Dissection of the Functional Domains of the Leishmania
Surface Membrane 3'-Nucleotidase/Nuclease, a Unique
Member of the Class I Nuclease Family*

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Class I nucleases are a family of enzymes that specifically hydrolyze single-stranded nucleic acids. Recently, we characterized the gene encoding a new member of this family, the 3'-nucleotidase/nuclease (Ld3'NT/NU) of the parasitic protozoan Leishmania donovani. The Ld3'NT/NU is unique as it is the only class I nuclease that is a cell surface membrane-anchored protein. Currently, we used a homologous episomal expression system to dissect the functional domains of the Ld3'NT/NU. Our results showed that its N-terminal signal peptide targeted this protein into the endoplasmic reticulum. Using Ld3'NT/NU-green fluorescent protein chimeras, we showed that the C-terminal domain of the Ld3'NT/NU functioned to anchor this protein into the parasite cell surface membrane. Further, removal of the Ld3'NT/NU C-terminal domain resulted in its release/secretion as a fully active enzyme. Moreover, deletion of its single N-linked glycosylation site showed that such glycosylation was not required for the enzymatic functions of the Ld3'NT/NU. Thus, using the fidelity of a homologous expression system, we have defined some of the functional domains of this unique member of the class I nuclease family.

Leishmania donovani is an important protozoan pathogen of humans that causes severe and often fatal visceral disease (visceral leishmaniasis or Kala azar) in the tropics and neotropics worldwide. This organism possesses a unique bifunctional enzyme into the parasite cell surface membrane. Further, removal of the Ld3'NT/NU C-terminal domain resulted in its release/secretion as a fully active enzyme. Moreover, deletion of its single N-linked glycosylation site showed that such glycosylation was not required for the enzymatic functions of the Ld3'NT/NU. Thus, using the fidelity of a homologous expression system, we have defined some of the functional domains of this unique member of the class I nuclease family.

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1 Tropical Disease Research, progress 1995–96: thirteenth program report of the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases.

2 The abbreviations used are: Ld3'NT/NU, L. donovani 3'-nucleotidase/nuclease; PBS, phosphate-buffered saline; SP, signal peptide; PCR, polymerase chain reaction; kb, kilobase; GFP, green fluorescent protein; TM, transmembrane domain; ConA, concanavalin A; PAGE, polyacrylamide gel electrophoresis; aa, amino acid; WT, wild type.

EXPERIMENTAL PROCEDURES

Parasite Culture and Transfection—L. donovani promastigotes, strain 1S, clone 2D (World Health Organization designation: MHOM/SD/62/1S-CL2D), and a lipopolysaccharide-deficient mutant (CSPO), kindly provided by Dr. Salvatore J. Turco, Department of Biochemistry, University of Kentucky Medical Center (10) derived from this clone were cultured as described previously (11). Log-phase promastigotes (2 × 10^7 cells/ml) were harvested by centrifugation at 2100 × g for 10 min at 4 °C. Cell pellets were washed in ice-cold phosphate buffer (PBS) (50 mM NaHPO_4, 150 mM NaCl, pH 7.4) by centrifugation as above. For transfection experiments, cells were resuspended in electroporation buffer (Hepes [ICN Biomedicals Inc., Aurora, OH], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, pH 7.0) to a density of 1 × 10^6 cells/ml. 500 μl of cell suspension were added to 2-mm gap electroporation cuvettes (BTX Inc., San Diego, CA) to which 20 μl of purified plasmid DNA (1 mg/ml in sterile 10 mM Tris, 2 mM EDTA [Quality Biological, Inc., Gaithersburg, MD], pH 8.0) was added. Cells were electroporated using a BTX ECM-600 electroporation system (BTX). Electroporation conditions were 800 V, 100 μF, 1200 Ω, and 100 μF.

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were: 475 V, 800 microfarads, 13 ohms, single pulse. Electroporated cells were incubated on ice for 10 min and then transferred into 5 ml of culture medium above and incubated at 26 °C for 24 h. Subsequently, the cells were harvested by centrifugation as above and resuspended in fresh culture medium containing 15 μM of Geneticin (G418, Life Technologies). The cells were then resuspended in a 1 kb PCR fragment, which was cloned into the Spe I restriction site (open) of the pKS NEO plasmid. The resulting (174 base pair) PCR fragment was cloned into the Spe I site of the pKS NEO cloning vector (T/A cloning system, Invitrogen). The resulting plasmid was then digested with the same enzymes as above. The orientation of the Spe I site in the pKS NEO vector to produce the pKS NEO 3′SP::GFP plasmid.

