (data not shown). These results are consistent with our initial observations concerning the sensitivity of the native/wild-type, promastigote-released/secreted nuclease to inhibition by DTT (see Fig. 1, panels A and B). In these immunoprecipitation assays, no nuclease activity was detected in samples reacted with NRS. The cumulative results of these antibody-based experiments strongly suggested that the single \(~35\)-kDa, DTT-sensitive nuclease produced and released by both wild-type L. donovani promastigotes and axenic amastigotes is encoded by this LdNuc\(^c\) gene.

Transfection of L. donovani Promastigotes with an Epitope-tagged LdNuc\(^c\) Gene Construct—Having shown that the anti-LdNuc\(^c\) antibody specifically recognized and immunoprecipitated the parasite wild-type released/secreted nuclease above, it was deemed necessary to determine whether the LdNuc\(^c\) gene in fact encoded a functional (DTT-sensitive) nuclease activity. To test this hypothesis, a homologous episomal expression system was devised. For these experiments, a chimeric construct was generated containing the complete ORF of the cloned LdNuc\(^c\) gene fused, in-frame, at its 3′-end with a sequence encoding a 9-aa HA epitope of the influenza virus. Subsequent to ligation into the pKSNEO leishmanial expression vector, this construct (designated subsequently as LdNuc\(^c::HA\)) was used to transfected L. donovani promastigotes (Fig. 5, panel A). Promastigotes transfected with the pKSNEO vector alone served as controls in all transfection experiments. Following drug selection, the growth kinetics of these transfected promastigotes was compared as described above. Results of these in vitro assays showed that promastigotes transfected with either the LdNuc\(^c::HA\) chimeric construct or the pKSNEO control plasmid had very similar growth kinetics (data not shown). Furthermore, nontransfected (“wild type”) L. donovani promastigotes, grown in complete medium lacking G418, displayed growth kinetics identical to those obtained with the transfectants above (data not shown). Taken together, our observations indicated that these episomal transfections did not appear to alter the characteristic growth kinetics of the parental L. donovani promastigote cell line. Subsequently, such transfected promastigotes were analyzed for their expression of the LdNuc\(^c::HA\) chimeric protein using Western blots, in situ activity gel assays, and immunofluorescence microscopy.

Expression of the LdNuc\(^c::HA\) Chimeric Protein in Transfected Parasites—Western blot analyses were performed to determine whether LdNuc\(^c::HA\)-transfected promastigotes synthesized and released/secreted the chimeric protein during their growth in vitro. For these experiments, transfected promastigotes were grown in chemically defined medium, and both lysates and cell-free culture supernatants from such cells were subjected to SDS-PAGE and subsequent Western blotting using either a mouse anti-HA monoclonal antibody or our rabbit anti-LdNuc\(^c\) peptide antibody. In such blots, the anti-HA antibody showed a band of reactivity with only the single \(~35\)-kDa LdNuc\(^c::HA\) chimeric protein present in both the lysates and cell-free culture supernatants of LdNuc\(^c::HA\)-transfected promastigotes (Fig. 5, panel B, LdNuc\(^c::HA\), lanes 1 and 3, respectively). The anti-HA antibody showed no reactivity with lysates or the culture supernatants from control pKSNEO-transfected promastigotes (Fig. 5, panel B, pKSNEO, lanes 2 and 4, respectively). Similarly, a control isotype-matched mouse immunoglobulin showed no reactivity with any of the samples tested in these assays (data not shown). As anticipated from our results above, in parallel blots, the rabbit anti-LdNuc\(^c\) peptide antibody showed only a single band of reactivity with the \(~35\)-kDa endogenous (i.e. native) nuclease present in both the lysates and culture supernatants of control pKSNEO-transfected promastigotes (Fig. 5, panel B, pKSNEO, lanes 2 and 4, respectively). Interestingly, this antibody gave even a stronger band of reactivity with lysates and cell-free culture supernatants of the LdNuc\(^c::HA\)-transfected parasites (Fig. 5, panel C, LdNuc\(^c::HA\), lanes 1 and 3, respectively). In contrast, control preimmune rabbit serum (NRS) showed no reactivity with any of the samples tested in these assays (data not shown).

