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The expression of biologically active human p53 in *Leishmania* cells: a novel eukaryotic system to produce recombinant proteins

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ABSTRACT

We have investigated the use of *Leishmania* cells as a novel eukaryotic expression system for the production of recombinant protein. These cells are easy to maintain, requiring no CO_2 incubator or shaker, and can be grown in standard tissue culture media. *Leishmania* cells can be readily transfected with plasmid DNA by electroporation and transformants selected with antibiotic resistance. Recent studies have shown that it is possible to express foreign genes in *Leishmania* for the purpose of understanding the biology of this protozoan cell. In the present study we report the use of this system as a means of producing a biologically functional human p53 protein. The conformation of the p53 protein is critical for its ability to bind specific DNA sequences. It is demonstrated that *Leishmania*-synthesized human p53 is phosphorylated and can bind specifically to its enhancer DNA sequence. These data demonstrate that *Leishmania* may represent a simple eukaryotic expression system for the production of biologically active recombinant proteins.

INTRODUCTION

Various eukaryotic and prokaryotic expression systems exist for the synthesis of recombinant proteins. However, inherent problems are associated with all of the currently available systems. For example, although it is relatively easy to express recombinant proteins in prokaryotic cells and the yields can be high, the resulting proteins are often not post-translationally modified correctly and are therefore not biologically active. In comparison, although recombinant proteins expressed in the available eukaryotic systems, such as baculovirus, may be correctly processed, this involves a number of steps and requires considerable expertise before it can be used efficiently. The use of yeast expression systems falls somewhere between the baculovirus and bacterial systems in that it is easier to use than the baculovirus system, but often the proteins are not correctly post-translationally processed.

In the present study we describe a novel eukaryotic expression system using the *Leishmania* protozoan (for reviews on *Leishmania* see 1,2) to express human p53 protein. *Leishmania* cells can be routinely transfected with plasmid DNA by electroporation and the transfected cells can be selected with antibiotics. This was first demonstrated by transfecting *L.enrettii* with a plasmid containing the bacterial neomycin resistance gene (*Neo*) and selecting transformants with G418 (3). We have chosen to analyse p53 in this system because the structure of the p53 protein is critical for its biological role as a tumour suppressor (for reviews on p53 see 4,5). The major biochemical activity of p53 is to bind a specific DNA enhancer sequence resulting in transcriptional activation of specific target genes (4,5). p53 is among the most interesting molecules with respect to the development of human tumours, since as many as 50% of all human tumours express mutated p53 protein arising through missense mutations of the *p53* gene. Mutant p53 proteins are structurally different from the wild-type protein and are generally biologically inactive because they lose the ability to bind DNA. Loss of wild-type p53 activity is widely accepted as representing a significant step toward the development of tumourigenesis. Considerable interest has focused on the structure–function relationship of p53 and it therefore represents an appropriate model to evaluate a novel eukaryotic expression system.

In the present study we demonstrate that it is possible to use *Leishmania* cells to express wild-type human p53 protein which is capable of binding specifically to its corresponding DNA enhancer sequence under conditions previously established for p53 DNA binding. This demonstrates that *Leishmania* cells are capable of correctly folding this complicated higher eukaryotic protein. These observations demonstrate that *Leishmania* represents a novel eukaryotic expression system for the following reasons. First, it is simple, requiring only one cloning vector which can be transfected directly into *Leishmania* cells. Second, *Leishmania* cells proliferate rapidly in suspension and can be grown without a CO_2 incubator or shaker. Third, this system is capable of protein phosphorylation, as revealed by the phosphorylation of p53 in *Leishmania* cells. Fourth, this system is capable of expressing biologically active eukaryotic proteins. Fifth, the yield of recombinant protein in *Leishmania* cells is comparable with that obtained in *Escherichia coli.* Finally, as in the case of wild-type p53, expression of this protein inhibits proliferation

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and/or induces apoptosis of higher eukaryotic cells. However, because *Leishmania* is a lower eukaryote it does not have the biochemical pathways influenced by powerful regulatory proteins such as p53. Therefore, this system is well suited to synthesizing proteins with important pharmacological properties which may be difficult to express in other higher eukaryotic cells. These observations make *Leishmania* an attractive alternative source of biologically active recombinant proteins.