**Purification of Soluble-expressed Ld3′nt/nu-L. donovani plasmodias transfectants with plasmid pKS NEO Ld3′nt/nu-L.**

The purifying rabbit sera above or their preimmune sera (normal rabbit serum) were used. Rabbit antiserum (rabbit No. 1336, anti-Leishmania spp.) was used to detect the parasite Ld3′nt/nu-L. donovani hybridoma supernatant. The cells were washed three times with buffer A containing 0.1% Triton X-100 (protein grade, Calbiochem). Affinity adsorbed material was eluted from these beads using buffer A containing 0.5 M imidazole (Sigma), dialyzed against buffer B containing 0.3 M imidazole (Sigma), dialyzed against buffer B containing 0.3 M 2-mercaptoethanol (Sigma) in a yeast expression system. Purification of Soluble-expressed Ld3′nt/nu-L. donovani plasmodias transfectants with plasmid pKS NEO Ld3′nt/nu-L. donovani hybridoma supernatant. The cells were washed three times with buffer A containing 0.1% Triton X-100 (protein grade, Calbiochem). Affinity adsorbed material was eluted from these beads using buffer A containing 0.5 M imidazole (Sigma), dialyzed against buffer B and stored at -20 °C. For nickel agarose bead adsorption, the pH of the cell-free culture supernatant was adjusted to 8.5 by the addition of 2 M NaOH. Ni2+–nitrilotriacetic acid–agarose beads (Qiagen Inc., Chatsworth, CA) were equilibrated in 20 mM Hepes, pH 8.0 (buffer A) and incubated overnight on a platform rocker at 4 °C with cell-free culture supernatants. Beads were subsequently washed three times with buffer A containing 0.1% Triton X-100 and the adsorbed proteins were eluted using buffer B containing 0.3 M 2-mercaptoethanol (Sigma) as substrate as described previously (1). Rabbit Antisera—Synthetic peptides were made (Genosys Biotechnology). The Woodlands, TX, containing amino acid residues 361–375 (CYLPKDRBFG) of the C-terminal domain of the Ld3′nt/nu-L. donovani hybridoma supernatant. These peptides were conjugated to keyhole limpet hemocyanin and used to immunize a New Zealand White rabbit (No. 1432) as described previously (1). The resulting antisera (anti-Ld3′nt/nu-L., anti-Ld3′nt/nu-L., anti-Ld3′nt/nu-L.) was used in preliminary experiments to specifically react with the parasite Ld3′nt/nu-L. donovani Western blot. A second antisera (rabbit No. 1432, anti-Ld3′nt/nu-L. specific), generated raised in a rabbit with a 20 μg/ml concentrations of Ld3′nt/nu-L. donovani western blot. The resulting precipitation of Glu21 to Tyr226, was described previously (1) and also used in the current study. In addition, a rabbit anti-GFP serum (CLONTECH) and an Escherichia coli recombinant GFP protein (CLONTECH) were used in these immunobaselines.

**3′-Nucleotidase Enzyme Assays—3′-Nucleotidase activity was measured in both promastigote cell lysates and in cell-free culture supernatants by test tube assays using 3′-adenosine monophosphate (3′-AMP, Sigma) as substrate as described previously (1). Rabbit Antisera—Synthetic peptides were made (Genosys Biotechnology). The Woodlands, TX, containing amino acid residues 361–375 (CYLPKDRBFG) of the C-terminal domain of the Ld3′nt/nu-L. donovani hybridoma supernatant. These peptides were conjugated to keyhole limpet hemocyanin and used to immunize a New Zealand White rabbit (No. 1432) as described previously (1). The resulting antisera (anti-Ld3′nt/nu-L., anti-Ld3′nt/nu-L., anti-Ld3′nt/nu-L.) was used in preliminary experiments to specifically react with the parasite Ld3′nt/nu-L. donovani Western blot. A second antisera (rabbit No. 1432, anti-Ld3′nt/nu-L. specific), generated raised in a rabbit with a 20 μg/ml concentrations of Ld3′nt/nu-L. donovani western blot. The resulting precipitation of Glu21 to Tyr226, was described previously (1) and also used in the current study. In addition, a rabbit anti-GFP serum (CLONTECH) and an Escherichia coli recombinant GFP protein (CLONTECH) were used in these immunobaselines.
according to Zlotnick et al. (16) or in situ staining of nuclease activity according to Bates (17).