Taken together, results of these Western blot experiments demonstrated the following: 1) that the LdNuc\(^c::HA\) chimeric gene construct was readily transcribed and translated into a single \(~35\)-kDa LdNuc\(^c::HA\) chimeric protein by transfected L.
donovani promastigotes, 2) that it was released/secreted by these transfectants during their growth in vitro, and 3) that the rabbit anti-LdNuc\(^{-}\) peptide antibody in fact recognized both the LdNuc\(^{-}\)-HA-expressed protein and the endogenous/native wild-type parasite nuclease. The latter is of relevance as it indicates the presence of the endogenous (i.e. native) LdNuc\(^{-}\) protein (i.e. encoded by the LdNuc\(^{-}\)-gene) possessed common structurally conserved antigenic epitopes.

**Immunofluorescence Analysis of Transfected Parasites**—Indirect immunofluorescence was used to visualize the distribution of LdNuc\(^{-}\) in L. donovani-transfected parasites. For experiments, transfected promastigotes were reacted with either anti-HA monoclonal antibody or our rabbit anti-LdNuc\(^{-}\) peptide antibody and subsequently reacted with either a FITC- or Texas Red-conjugated secondary antibody and examined by epi-fluorescence microscopy. Results obtained with the anti-HA antibody showed that the LdNuc\(^{-}\)-HA-expressed protein was intracellularly dispersed throughout the LdNuc\(^{-}\)-HA transfectants (Fig. 6, panel A2). Such staining is consistent with processing of the LdNuc\(^{-}\)-HA protein through the endoplasmic reticulum in these transfectants. Very similar IFA staining patterns have been reported for a variety of secretory proteins in these parasites (10, 28, 53). No fluorescence was detected with the anti-HA antibody in promastigotes transfected with the pKSneo control plasmid (Fig. 6, panel B2). Neither of the transfectants showed any reactivity with control isotype-matched normal mouse immunoglobulin used in these assays (data not shown).

In agreement with the anti-HA observations above, results obtained with the rabbit anti-LdNuc\(^{-}\) peptide antibody also showed that the LdNuc\(^{-}\)-HA-expressed protein presumably along with the endogenous wild-type enzyme were dispersed intracellularly throughout the LdNuc\(^{-}\)-HA transfectants (Fig. 6, panel C2). A similar IFA staining pattern, albeit with a lower and less intense fluorescence signal, was also obtained with this antibody for the intracellular distribution of the native/endogenous enzyme in pKSneo control transfecteds (Fig. 6, panel D2). The latter is consistent with IFA results obtained above with this antibody and nontransfected/wild-type promastigotes (cf. Fig. 4, panel B, panel A2). Presumably, this pattern of staining reflects the processing of the LdNuc\(^{-}\)-HA-expressed protein through the endoplasmic reticulum of these organisms. No fluorescence was detected in either LdNuc\(^{-}\)-HA- or pKSneo control-transfecteds treated with NRS from this rabbit (data not shown). Results of these IFA experiments showed the following: 1) the LdNuc\(^{-}\)-HA-expressed protein was synthesized and processed by the LdNuc\(^{-}\)-HA transfectants; and 2) that the LdNuc\(^{-}\)-HA-expressed protein had an apparent overlapping intracellular distribution with the native/endogenous protein.

**Functional Enzyme Activity Analyses of the LdNuc\(^{-}\)-HA-expressed Protein**—Our Western blot results above showed that the LdNuc\(^{-}\)-HA-expressed protein and the endogenous/native enzyme were dispersed intracellularly throughout in vitro, and 3) that the rabbit anti-LdNuc\(^{-}\) peptide antibody in fact recognized both the LdNuc\(^{-}\)-HA-expressed protein and the endogenous/native wild-type parasite nuclease. The latter is of relevance as it indicates the presence of the endogenous (i.e. native) LdNuc\(^{-}\) protein (i.e. encoded by the LdNuc\(^{-}\)-gene) possessed common structurally conserved antigenic epitopes.