MATERIALS AND METHODS

Culture conditions and transfections

Leishmania is a protozoan which can be grown in culture in suspension at 27°C without a CO_2 incubator or shaker. Although these protozoans are normally infective through the bite of a sandfly, if they are maintained in culture for extended periods they generally become avirulent. In addition, there are a number of strains which do not infect humans. For example, *L.tarentolae* only infects lizards and it can also readily be transfected. In the present study we have used an avirulent strain (1S2D) of *L.donovani* obtained from S.Turco (University of Kentucky). This strain has been adapted to grow in axenic culture and has lost the ability to infect mice or cultured macrophages, which is their exclusive host cell.

The *Leishmania* cells were cultured in RPMI medium (RPMI 1640 supplemented with 10% fetal bovine serum, 50 mM HEPES, pH 7.3, 100 U/ml penicillin, 100 U/ml streptomycin) in closed flasks (50 or 200 ml) at 27°C. *Leishmania* cells are routinely split 1:10 once a week in a sterile hood when they reach the stationary phase of growth at about 10^8 cells/ml. The proliferation rate of *Leishmania* cells when starting from 10^6–10^7 cells/ml is ~10^7/ml/day. We routinely maintain 10 ml cultures in 50 ml flasks. *Leishmania* cells can also be frozen by resuspending a pellet from a 10 ml late log phase culture in 1 ml 10% dimethylsulphoxide, 90% fetal calf serum and placing the cells in liquid nitrogen.

The wild-type *p53* cDNA (6) was inserted into the *Bam*HI site of the pALT-neo plasmid (3) to create plasmid pLsh-p53, which is shown in Figure 1. The size of the resulting pLsh-p53 plasmid was 7.5 kb. *Leishmania* cells were transfected with the pLsh-p53 or control pALT-neo plasmids by electroporation. Plasmid DNA was prepared for electroporation on CsCl/ethidium bromide density gradients (7). However, plasmid DNA prepared by commercially available columns, such as Qiagen, works equally well. Before electroporation the plasmid DNA can be sterilized by ethanol precipitation and resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) at 1 mg/ml. Late log phase *Leishmania* cells (10^8) from an ~10–20 ml culture were harvested by centrifugation (2200 r.p.m. for 5 min; Beckman J2-MC centrifuge, JA-17 rotor) and then washed once with cold phosphate-buffered saline (PBS). Cells were then resuspended in 1 ml filter-sterilized ice-cold high ionic strength electroporation buffer (21 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose). Aliquots (0.4 ml) were added to electroporation cuvettes (0.2 cm; BioRad, Richmond, CA) and left on ice for 5 min. Plasmid DNA (10–30 μg) was added to the cell suspension in the electroporation cuvette, mixed well and electroporated at 0.45 kV at 500 μF capacitance in a BioRad Gene Pulser. Electroporated cells were placed on ice for 10 min and then transferred to 10 ml RPMI medium, described above. After 24 h G418 was added to the culture medium to a final concentration of 8 μg/ml. The culture medium containing G418 was changed once a week until the cells grew to a density of 10^4/ml. Depending on the transfection efficiency, the selection process takes between 2 and 4 weeks and no cloning was necessary, since, as demonstrated in this paper, all the surviving cells expressed the plasmid-derived gene product of interest. Following establishment of the transfected cells the G418 concentration in the culture medium was routinely increased to 200 μg/ml or higher to increase the expression level of the transfected gene product of interest, as described in Results. In the presence of lower concentrations of G418 the transfected cells proliferated at a similar rate to wild-type cells, however, at 200 μg/ml G418 or higher the growth rate of the *Leishmania* cells slows to ~7 × 10^6/ml/day.