Microscopy—*L. donovani* promastigotes were fixed in suspension in 4% (w/v) paraformaldehyde (Sigma) in PBS for 20 min on ice, washed three times in PBS, and were allowed to attach to glass slides precoated with poly-L-lysine (Sigma). For direct fluorescence, cells were mounted in PBS. Images were acquired using a Zeiss AxioPlan microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence and a cooled CCD camera (Photometrics, Tucson, AZ). Fluorescence was detected using fluorescein isothiocyanate excitation/barrier filters. Cells were also examined by confocal microscopy using a Zeiss LSM 410 system (Zeiss). For indirect immunofluorescence, cells were blocked for 30 min in 1% (w/v) bovine serum albumin (United States Biochemical Co., Cleveland, OH) in PBS and incubated 1 h with either the anti-Ld3’NT/NU-specific antibody (No. 1336) serum or the anti-Ld3’NT/NU C-terminal specific antibody (No. 1432, Ld3’ NT/Term, lanes 1−3) with E. coli recombinant GFP protein (lanes 1 and 1’), lysates of promastigotes transfected with either the [pKS N3’SP:GFP] plasmid (lanes 2 and 2’) or the [pKS N3’SP:GFP::TM] plasmid (lanes 3 and 3’). D, fluorescence image showing cell surface localization of the 3’SP::GFP::TM chimeric protein in C3PO promastigotes transfected with the [pKS N3’SP::GFP::TM] plasmid and its accumulation in their flagellar reservoirs.

**RESULTS**

**Episomal Expression of the Ld3’NT/NU**—Previously, we characterized the gene encoding Ld3’NT/NU (1). The deduced protein of this gene (Fig. 1A) contains a putative hydrophobic SP (aa residues Met1-Ala2-Arg3-Ala4-Arg5-Phe6-Leu7-Gln8-Leu9-Leu10-Leu11-Val12-Thr13-Leu14-Thr15-Leu16-Leu17-Ser18-Thr19-Ala20-Ala21-Ala22-Pro23-Val24-Ser25-Ala26), an N-linked glycosylation site (aa residues Asn292), and a TM (aa residues Ala334-Ala335-Val336-Thr337-Ala338-Il339-Val340-Ala341-Val342-Ala343-Leu344-Phe345-Ile346-Ala347-Gly348-Ile349-Ile350-Ile351-Ala352-Thr353-Leu354-Glu355-Val356-Leu357-Ala358-Leu359). To confirm that the cloned Ld3’NT/NU gene product was targeted to the cell surface membrane of *L. donovani*, the nucleotide sequence corresponding to the deduced protein shown in Fig. 1A was cloned into the [pKS N3’OEI] leishmanial expression plasmid (14). *L. donovani* wild type (WT) and C3PO promastigotes transfected with the [pKS N3’OEI] plasmid (lane 2). B, indirect immunofluorescence showing cell surface reactivity of the anti-Ld3’NT/NU-specific antibody (No. 1336) with C3PO transfected with [pKS N3’NT/NU] plasmid (lane 2). C, Western blot showing the reactivity of either anti-GFP-specific antibody (α-GFP, lanes 1 and 3) or anti-Ld3’NT/NU C-terminal specific antibody (No. 1432, α-Ld3’ NT/Term, lanes 1−3) with E. coli recombinant GFP protein (lanes 1 and 1’), lysates of promastigotes transfected with either the [pKS N3’SP::GFP] plasmid (lanes 2 and 2’) or the [pKS N3’SP::GFP::TM] plasmid (lanes 3 and 3’). D, fluorescence image showing cell surface localization of the 3’SP::GFP::TM chimeric protein in C3PO promastigotes transfected with the [pKS N3’SP::GFP::TM] plasmid and its accumulation in their flagellar reservoirs. Western blots showed that the anti-Ld3’NT/NU-specific antibody (No. 1336) reacted with a single 43-kDa protein in both transfected controls and [pKS N3’NT/NU] transfected promastigotes. In control promastigotes, this 43-kDa band reflects the endogenous Ld3’NT/NU (Fig. 2A, lane 1). The enhanced reactivity seen with this antibody in [pKS N3’NT/NU] transfected cells represents the episomal overexpression of the Ld3’NT/NU protein (Fig. 2A, lane 2). Identical results were obtained with both WT (not shown) and C3PO promastigotes in these experiments. Normal rabbit sera showed no reactivity in these Western blots (data not shown).