**Immunofluorescence Analysis of Transfected Parasites**—Indirect immunofluorescence was used to visualize the distribution of LdNuc\(^{-}\) in L. donovani-transfected parasites. For experiments, transfected promastigotes were reacted with either anti-HA monoclonal antibody or our rabbit anti-LdNuc\(^{-}\) peptide antibody and subsequently reacted with either a FITC- or Texas Red-conjugated secondary antibody and examined by epi-fluorescence microscopy. Results obtained with the anti-HA antibody showed that the LdNuc\(^{-}\)-HA-expressed protein was intracellularly dispersed throughout the LdNuc\(^{-}\)-HA transfectants (Fig. 6, panel A2). Such staining is consistent with processing of the LdNuc\(^{-}\)-HA protein through the endoplasmic reticulum in these transfectants. Very similar IFA staining patterns have been reported for a variety of secretory proteins in these parasites (10, 28, 53). No fluorescence was detected with the anti-HA antibody in promastigotes transfected with the pKSneo control plasmid (Fig. 6, panel B2). Neither of the transfectants showed any reactivity with control isotype-matched normal mouse immunoglobulin used in these assays (data not shown).

In agreement with the anti-HA observations above, results obtained with the rabbit anti-LdNuc\(^{-}\) peptide antibody also showed that the LdNuc\(^{-}\)-HA-expressed protein presumably along with the endogenous wild-type enzyme were dispersed intracellularly throughout the LdNuc\(^{-}\)-HA transfectants (Fig. 6, panel C2). A similar IFA staining pattern, albeit with a lower and less intense fluorescence signal, was also obtained with this antibody for the intracellular distribution of the native/endogenous enzyme in pKSneo control transfecteds (Fig. 6, panel D2). The latter is consistent with IFA results obtained above with this antibody and nontransfected/wild-type promastigotes (cf. Fig. 4, panel B, panel A2). Presumably, this pattern of staining reflects the processing of the LdNuc\(^{-}\)-HA-expressed protein through the endoplasmic reticulum of these organisms. No fluorescence was detected in either LdNuc\(^{-}\)-HA- or pKSneo control-transfecteds treated with NRS from this rabbit (data not shown). Results of these IFA experiments showed the following: 1) the LdNuc\(^{-}\)-HA-expressed protein was synthesized and processed by the LdNuc\(^{-}\)-HA transfectants; and 2) that the LdNuc\(^{-}\)-HA-expressed protein had an apparent overlapping intracellular distribution with the native/endogenous protein.

**Functional Enzyme Activity Analyses of the LdNuc\(^{-}\)-HA-expressed Protein**—Our Western blot results above showed that the LdNuc\(^{-}\)-HA-chimeric protein was synthesized and released/secreted by transfected promastigotes; however, it was important to demonstrate that this expressed protein actually possessed functional nuclease activity. To address this, in initial experiments, transfected promastigotes were grown in chemically defined medium, and aliquots of their concentrated culture supernatants were analyzed directly for nuclease activity in SDS-polyacrylamide/poly(A) substrate gels, as above. Results of
these gel assays showed that culture supernatants from pKSNEO control transfecants contained a single ~35-kDa band of nuclease activity (Fig. 7, panel A, lane 1) comparable with that synthesized and released/secreted by wild-type, non-transfected promastigotes (see Fig. 1, panel B, lane 2). In comparison, supernatants from LdNuc::HA-transfected parasites contained a much more prominent/enhanced ~35-kDa band of nuclease activity (Fig. 7, panel A, lane 2). The latter band presumably reflects the sum total of both the LdNuc::HA-expressed and the endogenous parasite-enzyme activities released/secreted by these transfecants. It is of relevance to point out that the activity of the LdNuc::HA-expressed protein, like the activity of the native enzyme, was completely abolished by DTT treatment (Fig. 7, panel A', lanes 2 and 1, respectively). These results indicated that one or more disulfide bonds are necessary for maintenance of the functional activity of both the LdNuc::HA-expressed protein and the native/wild-type enzyme. The latter observations are in agreement with our structural analyses which showed that the mature LdNuc::de-reduced protein possessed four cysteine residues (see Fig. 3, panel A) and suggested that some of those might be involved in disulfide bridges.