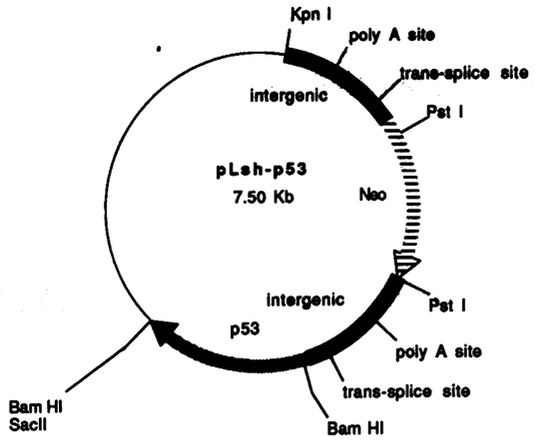

Figure 1. Map of the plasmid pLsh-p53. The *Leishmania* tubulin intergenic regions are shown as black boxes. The *Neo* gene is the striped box. The *p53* gene is the spotted box. The pBluescript plasmid sequences are shown as a line. *Trans*-splicing acceptor sites and poly(A) addition signal sites are indicated. The cloning of *p53* into this plasmid is described in the Results section. This plasmid is ~7.5 kb in length.

Northern and Southern blot analysis

Total RNA was isolated from promastigotes by the guanidine isothiocyanate method with RNAzol (Cinna/Biotec Laboratories International Inc., Friendswood, TX). Northern blot analysis of *Leishmania* RNA was carried out as previously described (8) using 10 μg RNA. For Southern blot analysis 10 μg total DNA was digested to completion with restriction enzymes (BRL) and the fragments separated on 0.7% agarose gels and transferred to nylon membranes (Hybond-N, Amersham) as previously described (8). For both the Northern and Southern blot analyses the hybridizations were carried out for 18 h at 60°C in a solution containing 1% SDS, 1 M NaCl, 10% dextran sulphate and 50 μg/ml salmon sperm DNA. The membranes were washed twice in 2× SSC for 5 min at room temperature, washed twice in 2× SSC, 1% SDS at 60°C for 30 min and autoradiographed on Kodak X-Omat films with intensifying screens. The DNA probe was a human *p53* cDNA 2.0 kb *Bam*HI fragment (6) which was purified following restriction enzyme digestion and agarose gel electrophoresis.

Western blot analysis of p53 synthesized in *Leishmania* and *E.coli*

Approximately 10^8 Leishmania cells were collected by centrifugation, washed in PBS and lysed in 200 µl lysis buffer [0.25 M Tris–HCl, pH 8.0, 0.5% Triton X-100, 1 mM phenylmethylsulphonyl flouride (PMSF)] for 10 min on ice. Lysed cells were centrifuged at 14 000 r.p.m. for 10 min in a microcentrifuge and the protein concentration determined in the supernatant. Samples were then mixed with SDS–PAGE sample buffer and subjected to Western blot analysis as previously described (8). *Escherichia coli* (DH5α) transformed with plasmid pUR-291.5 (9) (which expresses a p53–β-galactosidase fusion protein) were cultured overnight in LB media containing 50 µg/ml ampicillin and 100 µg/ml IPTG. Cells were collected by centrifugation, washed in PBS and resuspended in the same lysis buffer (0.25 M Tris–HCl, pH 8.0, 0.5% Triton X-100, 1 mM PMSF), sonicated and then the protein concentration determined. Samples mixed with SDS–PAGE sample buffer were boiled for 3 min, then loaded onto the gel and subjected to SDS–PAGE followed by Western blot analysis using monoclonal antibodies PAb1801 and PAb1802, which are specific for human p53, as previously described (10). In some instances, as a positive control for human p53 protein, SV40-transformed human fibroblast cells, VA13 or HT1080 human fibrosarcoma cells (obtained from ATCC) were used. These cells have high levels of p53 and in the case of VA13 cells they contain the SV40 large T protein which stabilizes p53. The VA13 cells were lysed and the p53 protein concentrated by immunoprecipitation as previously described (9,10), then subjected to Western blot analysis as described above.

DNA binding assay

DNA binding assays were carried out as previously described for measuring p53 DNA binding activity in human cells (11–13). *Leishmania* cells (200 ml) grown to late log phase were harvested, washed in PBS and then resuspended in 0.6 ml cold lysis buffer [20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton-X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 100 µg/ml aprotinin]. The lysed cells were centrifuged for 5 min at 2000 r.p.m. and 4°C and the pellet containing the nuclei were resuspended in 200 µl nuclear extraction buffer (identical to the lysis buffer with 500 mM NaCl). Nuclei were gently rocked for 1 h at 4°C and then submitted to centrifugation at 15 000 r.p.m. for 10 min and the supernatant containing the nuclear extract was aliquoted and stored at –70°C.