The *L. donovani* promastigotes above were also analyzed by light microscopy. By phase contrast microscopy, all of these transfected cells showed the typical pyriform morphology of flagellated *Leishmania* promastigotes. Both controls and transfected promastigotes were treated with the anti-Ld3’NT/NU-specific antibody (No. 1336) to visualize by indirect immunofluorescence the cell surface localization of the Ld3’NT/NU protein. Although both WT and C3PO promastigotes (untransfected) possess externally oriented cell surface membrane 3’-nucleotidase enzyme activity, as previously demonstrated by both subcellular fractionation and fine structure cytochemistry (18, 19), neither showed significant fluorescence with the anti-Ld3’NT/NU-specific antibody (No. 1336). The weak fluorescence signal obtained with this antibody presumably reflects the low level of endogenous Ld3’NT/NU protein expressed on the cell surface of these parasites (i.e. number of protein copies/cell). In contrast, promastigotes transfected with the [pKS N3’NT/NU] plasmid, WT (not shown), and C3PO (Fig. 2B) showed bright cell surface immunofluorescence with the anti-Ld3’NT/NU-specific antibody (No. 1336). Such distinct fluorescence presumably reflects the increased copy number of this protein present on the cell surface of these parasites. The latter observation was confirmed using confocal fluorescence microscopy. These results demonstrated that the Ld3’NT/NU-expressed protein was targeted to the parasite cell surface as presented. D. M. Dwyer, unpublished data.
detected by the anti-\textit{Ld}3\textsuperscript{NT/NU}-specific antibody (No. 1336). Further, results of multiple observations showed that C3PO transfectants had significantly more cell surface fluorescence than WT transfectants. The difference in fluorescence intensity between these transfectants could represent the relative amounts of \textit{Ld}3\textsuperscript{NT/NU} proteins expressed on their cell surfaces. This might also explain the apparent lack of reactivity of the anti-Ld3\textsuperscript{NT/NU}-specific antibody (No. 1336) with untransfected cells. Normal rabbit sera controls showed no reactivity with any cell types used in these experiments.

\textit{Expression of Ld3\textsuperscript{NT/NU}-GFP Chimeric Proteins by \textit{L. donovani} Promastigotes}—To demonstrate that the C-terminal region of the \textit{Ld3\textsuperscript{NT/NU}} functions as a membrane anchor domain, \textit{Ld3\textsuperscript{NT/NU}}-GFP chimeric proteins were expressed in \textit{L. donovani} promastigotes. To that end, nucleotide constructs encoding the two \textit{Ld3\textsuperscript{NT/NU}}-GFP chimeric proteins described below were cloned into the [pKS NEO] leishmanial expression plasmid as above. In one of these chimeric proteins (3'SP::GFP::TM, Fig. 1B), GFP was substituted for aa residues Glu\textsuperscript{52} to Ser\textsuperscript{333} of the \textit{Ld3\textsuperscript{NT/NU}}. Thus, its N terminus contained the first 51 aa residues of the \textit{Ld3\textsuperscript{NT/NU}} including the SP (aa residues Met\textsuperscript{1} to Ala\textsuperscript{26}). The C terminus of this chimeric protein contained the entire C-terminal region (aa residues Ala\textsuperscript{334} to Leu\textsuperscript{377}) of the \textit{Ld3\textsuperscript{NT/NU}} including its putative anchor domain (TM, aa residues Ala\textsuperscript{334} to Leu\textsuperscript{377}). The second \textit{Ld3\textsuperscript{NT/NU}}-GFP chimeric protein (3'SP::GFP, Fig. 1C) was identical to the first one except that it lacked the entire C-terminal region (aa residues Ala\textsuperscript{334} to Leu\textsuperscript{377}) of the \textit{Ld3\textsuperscript{NT/NU}}. Transfected promastigotes were grown under increasing concentrations of G418 up to 250 \mu g/ml, and lysates of such cells were analyzed by SDS-PAGE and Western blotting. In such blots, the anti-GFP-specific antibody showed strong reactivity with a control ~30-kDa \textit{E. coli} recombinant GFP protein (Fig. 2C, lane 1). That antibody also reacted with a single ~30-kDa protein in both WT (not shown) and C3PO (Fig. 2C, lane 2) promastigotes transfected with the [pKS NEO 3'SP::GFP] plasmid. The anti-GFP-specific antibody also reacted with an ~35-kDa protein and to a lesser extend with an ~32-kDa protein in cells transfected with the [pKS NEO 3'SP::GFP::TM] plasmid (Fig. 2C, lane 3). The latter presumably represents a proteolytic degradation product of the ~35-kDa protein. Further, this ~35-kDa protein was also recognized by our anti-Ld3\textsuperscript{NT/NU} C-terminal specific (No. 1432) antibody (Fig. 2C, lane 3) demonstrating that these cells expressed the 3'SP::GFP::TM chimeric protein. In contrast, the anti-Ld3\textsuperscript{NT/NU} C-terminal specific (No. 1432) antibody showed no reactivity with either parasites transfected with the [pKS NEO 3'SP::GFP] plasmid (Fig. 2C, lane 2) or with the control \textit{E. coli} recombinant GFP protein (Fig. 2C, lane 1). Normal rabbit sera controls showed no reactivity in these Western blot assays (data not shown).