By having shown that our rabbit anti-LdNuc antibody recognized and immunoprecipitated the ~35-kDa wild-type L. donovani released/secreted nuclease activity above (see Fig. 4, panel C), it was important to demonstrate that antibody would also immunoprecipitate the LdNuc::HA-expressed protein and that such immunoprecipitates would possess functional nuclease activity. For these experiments, concentrated culture supernatants from transfected parasites were subjected to immunoprecipitation with the anti-LdNuc antibody and subsequently analyzed for their nuclease activity in SDS-polyacrylamide zymogram gels, as above. Results of these assays showed that the anti-LdNuc antibody immunoprecipitated the single ~35-kDa band of nuclease activity present in the culture supernatants of pKSNEO control transfecants (Fig. 7, panel B, lane 1). The latter band of activity basically reflects the endogenous/native enzyme activity produced by these parasites. In comparison, immunoprecipitates from LdNuc::HA transfecants with this antibody showed a much more prominent/enhanced ~35-kDa band of nuclease activity (Fig. 7, panel B, lane 2). Presumably, the latter reflects the sum total of the nuclease activities of the LdNuc::HA-expressed enzyme and the native/wild-type enzyme, which were both immunoprecipitated by the anti-LdNuc antibody. It is important to point out that the nuclease activity in such immunoprecipitates was completely abrogated following treatment with DTT (Fig. 7, panel B', lanes 2 and 1, respectively). In control assays, preimmune rabbit serum (NRS) showed no reactivity with any of the samples tested (data not shown). Taken together, results of these immunoprecipitation experiments showed that the LdNuc-
HA-expressed protein did in fact possess functional, DTT-sensitive nuclease activity comparable with that of the native wild-type parasite released/secreted nuclease.

Coupled immunoprecipitation/zymogram gel analyses were also carried out using an anti-HA antibody to definitively demonstrate the functional nuclease activity of the *LdNuc*<sup>+</sup>-HA-expressed protein. For such experiments, culture supernatants from transfected parasites were subjected to immunoprecipitations using a mouse anti-HA immunoaffinity bead matrix, and the resulting immune complexes were analyzed for their nuclease activity using poly(A)-containing SDS-polyacrylamide substrate gels, as above. Results of these assays showed that the anti-HA antibody specifically immunoprecipitated a single ∼35-kDa band of nuclease activity from the culture supernant of *LdNuc*<sup>+</sup>-HA transfectants (Fig. 7, panel C, lane 2). In contrast, as anticipated, no activity was detected in anti-HA immunoprecipitates obtained from culture supernatants of *pKSNEO* control transfectants (Fig. 7, panel C, lane 1). Similarly, no detectable nuclease activity was observed with immunoprecipitates obtained from parallel samples reacted with an isotype-matched normal mouse control immunoglobulin (data not shown). It is of significance to note that the nuclease activity immunoprecipitated from the *LdNuc*<sup>+</sup>-HA transfectants with the anti-HA antibody was completely abolished by DTT treatment (Fig. 7, panel C', lane 2). These results definitively demonstrated that the 35-kDa *LdNuc*<sup>+</sup>-HA-expressed protein released/secreted by the *LdNuc*<sup>+</sup>-HA transfectants did in fact possess functional nuclease activity that, like the endogenous wild-type parasite enzyme, was completely inhibited following treatment with DTT.

The cumulative results of these coupled immunoprecipitation-zyogram activity gel analyses clearly showed that the cloned *LdNuc*<sup>+</sup> gene encodes a functional, DTT-sensitive nuclease activity directly comparable with that synthesized and released/secreted by wild-type *L. donovani* parasites.

