Characterization of developmentally-regulated nucleases in promastigotes and amastigotes of *Leishmania mexicana*

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(Received 16 November 1992; accepted 17 November 1992)

**Abstract:** The parasitic protozoon *Leishmania mexicana* was examined for the presence of 3'-nucleotidase/nuclease using substrate SDS-PAGE. Two activities were detected: one with an apparent molecular mass of 40 kDa, and a doublet of 29/31 kDa. The two enzymes showed differences in their levels of expression in the two life-cycle stages of parasite examined: the 29/31 kDa doublet was detected at 60-fold higher levels in the pathogenic amastigote stage, but was also expressed by promastigotes, whereas the 40 kDa activity was only detected in promastigotes. Both were capable of hydrolysing a variety of substrates including 3'-AMP and poly(A). However, the 29/31 kDa form showed a broader, unique substrate specificity in that it was also capable of digesting double-stranded RNA and DNA.

**Key words:** *Leishmania mexicana*; Nucleotidase; Nuclease

**Introduction**

Most parasitic protozoa are incapable of synthesising purines de novo and must rely on their hosts for these essential nutrients [1]. In *Leishmania* species one enzyme which has been proposed to play a role in the acquisition of purines is 3'-nucleotidase/nuclease [2,3]. This is an externally oriented ectoenzyme present in the surface membranes of the promastigote form of *L. donovani* and *L. mexicana* [4–10]. Since an equivalent activity has not been found in mammalian cells, the parasite enzyme may represent a new chemotherapeutic target. However, these previous studies have almost exclusively been conducted on promastigotes, the form of the parasite found in the sandfly vector. The occurrence and function of an equivalent activity in amastigotes, the intracellular pathogenic stage found in the mammalian host and the ultimate target of chemotherapy, are less certain. However, enzyme activity and cytochemical data suggesting a surface activity have been reported for *L. mexicana* amastigotes [7]. The aim of this study was to investigate the occurrence of 3'-nucleotidase/nuclease activity in *L. mexicana* promastigotes and amastigotes using substrate SDS-PAGE.
Materials and Methods

Parasites

*Leishmania mexicana* (WHO designation MNYC/BZ/62/M379) was maintained in female CBA mice and intracellular amastigotes isolated as previously described [11]. Promastigotes and amastigotes were axenically cultured and harvested at the late logarithmic phase of growth [12,13]. Cell pellets were used immediately or stored at −70°C.

Substrate SDS-PAGE

*Nuclease gels.* Cell pellets were lysed in 38 mM Tris/100 mM glycine pH 8.5, 1% (w/v) SDS, 50 μg ml−1 leupeptin at 5 × 10⁸–10⁹ cells/ml. The resulting lysates were then mixed with an equal volume of double strength non-reducing gel sample buffer [14]. All samples were incubated in a boiling water bath for 2 min and allowed to cool, prior to electrophoresis.

SDS-PAGE with a 10% resolving gel and discontinuous buffer system was performed using standard methods [14], but including poly(A) at 0.3 mg ml⁻¹, final concentration, in the resolving gel. After electrophoresis, gels were washed by gentle agitation in 0.1% (v/v) Triton X-100/100 mM HEPES, pH 8.5, to enable renaturation of enzyme activity, then incubated at 37°C in the same buffer for a further 1–2 h [15]. Each gel was then fixed in 7.5% (v/v) acetic acid for 10 min, washed in distilled water for 3 × 10 min, stained with 0.2% (w/v) Toluidine blue in 10 mM HEPES, pH 8.5, for 15 min, and destained with distilled water [16]. Regions of enzyme activity were revealed by digestion of poly(A) and appeared as clear bands in a dark blue staining gel.

Alternative polynucleotide substrates were used at the same concentration with the following exceptions: poly(dA) 61.6 μg ml⁻¹; poly(dT) 12.3 μg ml⁻¹; poly(dA)-poly(dT) 83.3 μg ml⁻¹; poly(dG)-poly(dC) 33.3 μg ml⁻¹. In experiments where the pH was varied, the buffer was modified to 0.1% Triton X-100 in 50 mM HEPES and 50 mM MES at appropriate pH.