\textit{L. donovani} WT and C3PO promastigotes transfected with the above plasmids or the control [pKS NEO] expression plasmid were examined by epifluorescence and confocal fluorescence microscopy. Such observations revealed that WT (not shown) and C3PO (Fig. 2D) promastigotes transfected with the [pKS NEO 3'SP::GFP::TM] plasmid had bright cell surface fluorescence. These results demonstrated that the 3'SP::GFP::TM chimeric protein was targeted to the cell surface membrane of transfected parasites. In addition, GFP was also seen to accumulate within the flagellar reservoirs of these transfected cells. In contrast, promastigotes transfected with the [pKS NEO 3'SP::GFP] plasmid showed only diffuse intracellular fluorescence reflecting the processing of GFP within the endoplasmic reticulum (data not shown). Further, the latter transfectants released/secreted soluble GFP into their culture supernatant, which was detected by Western blots with the anti-GFP antibody (data not shown). Control promastigotes transfected with the [pKS NEO] expression plasmid alone showed no detectable cellular fluorescence. Cumulatively, these results demonstrated that the C-terminal domain of the \textit{Ld3\textsuperscript{NT/NU}} functioned to anchor this enzyme into the cell surface membrane of these parasites.

\textit{Expression of a Truncated Ld3\textsuperscript{NT/NU} in \textit{L. donovani}}—To determine whether the C-terminal region (aa residues Ala\textsuperscript{334} to Leu\textsuperscript{377}) of the \textit{Ld3\textsuperscript{NT/NU}} was necessary for its enzymatic activities, \textit{L. donovani} promastigotes were transfected with an expression plasmid [pKS NEO \textit{Ld3\textsuperscript{nt/nu}}] encoding a truncated \textit{Ld3\textsuperscript{NT/NU}}. In this truncated protein (\textit{Ld3\textsuperscript{nt/nu}}), the C-terminal region of the \textit{Ld3\textsuperscript{NT/NU}} was replaced by six histidine residues (His)\textsubscript{6} (Fig. 1D). Both [pKS NEO \textit{Ld3\textsuperscript{nt/nu}}]-transfected promastigotes and those transfected with the control [pKS NEO] plasmid were grown in the presence of 250 \mu g/ml G418 and analyzed by SDS-PAGE. Lysates of these cells were probed in Western blots with the rabbit anti-Ld3\textsuperscript{NT/NU} specific antibody (No. 1336) or normal rabbit serum. Results of these assays showed that parasites transfected with the control [pKS NEO] plasmid contained only a single 43-kDa band corresponding to the endogenous \textit{Ld3\textsuperscript{NT/NU}} (Fig. 3A, lane 1). Promastigotes transfected with the [pKS NEO \textit{Ld3\textsuperscript{nt/nu}}] plasmid showed a similar 43-kDa band of endogenous \textit{Ld3\textsuperscript{NT/NU}} and an additional band of ~38 kDa (Fig. 3A, lane 2). The latter demonstrated that such transfectants synthesized the truncated \textit{Ld3\textsuperscript{nt/nu}} protein encoded by the [pKS NEO \textit{Ld3\textsuperscript{nt/nu}}] plasmid. Lysates of neither transfectant showed any reactivity with normal rabbit serum (not shown). 3'-Nucleotidase activity present in lysates of transfected promastigotes was visualized by \textit{in situ} staining of SDS-PAGE gels. Results showed that lysates of each transfected cell line contained a 43-kDa band of activity reflecting the endogenous \textit{Ld3\textsuperscript{NT/NU}} enzyme (Fig. 3B, lanes 1 and 2). In addition, the [pKS NEO \textit{Ld3\textsuperscript{nt/nu}}] transfected promastigotes showed an ~38-kDa band of 3'-nucleotidase activity corresponding to the \textit{Ld3\textsuperscript{nt/nu}} expressed protein (Fig. 3B, lane 2). Results obtained from similar gels stained for nuclease activity showed that lysates of both transfected cell types contained a 43-kDa band of enzyme activity corresponding to the endogenous \textit{Ld3\textsuperscript{NT/NU}} (Fig. 3C, lanes 1 and 2). Further, lysates of para-
sites transfected with the \([pKS NEO Ld3\,nt/nu]\) plasmid also showed a ~38-kDa band of nuclease activity corresponding to the \(Ld3\,nt/nu\) expressed protein (Fig. 3C, lane 2). Taken together these results demonstrated for the first time, that the \(Ld3\,NT/NU\) gene encodes a bifunctional protein, which has both 3'-nucleotidase and nuclease activities. Further, they demonstrated that the C-terminal domain of this protein was not required for either its 3'-nucleotidase or its nuclease activities.