**Activity of the *LdNuc*<sup>+</sup>-HA-expressed Protein with Synthetic Polynucleotide Substrates**—From the results outlined above, it is evident that the wild-type parasite released/secreted nuclease and the *LdNuc*<sup>+</sup>-HA-expressed enzyme both readily hydrolyzed the poly(A) substrate present in zymogram gels. It is of interest to note that, in initial experiments, we found that the 35-kDa, DTT-sensitive nuclease released/secreted by wild-type promastigotes was capable of also hydrolyzing various other synthetic polynucleotide substrates (i.e. poly(U) > poly(I) > poly(C) but not poly(G); data not shown). Therefore, it was of relevance to determine whether the *LdNuc*<sup>+</sup>-HA-expressed nuclease also possessed such polynucleotide hydrolyase activity. To test this, anti-HA immunoprecipitates obtained as above from the culture supernatants of *LdNuc*<sup>+</sup>-HA and *pKSNEO* control transfectants were subsequently analyzed for their nuclease activity using SDS-polyacrylamide zymogram gels containing various individual polynucleotide substrates (i.e. poly(C), poly(I), poly(U), or poly(G)). Results of these assays showed that the anti-HA immunoprecipitates from *LdNuc*<sup>+</sup>-HA transfectants possessed a single ∼35-kDa band of nuclease activity, which, like the native enzyme, was capable of hydrolyzing three of the polynucleotide substrates tested, i.e. poly(U) > poly(I) > poly(C) (Fig. 8, lane 1 in panels A–C, respectively). In contrast, such anti-HA immunoprecipitates failed to show any detectable nuclease activity with poly(G) in such zymogram gels (Fig. 8, panel D, lane 1). As anticipated, the anti-HA immunoprecipitates obtained from culture supernatants of *pKSNEO* controls showed no nuclease activity with any of the polynucleotide substrates tested in these assays (Fig. 8, lane 2 in panels A–D). Likewise, immunoprecipitates from both *LdNuc*<sup>+</sup>-HA and *pKSNEO* control transfectants with normal mouse control immunoglobulin also failed to show any nuclease activity in these polynucleotide zymogram gels (data not shown).

Taken together, results of these coupled immunoprecipitation-activity gel analyses showed that the *LdNuc*<sup>+</sup>-HA-expressed nuclease, like the native, wild-type parasite released/secreted enzyme, was capable of hydrolyzing a variety of different synthetic polynucleotide substrates.

**Activity of the *LdNuc*<sup>+</sup>-HA-expressed Enzyme with Nucleic Acid Substrates**—The functional activities of the *LdNuc*<sup>+</sup>-HA-expressed nuclease were also evaluated using several different nucleic acid substrates. In these assays, RNase activity was assessed using an *in vitro*-transcribed [∼700 nt] RNA as substrate, whereas DNase activities were analyzed using both a single-stranded M13mp18 phage DNA (ssDNA) and double-stranded M13mp18 RF I, DNA (dsDNA) as substrates. In preliminary experiments, concentrated culture supernatants from wild-type *L. donovani* promastigotes were found to hydrolyze each of these nucleic acid substrates (data not shown). Those results suggested that the wild-type parasite released/secreted nuclease had both RNase and DNase activities. To determine whether the *LdNuc*<sup>+</sup>-HA-expressed enzyme also possessed such RNase and DNase activities, culture supernatants from both *LdNuc*<sup>+</sup>-HA- and *pKSNEO* control transfected parasites were subjected to immunoprecipitations with the anti-HA antibody affinity bead matrix, as above. Aliquots of the resulting bead-bound immune complexes were reacted with the RNA, ssDNA, and dsDNA substrates above, in buffer at either pH 8.5 or pH 5 for 30 min at 37 °C. Following such incubation, RNA-containing samples were separated in TBE-polyacrylamide gels.
and stained with SYBR Green II, whereas the DNA-containing samples were separated in E-Gels®, and all gels were visualized by UV transillumination. Results of those assays showed that immunoprecipitates obtained from culture supernatants of *L. donovani*::HA transfectants with the anti-HA antibody readily hydrolyzed not only the RNA (Fig. 9, panel A, lane 1) but also both the ssDNA (Fig. 9, panel B, lane 1) and the dsDNA (Fig. 9, panel C, lane 1) substrates tested in these experiments. It is of interest to note that each of these nucleic acid substrates appeared to be more efficiently hydrolyzed at acidic pH (i.e. pH 5.0) than under alkaline conditions (i.e. at pH 8.5, data not shown). The latter observations suggest that this parasite enzyme has a “relatively broad” pH tolerance and thus could function within the diverse micro-environments of both its sandfly vector and mammalian hosts. As anticipated, anti-HA immunoprecipitates obtained from culture supernatants of *L. donovani*::HA immunoprecipitated with the anti-HA antibody readily hydrolyzed any of these RNA, ssDNA, or dsDNA substrates (lanes 2 in Fig. 9, panels A–C, respectively). Similarly, in parallel control reactions, immunoprecipitates obtained from culture supernatants of either *LdNuc*::HA or pKSNEO transfectants with normal mouse immunoglobulin also failed to show any RNase or DNase activity in these assays (data not shown).