For the DNA binding assays 6 µl nuclear extract (10 µg protein) was mixed with 1 µg sonicated salmon sperm DNA and 14 µl binding buffer (100 mM NaCl, 20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM DTT, 1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 0.1% Triton X-100, 20% glycerol). Following incubation for 10 min at room temperature 5 ng ^{32}P end-labelled double-stranded p53 target oligonucleotide (5′-GGGCATGTCCGGGCATGTCC-3′) was added and incubated at room temperature for an additional 20 min. In some instances, as indicated in the text, competing amounts of unlabelled oligonucleotide (50–250 ng unlabelled competitor p53 oligonucleotide DNA or 200 ng unlabelled AP1 enhancer oligonucleotide as a non-competitive control) was also included. For the supershift experiments 0.5 µg purified Mab PAb 421 (Oncogene Science) was added to the nuclear extract prior to addition of the salmon sperm DNA and the binding buffer in order to stimulate p53 binding, as previously described (11). As a positive control for DNA binding human fibrosarcoma HT1080 cells, which contain relatively high levels of wild-type p53, were used as previously described (13). DNA–protein complexes were subjected to electrophoresis at 140 V on 4% polyacrylamide gels in 1× TBE buffer (90 mM Tris–borate, 2 mM EDTA) at 4°C, followed by autoradiography.

Indirect immunofluorescence

Promastigotes containing plasmid pLsh-p53 or pALT-neo were fixed and stained for immunofluorescence as follows. Cells were spread onto acid-washed slides, fixed with methanol and then pre-incubated at room temperature for 45 min in PBS containing 10% fetal calf serum (FCS) and 0.1% Triton X-100. Subsequent incubations were carried out in a humidified chamber with antibodies diluted in PBS containing 3% FCS and 0.1% Triton X-100. A cocktail of anti-p53 monoclonal antibodies PAb 1801, 1802 and 122 was used. The slides were incubated with primary antibody overnight at 4°C and washed four times in PBS containing 0.1% Triton X-100. Slides were then incubated for 4 h at room temperature with fluoresceinated goat anti-mouse IgG–FITC conjugate (Sigma) diluted 1:100. Slides were washed twice in PBS containing 0.1% Triton X-100 and four times in PBS alone. Slides were then counterstained in Evans blue, mounted, viewed under a fluorescent microscope and photographed.

Immunoprecipitation analysis of phosphorylated p53

Late log stage *Leishmania* cells (50 ml) were harvested by centrifugation, washed once in labelling medium, 1× RPMI medium 1640 without L-glutamine or sodium phosphate (Gibco BRL catalogue no. 11877-024), 20 mM HEPES, pH 7.3, 0.1% fetal bovine serum. Cells were then resuspended in 2.5 ml labelling medium followed by addition of 2.5 mCi ^{32}P and incubated for 4 h at room temperature. p53 was then subjected to immunoprecipitation essentially as described previously for human cells (9,10). Labelled cells were harvested by centrifugation, washed once in PBS and resuspended in 1.0 ml NP-40 lysis buffer (1.0% v/v NP-40, 150 mM NaCl, 20 mM Tris–HCl, pH 8.0) and left on ice for 30 min. The cell debris was removed by centrifugation and 1 µg anti-p53 Mab PAb 421 and 40 µl 10% v/v protein A–agarose beads were added to the remaining supernatant and allowed to rotate at 4°C for 1 h. The immunoprecipitates were collected by centrifugation and washed four times with 1 ml NET/gel buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.05% NP-40, 0.02% NaN_3, 0.25% gelatin). Samples were then resuspended in SDS–PAGE sample buffer and subjected to electrophoresis and autoradiography as previously described (9,10). As a control for the migration of p53 in this analysis p53 was also labelled with [^{35}S]methionine in an *in vitro* transcription/translation system as previously described (14).