**Extracellular Release of the Soluble \(Ld3\,nt/nu\) Expression Protein**—Having established that the C-terminal domain of the \(Ld3\,NT/NU\) was involved in anchoring it into the surface membrane of the parasite, it was hypothesized that the truncated \(Ld3\,nt/nu\) would be a soluble, and possibly, a released/secreted protein. To address this question, cell-free culture supernatants from promastigotes transfected with \([pKS NEO Ld3\,nt/nu]\) or the control \([pKS NEO]\) plasmids were assayed by Western blot and by SDS-PAGE in lanes 1 and 2, respectively. A. Western blot showing the reactivity of a rabbit anti-\(Ld3\,NT/NU\)-specific antibody (No. 1336) with both transfectants. B, in situ staining of 3'-nucleotidase activity present in supernatants of both transfectants. C, in situ staining for nuclease activity present in supernatants of both transfectants. The molecular masses (in kDa) of protein standards are given at the left of this figure.

![Fig. 4. Kinetics of soluble 3'-nucleotidase activity released by promastigotes during their growth in vitro. A. 3'-nucleotidase activity present in cell-free culture supernatants of \(L.\ donovani\) promastigotes transfected with either the \([pKS NEO Ld3\,nt/nu]\) or control \([pKS NEO]\) plasmid over a 60-h time course. Enzyme activity was measured using a colorimetric assay and is expressed as nmol/min/ml. The inset shows the growth of the two transfected cell types (y axis, cell number \(\times 10^4/ml\) over the same time course. B, SDS-PAGE gel stained in situ for the 3'-nucleotidase activity present in aliquots of cell-free culture supernatants from \([pKS NEO Ld3\,nt/nu]\)-transfected promastigotes during their growth in vitro as above. C, Western blot showing the reactivity of a rabbit anti-\(Ld3\,NT/NU\)-specific antibody (No. 1336) with samples identical to those used in B. The apparent molecular mass of a ~37.6-kDa protein standard is shown at the left in B and C.](image)

![Fig. 5. SDS-PAGE analyses of cell-free culture supernatants from \(L.\ donovani\) promastigotes. Nickel-agarose, affinity purified proteins from culture supernatants of promastigotes transfected with either a \([pKS NEO]\) control or the \([pKS NEO Ld3\,nt/nu]\) plasmid were analyzed by SDS-PAGE in lanes 1 and 2, respectively. A. Western blot showing the reactivity of a rabbit anti-\(Ld3\,NT/NU\)-specific antibody (No. 1336) with both transfectants. B, in situ staining of 3'-nucleotidase activity present in supernatants of both transfectants. C, in situ staining for nuclease activity present in supernatants of both transfecnts. The molecular masses (in kDa) of protein standards are given at the left of this figure.](image)
protein presumably reflects a proteolytic product of the mature \( \sim 38\)-kDa released protein. Further, results obtained from such samples using in situ stained gels demonstrated that the \( \sim 38/37\)-kDa protein doublet had both 3'-nucleotidase (Fig. 5B, lane 2) and nuclease activities (Fig. 5C, lane 2), respectively. Results of assays with material from control, [pKS NEO]-transfected promastigotes showed no reactivity in Western blots with the rabbit anti-Ld3'NT/NU-specific serum (No. 1336, Fig. 5A, lane 1). Similarly, such control material showed neither 3'-nucleotidase (Fig. 5B, lane 1) nor nuclease activity (Fig. 5C, lane 1) in in situ stained gels. Taken together, these results demonstrated that the soluble released Ld3'nt/nu* possessed a histidine tag and therefore was the product of the [pKS NEO Ld3'nt/nu*] plasmid. Further, the Ld3'nt/nu* signal peptide must target the nascent protein into the endoplasmic reticulum for it to be released from these cells presumably via default into the parasite secretory pathway.