Taken together, the combined results of these coupled anti-HA immunoprecipitation-nucleic acid hydrolysis-gel assays clearly demonstrated that the *LdNuc*::HA-expressed enzyme possessed both functional ribonuclease and deoxyribonuclease activities congruent with those of the native wild-type parasite released/secreted nuclease.

**DISCUSSION**

Species of the pathogenic protozoan parasite *Leishmania* are responsible for causing over 2 million new cases of human cutaneous, mucocutaneous, and fatal visceral disease per year worldwide (1). All *Leishmania* parasites are purine auxotrophs (i.e. they are incapable of synthesizing the purine ring *de novo*). Thus, they are totally dependent upon their hosts to provide/supply these essential nutrients that are critical for their survival, growth, and development. To both access and acquire these essential purines from their hosts, *Leishmania* parasites have evolved a cell surface membrane [purine] salvage pathway that includes a unique ≥40-kDa enzyme, *i.e.* a bi-functional, 3’-nucleotidase/nuclease and several purine nucleoside and nucleo-base transporters (5–7, 9, 54–57). In contrast, however, to date no evidence exists that these organisms are capable of synthesizing and releasing/secretion any soluble, purine salvage pathway enzymes into their host environments. In light of this, it was of interest to investigate whether any such nucleic acid hydrolyase activities might be produced and released/secreted by these organisms. Thus, in this study, using SDS-polyacrylamide/poly(A)-zymogram gels, we demonstrated that *L. donovani* promastigotes did, in fact, constitutively synthesize and release/secrete a single soluble, DTT-sensitive ~35-kDa nucleolytic activity into their culture supernatants during their growth *in vitro*. Furthermore, we showed that this ~35-kDa, DTT-sensitive nuclease was also synthesized by both *L. donovani* in vitro-grown axenic amastigotes as well as by in vivo-derived amastigotes isolated from infected hamster spleen tissue. Taken together, these observations indicated that this parasite nuclease was constitutively produced throughout the developmental life cycle of these organisms suggesting that it might be essential for their survival. Therefore, it was of interest to further characterize the properties of this parasite released/secreted enzyme. Thus, in preliminary experiments, multiple different approaches were used in attempts to purify the ~35-kDa secretory nuclease from concentrated parasite culture supernatants. Results of those experiments showed, however, that the actual amount of protein that could be obtained from such samples was far below the level needed for these studies. Therefore, we adopted an alternative, molecularly directed approach to identify the gene encoding this unique parasite enzyme. To that end, by using a PCR-based approach, we isolated and characterized a 951-bp ORF (*LdNuc*) encoding a 316-aa deduced protein with a calculated molecular mass of 35,003 Da. Analyses of the *LdNuc*-deduced protein, using various structural algorithms, suggested that it possessed features typical of a soluble released/secreted protein (*i.e.* the presence of a putative N-terminal signal peptide for targeting into the ER, two potential N-linked glycosylation sites, overall hydrophilicity, the absence of both membrane anchor domains, and ER-retention motifs). These predicted structural properties are in good agreement with our experimental observations that demonstrated that the native 35-kDa wild-type nuclease was glycosylated (*i.e.* mannosylated) and that it was constitutively released/secreted by *L. donovani* parasites during their growth.
in vitro. Furthermore, BLAST-P analyses of the LdNuc<sup>c</sup>-deduced aa sequence showed that it had homologies to a variety of nucleases from diverse sources (7–9, 22, 34–36, 38–41). It is of significance to point out that results of Pfam data base comparisons showed that the LdNuc<sup>c</sup>-deduced protein belongs to the P1/S1 family of class I nucleases, which include the prototype P1 and S1 nucleases of *P. citrinum* and *A. oryzae*, respectively (40, 41). It has been reported that many class I nuclease family members possess five conserved blocks of aa residues designated as domains I–V (8). Results of our sequence analyses showed that all five of these domains were also present in the LdNuc<sup>c</sup>-deduced protein. In addition, some of these class I nuclease family members have also been shown to possess intra-chain disulfide bonds, which are critical for maintaining the enzymatic functions of these proteins (40, 41). In that regard, structural analyses of the mature LdNuc<sup>c</sup>-deduced protein showed that it possessed four cysteine residues that could be involved in intra-chain disulfide bonding. It is important to note that such disulfide bonds were in fact found to be essential for the function of the native/wild-type 35-kDa parasite enzyme because treatment with reducing agents (i.e. DTT) abolished its nuclease activity. Furthermore, it has also been reported that some microbial members of the P1/S1 family are typically soluble, secreted enzymes (47–51), which function extracellularly to cleave RNA and single-stranded DNA without any apparent nucleo-base preference/specificity (reviewed in Ref. 58). These properties are in agreement with those we determined experimentally for the native, wild-type, released/secreted parasite 35-kDa nuclease.