*Nucleotidase gels.* The general procedure used was similar to that described above with the following modifications. Poly(A) or other polynu-

![Image of Substrate SDS-PAGE of *L. mexicana* promastigote and amastigote samples. Panel 1 shows a nuclease gel using poly(A) as a substrate and panel 2 a nucleotidase gel using 3'-AMP. Each lane was loaded with the equivalent of 10⁷ amastigotes (a) or 10⁷ promastigotes (b). The positions of molecular mass standards are indicated on the right: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa.](image-url)
cleotide substrates were usually omitted from the resolving gel, although it was possible to include these if desired, for example to process parts of the same original gel in different ways. After renaturation each gel was incubated for 1–2 h in 0.1% (v/v) Triton X-100/100 mM HEPES, pH 8.5, containing 2.5 mM 3'-AMP. Each gel was then rinsed briefly in distilled water, stained for released phosphate by incubation with Malachite green reagent (see below) for 30 min, washed and stored in distilled water. Malachite green reagent was prepared on the day of use as follows: 0.045% (w/v) Malachite green in distilled water was mixed with 4.2% (w/v) ammonium molybdate · 4H₂O in 4 M HCl, at a ratio of 3 : 1. This mixture was allowed to stand for 15 min at room temperature, then filtered through a Whatman no. 1 filter paper and Tween-20 added to 0.05% (v/v) final concentration [17]. Regions of activity were revealed by dark green staining bands on a yellow/light green background. Alternative nucleotidase substrates were used at 2.5 mM.

Densitometry. This was performed using a Hoefer GS 300 scanning densitometer and associated software (Hoefer Scientific Instruments, Newcastle-under-Lyme, Staffs., UK). Peak areas were measured using the data smoothing, vertical invert, curved baseline and manual integration functions.

Protein determinations

Protein concentrations were determined by the Pierce bicinchoninic acid method using bovine serum albumin, fraction V as a standard (BCA assay: Life Science Laboratories, Luton, UK).

Results

Analysis of promastigotes and amastigotes of *Leishmania mexicana* by substrate SDS-PAGE revealed two forms of 3'-nucleotidase/nuclease: a band of apparent molecular mass 40 kDa and a doublet of 29/31 kDa (Fig. 1). The greatest activity of both forms was shown by renaturation and incubation at pH 8.5, activity decreasing with pH until at pH 5.5 and below no activity could be detected (data not shown). In these initial experiments and all those described below, the 29 kDa and 31 kDa bands behaved in apparently identical fashion and consequently are referred to as a 29/31 kDa doublet. The 40 kDa form was only detected in promastigote samples, whereas the 29/31 kDa doublet, although detectable in promastigote samples, was found at much higher levels in amastigotes, as judged by the intensity of the bands produced (Fig. 1). The relative amounts of each enzyme form were estimated using scanning densitometry. These results indicated that amastigotes had approximately 10-fold more total nuclease activity against poly(A) than promastigotes per cell (summing all peaks). This is equivalent to a 27-fold greater relative specific activity (4.6 mg cell protein/10⁹ amastigotes and 12.2 mg/10⁹ promastigotes). The individual 29/31 kDa form was expressed at approximately 60-fold higher levels in amastigotes than promastigotes. Comparing the same samples in nucleotidase and nuclease gels, it appeared that the 40 kDa form utilised 3'-AMP as a substrate more efficiently than poly(A), whereas the 29/31 kDa form preferred poly(A) as a substrate over 3'-AMP (Fig. 1). In some experiments different parts of the same gel or different parts of the same lane were processed for nuclease and nucleotidase activity. The bands detected in each case were of identical mobility (data not shown).

The 40 kDa form exclusive to promastigotes was detected in parasites which were axenically cultured. However, the 29/31 kDa doublet, although present in promastigote samples, was expressed at higher levels in amastigotes. Since the latter were isolated from infected animals, experiments such as that illustrated in Fig. 2 were performed to confirm the parasite origin of the doublet. An equivalent activity could not be detected in mouse peritoneal exudate cells, a commonly used experimental host cell for amastigotes (Fig. 2, lane 1). Further, the pattern observed was identical in crude preparations of lesion amastigotes which contained host cells and contaminants, to that seen with purified lesion amastigotes (lacking host cells) or axenically cultured amastigotes (Fig. 2, lanes 2–4). These results demonstrated that the 29/31 kDa doublet did
The substrate specificity of the two enzyme forms are summarised in Table 1. Each substrate was tested at least twice with both a promastigote and an amastigote sample. With all the nucleotide substrates both enzymes gave identical results. However, when a variety of polynucleotides were examined in nuclease gels some differences in activity were noted. Both enzyme forms used poly(A), poly(U) and calf liver RNA as substrates, but not poly(G). However, the 29/31 kDa doublet was also capable of degrading poly(C), and double-stranded RNA in the form of poly(A)-poly(U). Further differences were found with polydeoxynucleotides. Although both the 40 kDa and 29/31 kDa forms hydrolysed poly(dT) and calf thymus single-stranded DNA, only the 29/31 kDa form used poly(dA) and various types of double-stranded DNA as substrates.