Glycosylation of the Ld3'nt/nu* Expressed Protein—The native Ld3'NT/NU has been shown to be an N-linked glycoprotein that binds to ConA beads (1, 20). Further, we showed that the Ld3'NT/NU gene-deduced protein contained a single N-linked glycosylation site at asparagine 293 (Fig. 1A) (1). In the current study, we demonstrated that the release/secreted Ld3'NT/NU possessed both 3'-nucleotidase and nuclease activities and in preliminary experiments found that it bound to ConA beads. Thus to ascertain whether N-linked glycosylation was necessary for the enzymatic functions of the Ld3'nt/nu*, an expression plasmid encoding a mutated Ld3'nt/nu* sequence was constructed. In that plasmid [pKS NEO Ld3'nt/nu*Asn], the “AAC” codon corresponding to the putative glycosylation site, Asn\(^{293}\), was substituted by a “CAG” sequence encoding a Gln residue. This single amino acid substitution should result in the loss of the single N-linked glycosylation site of the Ld3'nt/nu* (Fig. 1E). L. donovani promastigotes were transfected with the [pKS NEO Ld3'nt/nu*Asn] plasmid and selected for growth in the presence of 250 \( \mu\)g/ml G418. Cell-free culture supernatants from these transfected cells and from [pKS NEO Ld3'nt/nu*]-transfected promastigotes were both shown to contain 3'-nucleotidase activity in colorimetric assays. Aliquots of these culture supernatants were also adsorbed with ConA-Sepharose beads and with nickel-agarose beads. Material eluted from such beads was subjected to SDS-PAGE and analyzed by Western blot and by in situ staining for 3'-nucleotidase and nuclease activities. As predicted from our preliminary experiments above, Western blot results showed that the rabbit anti-Ld3'NT/NU-specific serum (No. 1336) reacted with the \( \sim 38\)-kDa Ld3'nt/nu* protein present in the ConA-eluted material from the [pKS NEO Ld3'nt/nu*]-transfected cells (Fig. 6A, lane 1). Further, the Ld3'nt/nu* protein present in such ConA-eluted material showed both 3'-nucleotidase (Fig. 6B, lane 1) and nuclease (Fig. 6C, lane 1) activities. In contrast, ConA-eluted material from the [pKS NEO Ld3'nt/nu*Asn] transfected parasites showed no reactivity in Western blots probed with the rabbit anti-Ld3'NT/NU-specific serum (No. 1336, Fig. 6A, lane 2) and showed no reactivity in gels stained in situ for 3'-nucleotidase (Fig. 6B, lane 2) or nuclease (Fig. 6C, lane 2) activity. The latter results indicated that the Ld3'nt/nu*Asn protein did not bind to ConA beads. However, the Ld3'nt/nu*Asn protein was bound and eluted from nickel-agarose beads as it reacted with the rabbit anti-Ld3'NT/NU-specific serum (No. 1336) in Western blots (Fig. 6A, lane 4). In such blots this antibody reacted with a \( \sim 37\)-kDa protein and to a lesser extent with a protein of \( \sim 35\) kDa. The \( \sim 35\)-kDa protein presumably reflects a proteolytic product of its \( \sim 37\)-kDa Ld3'nt/nu*Asn precursor. Further, the \( \sim 37\)- and \( \sim 35\)-kDa proteins had both 3'-nucleotidase (Fig. 6B, lane 4) and nuclease activities (Fig. 6C, lane 4), respectively. As expected, nickel-agarose-eluted material from [pKS NEO Ld3'nt/nu*] cells (i.e., Ld3'nt/nu*) gave positive results in Western blots and in in situ stained gels (Fig. 6, A–C, lane 3). Taken together, these results demonstrated that the Ld3'nt/nu* was glycosylated at Asn\(^{293}\) and that the apparent molecular mass of its N-linked glycan was \( \sim 1–2\) kDa. Further, these results indicated that such N-linked glycosylation was not essential for the enzymatic functions of the Ld3'nt/nu*.

**DISCUSSION**

Class I nucleases are a family of enzymes from diverse sources (e.g., plants, fungi, and protozoa) that specifically hydrolyze single-stranded DNA and RNA (3, 5, 21). It has been reported that these nucleases play a major biological role in cell growth and division by generating free nucleosides and phosphate from nucleic acid substrates (2, 5). Although various members of this family have been identified, the structure of only one of these has been studied in detail (i.e., the P1 fungal nuclease) \((8–9)\). To date, however, none of these nucleases have been dissected using molecular/recombinant DNA techniques to address the functionality of their structural constituents. In that regard, we used a homologous episomal expression system from a parasitic protozoan, *L. donovani*, to delineate the functional domains of one member of this family, the unique *L. donovani* surface membrane Ld3'NT/NU. This homologous transfection system was used to ensure that processing of the expressed Ld3'NT/NU protein would be equivalent to that of its native endogenous cell surface homolog. The Ld3'NT/NU gene-deduced protein was previously shown to contain sequences reflecting a signal peptide, an N-linked glycosylation site, and a hydrophobic transmembrane domain \( (1)\). These sequences were presumed responsible for targeting the nascent protein into the endoplasmic reticulum, facilitating its processing and enzymatic activities, and for anchoring the mature protein into the parasite surface membrane, respectively. To address these predictions experimentally, various Ld3'NT/NU gene constructs were expressed in *L. donovani* promastigotes, and the resulting expressed proteins were analyzed with regard to their localization and enzymatic activities. Further, the Ld3'NT/NU signal peptide sequence was included in all constructs to ensure the proper translocation of the ex-
pressed proteins into the endoplasmic reticulum. This was done because our previous observations demonstrated that a truncated Ld3′NT/NU protein lacking a signal peptide was expressed solely in the cytoplasm of transfected cells and was toxic to these parasites (13).