It is of interest to point out that previously several other trypanosomatid proteins have been shown to belong to the P1/S1 family of class I nucleases. In contrast to the soluble/secretory nature of the *L. donovani* 35-kDa released/secreted nuclease described in this study, those trypanosomatid P1/S1 family members were shown to have very distinct subcellular localizations. The latter include the following: 1) a 40-kDa bi-functional, 3′-nucleotidase/nuclease anchored in the cell surface membranes of several pathogenic species of *Leishmania* (i.e. *L. donovani* and *Leishmania mexicana* (5, 7, 9)) as well as *Crithidia luciliae* (8), and 2) a P-4 antigen/nuclease specifically localized to the endoplasmic reticulum of *Leishmania pifanoi* amastigote forms (39). In addition, a related P1/S1 gene homolog has been reported from an Iranian isolate of *L. major*, but no functional enzyme activity or subcellular localization was demonstrated for the product of that gene (22).

Results of our Southern blot analyses suggested that two copies of the LdNuc<sup>c</sup> gene were present in the *L. donovani* genome. Interestingly, two tandemly arranged, almost identical copies of a P1/S1 nuclease (i.e. homologs of the LdNuc<sup>c</sup> gene) have also been annotated (i.e. on chromosome 30) in *L. major* (Friedlin). In contrast, in the GeneDB data base only a single copy of such a homolog has been annotated in the genomes of *Leishmania infantum* and *Leishmania braziliensis*. Our Northern blot analyses demonstrated that LdNuc<sup>c</sup> mRNA was transcribed by both wild-type *L. donovani* promastigotes and axenic amastigotes. Furthermore, results obtained in Western blots and in our coupled immunoprecipitation/SDS-polyacrylamide/poly(A)-zymogram gel assays with the anti-LdNuc<sup>c</sup> antibody (i.e. raised against an antigenic epitope of the LdNuc<sup>c</sup>-deduced protein) showed that it specifically recognized and immunoprecipitated the native/wild-type, 35-kDa nuclease produced by both of these parasite developmental forms. Those results indicated that LdNuc<sup>c</sup> mRNA was, in fact, translated into a functionally active, 35-kDa, DTT-sensitive nuclease that is constitutively expressed and released/secreted by both *L. donovani* promastigotes and axenic amastigotes.