The effects of a variety of potential inhibitors in substrate SDS-PAGE are summarised in Table 2. Nuclease gels with poly(A) as substrate were incubated with a variety of potential inhibitors, all at 1 mM concentration. The results are expressed as ‘+’ if complete inhibition resulted (i.e. no activity detected), ‘+/−’ for partial inhibition, and ‘−’ for no apparent effect compared to controls.
Table 2. The properties of both enzyme forms appeared to be essentially identical. 1 mM CoCl₂ resulted in partial inhibition, which was complete when the concentration was raised to 10 mM.

Discussion

Previous studies have demonstrated the presence of nucleotidase, RNAase and DNAase activities in *L. mexicana* [7,18]. However, the apparent molecular masses of the enzymes responsible, the question of whether these activities were catalysed by the same or different enzymes and whether the individual enzymes expressed varied between different life-cycle stages were not determined. In this study application of substrate SDS-PAGE to whole cell lysates of *L. mexicana* promastigotes and amastigotes revealed the presence of two forms of 3'-nucleotidase/nuclease with apparent molecular masses of 40 kDa and 29/31 kDa.

In terms of their enzymatic properties, both enzyme forms were capable of hydrolysing, after renaturation, a variety of 3'-ribonucleotide and polynucleotide substrates, and responded similarly to a range of potential inhibitors. Some significant differences were observed, however, with the 29/31 kDa doublet being capable of hydrolysing additional polynucleotide substrates, including DNA (Table 1). The ability of an apparently single enzyme activity to hydrolyse ribonucleotides, single-stranded or double-stranded RNA and DNA is unusual, but has been reported before for some plant and fungal nucleases [19,20]. There were no substrates amongst those tested which were used exclusively by the 40 kDa form. Neither enzyme was affected by fluoride or tartrate, both phosphatase inhibitors, or ammonium molybdate which inhibits a distinct 5'-nucleotidase activity present in *L. donovani* promastigotes [9,10]. Thus, the promastigote-specific 40 kDa form is likely to be the *L. mexicana* equivalent of this surface-membrane enzyme which probably functions in purine salvage from the sandfly host.

The range of substrates used, inhibitor sensitivity, apparent molecular mass and fractionation into membrane pellets (data not shown) of the 40 kDa form, are similar to those previously reported for 3'-nucleotidase/nuclease isolated from *L. donovani* promastigotes [9,10]. Thus, the promastigote-specific 40 kDa form is likely to be the *L. mexicana* equivalent of this surface-membrane enzyme which probably functions in purine salvage from the sandfly host. Thus, the apparent absence of the surface membrane enzyme in amastigotes is a significant result, if it is corroborated by further experiments and proves to be a general feature of *Leishmania* species. The 29/31 kDa enzyme may represent a soluble homologue as it fractionated predominantly into supernatant fractions of amastigotes and promastigotes (data not shown). These considerations suggest that the role(s) played by these two enzymes in purine salvage and/or metabolism of nucleic acids are different.

The current study is the first to demonstrate 3'-nucleotidase/nuclease activity in amastigotes. The developmentally regulated expression of the different enzyme forms make these potentially useful biochemical markers for promastigotes and amastigotes. The unusual properties exhibited by the activities described in this report warrant further investigation and purification of the enzymes concerned, especially of the novel 29/31 kDa form expressed by the pathogenic amastigote stage. This will enable kinetic characterization of these enzymes, investigation of their roles in parasite biology and assessment of their chemotherapeutic potential.
Acknowledgements

I thank the Caledonian Research Foundation and the Royal Society of Edinburgh for financial support and G. Tobashnick for technical assistance.

References