Among the class I nuclease, only the Ld3′NT/NU has been shown to be a surface membrane-anchored protein (1, 20). To demonstrate that its unique C-terminal domain functioned as a membrane anchor, Ld3′NT/NU-GFP chimeric proteins containing this C-terminal domain were expressed in L. donovani promastigotes. Fluorescence microscopy analysis showed that the 3′SP::GFP::TM chimera was expressed on the cell surface membrane of transfected parasites. These results demonstrated that the C-terminal portion of the Ld3′NT/NU functions as the membrane-anchoring domain for this cell surface protein. With regard to targeting of the 3′SP::GFP::TM protein to the parasite cell surface, we hypothesize that it might traffic by default through the parasite secretory pathway or be targeted via Ld3′NT/NU signals remaining within this chimeric protein. Further, to determine whether the C-terminal domain of the Ld3′NT/NU was involved in the enzymatic functions of this protein, a truncated Ld3′NT/NU protein lacking its C-terminal domain was expressed in L. donovani promastigotes. Such transfected cells expressed an ∼38-kDa protein (Ld3′nt/nu*), which had both 3′-nucleotidase and nuclease activities. Further, the latter was released in active form into the culture supernatant of these cells during their growth in vitro. These results demonstrated that the C-terminal domain of the Ld3′NT/NU was not required for the enzymatic activities of this protein. Moreover, because the Ld3′nt/nu* lacked a membrane anchor, we postulate that it was released from transfected cells either via default into the secretory pathway of the parasite or targeted into this pathway by signals encoded within this protein. Similar conclusions pertain to the 3′SP::GFP protein expressed by transfected promastigotes. Our cumulative results indicated that the N-terminal Ld3′NT/NU signal peptide translocated the various expressed proteins into the endoplasmic reticulum of transfected cells. These results are in agreement with our previous observations concerning the function of the Ld3′NT/NU signal peptide (13). A similar hydrophobic signal peptide has been deduced from the gene encoding the secreted S1-nuclease from Aspergillus oryzae (22) and must serve a similar endoplasmic reticulum-targeting function for the processing of this fungal enzyme.

Like most other members of the class I nuclease family, the Ld3′NT/NU is a glycoprotein (1, 20). This protein contains a single putative N-linked glycosylation site at asparagine residue 293 (Fig. 1A) (1). In the current study, we confirmed that this site was used for N-linked glycosylation and that the size of its glycans was ∼1–2 kDa. These results are in agreement with those of Campbell et al. (4) who showed that N-glycanase treatment of the native Ld3′NT/NU resulted in a decrease of ∼2 kDa in its apparent molecular mass. These authors also reported that such treatment of the Ld3′NT/NU resulted in the loss of its enzymatic activity. They concluded that the glycans was either essential for the enzymatic function of this protein or was necessary for its ability to remain after SDS-PAGE. In contrast, we showed that the Ld3′nt/nu*Asn→ expressed protein possessed both endogenous 3′-nucleotidase and nuclease activities. Thus, our results demonstrated that N-linked glycosylation per se was not absolutely required for the enzymatic functions of the mature Ld3′NT/NU. Whereas most other class I nuclease are also glycosylated, whether such carbohydrate side chains play any role in effecting their enzymatic activities remains to be examined. However, N-linked glycosylation could play other important roles in the intracellular processing of the Ld3′NT/NU and other class I nuclease, e.g. interactions with endoplasmic reticulum chaperons such as calreticulin or Bip (23–25). Such interactions are presumably important for the proper folding trafficking of these proteins and in targeting the Ld3′NT/NU to the parasite cell surface membrane.

In this study, using a recombinant molecular approach, we identified some of the functional domains of a unique, surface membrane-anchored, class I nuclease of a parasitic protozoan. A truncated form of this parasite cell surface protein was successfully produced by homologously transfected cells as a released/secreted active enzyme. The availability of such a soluble, extracellular, glycosylated enzyme should prove useful for future immunological and fine structural studies of this unique class I nuclease.

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