Taken together, the foregoing suggested that the cloned LdNuc<sup>c</sup> gene encoded the native/wild-type, 35-kDa parasite released/secreted nuclease. To test/confirm this hypothesis, it was necessary to demonstrate that the LdNuc<sup>c</sup> gene actually encoded a functionally active nuclease with properties characteristic of those determined for the native/wild-type parasite enzyme. To that end, we used a homologous episomal expression system to produce an HA-tagged LdNuc<sup>c</sup> chimeric protein. Results of Western blot analyses with both the anti-LdNuc<sup>c</sup> and the anti-HA antibodies showed that LdNuc<sup>c</sup>-HA-transfected parasites readily synthesized and released/secreted the 35-kDa LdNuc<sup>c</sup>-HA chimeric protein during their growth *in vitro*. Furthermore, the results of our coupled immunoprecipitation/SDS-polyacrylamide nuclease-zymogram activity gel assays with both of these antibodies showed that the 35-kDa LdNuc<sup>c</sup>-HA-expressed protein did in fact possess functionally active, DTT-sensitive nuclease activity homologous to the native/wild-type enzyme. Results of such coupled immunoprecipitation/zymogram gel analyses also demonstrated that the LdNuc<sup>c</sup>-HA chimeric enzyme, like the native/wild-type nuclease, could hydrolyze, in addition to poly(A), a variety of other synthetic polynucleotide substrates (i.e. poly(U), poly(I), and poly(C) but not poly(G)). The apparent inability of the parasite LdNuc<sup>c</sup> enzyme to hydrolyze poly(G) might be due to the secondary structure of this polynucleotide polymer. Similar observations have also been reported for several other class I nuclease family members (59–61). Although such synthetic polynucleotides served as very useful test substrates in these experiments, it was essential, however, to show that the parasite LdNuc<sup>c</sup> nuclease could in fact hydrolyze naturally occurring nucleic acid substrates. In that regard, the results of our coupled anti-HA immunoprecipitation/substrate hydrolysis assays clearly demonstrated that the LdNuc<sup>c</sup>-HA-expressed nuclease, just like the native/wild-type enzyme, was capable of hydrolyzing not only RNA but also both ssDNA and dsDNA substrates and that such hydrolysis occurred under both acidic (pH 5.0) and alkaline conditions (pH 8.5). This apparent "broad pH tolerance" suggests that the parasite enzyme could function within the diverse micro-environments of its hosts.

In summary, our cumulative results strongly suggest that the LdNuc<sup>c</sup> gene in fact encodes the native/endogenous *L. donovani*, DTT-sensitive, 35-kDa secretory nuclease identified in this study. As indicated above, all *Leishmania* parasites are purine auxotrophs that reside and multiply in highly restricted micro-environments within their hosts (i.e. in the gut lumen of their sandfly vectors and in the phagolysosomal compartments of mammalian macrophages). We hypothesize that within such compartments, the leishmanial 35-kDa secretory nuclease could function at a distance away from the parasite to hydrolyze and mobilize host-derived nucleic acids. Presumably, this
secretory nuclease would act in concert with other parasite purine salvage enzymes (e.g. the surface membrane 3’-nucleotidase/nuclease) and nucleoside/nucleo-base transporters to facilitate the acquisition of such essential nutrients by these organisms. In that context, the results of this study should facilitate future investigations into the role(s) that the LdNuc secretory enzyme might play in the survival, growth, and development of this important human pathogen.

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