RESULTS

p53 expression vector

A human *p53* 2.0 kb cDNA containing the entire coding sequence (6) was ligated into the *Bam*HI site downstream of the *Leishmania* tubulin gene intergenic sequence in the pALT-neo plasmid, which has been previously described (3). The p53-expressing plasmid (designated pLsh-p53) is shown in Figure 1. Unlike higher eukaryotic cells, there is currently no conclusive evidence

that *Leishmania* genes are under the control of specific promoter and enhancer sequences. In fact, the upstream α-tubulin intergenic sequence in vector pALT-neo can be substituted by non-*Leishmania* sequences containing a polypyrimidine tract followed by a *trans*-splice acceptor site, such as the AG dinucleotide (M.Ouellette personal communication; 15) and this would still result in expression of the downstream gene. The α-tubulin intergenic regions function largely through providing *trans*-splicing acceptor sites and poly(A) addition signals, rather than containing specific promoter sequences. In practical terms, the pyrimidine tract/AG sequence can be considered as an appropriate upstream sequence for the expression of foreign genes in *Leishmania* cells and these sequences are provided by the α-tubulin intergenic regions, as indicated in the vector shown in Figure 1. More detailed descriptions of gene expression in trypanosomatid protozoans can be found in Agabian, Clayton and LeBowitz *et al.* (16–18).

Transfection, selection and expression of the *p53* gene in *Leishmania* cells

The pLsh-p53 plasmid was transfected into *Leishmania* cells by electroporation and transfected cells were placed directly into media containing G418 as described in Materials and Methods. In this manner transfected cells were grown as a pooled population and this process takes of the order of 2–4 weeks, at which time there are ~10^6 G418-resistant cells/ml culture. We have observed that there is no need to plate out cells and select individual clones, since the G418-resistant population of cells consistently contain and express the transfected gene. This approach makes the development of transformed *Leishmania* cells simple and efficient. The presence of the transfected *p53* gene in the G418-resistant population of cells is demonstrated by Southern blot analysis (Fig. 2A). Digestion with *Pst*I resulted in a major *p53* hybridizing fragment of 6.7 kb. Digestion with *Sac*II (single cutter for plasmid pLsh-p53) resulted in a major fragment of 7.5 kb and, as expected, a 2.0 kb *p53* sequence hybridizing band was observed when the DNA was digested with *Bam*HI (Fig. 2A). Similarly, hybridization with a *Neo*-specific probe resulted in a 7.5 kb *Sac*II band and a 5.5 kb *Bam*HI band (data not shown). These data argue that the pLsh-p53 plasmid is episomal in the transfected cells and this is consistent with a previous report (3) showing that this plasmid remains episomal in transfected cells.

Northern blot analysis verified that the human *p53* gene was transcribed in the transfected *Leishmania* cells. As shown in Figure 2B, the human *p53* gene was transcribed at a high level in these cells, whereas there was no hybridization signal in the control G418-resistant cells containing the same plasmid without the *p53* gene. This confirmed that the human *p53* gene was expressed in the relevant *Leishmania* cells. A major band at 5.5 kb and two minor bands at ~2.8 and 7.0 kb were detected with the *p53* probe from total RNA isolated from these cells. These data are consistent with the polyadenylation occurring within the *Leishmania* tubulin intergenic sequences to yield the two larger mRNA species and also within the plasmid sequence to yield the smaller mRNA species.

Detection of the p53 protein in *Leishmania* cells

Western blot analysis was performed to determine whether human p53 protein was present in the transfected cells. As shown

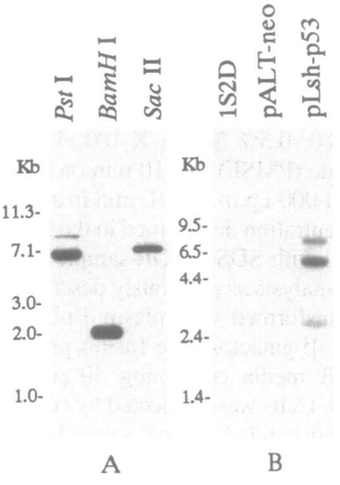

Figure 2. (**A**) Southern blot analysis of the human *p53* gene in *Leishmania* cells transfected with the pLsh-p53 plasmid. DNA (10 μg) was digested with *Bam*HI, *Pst*I and *Sac*II as indicated in each lane. The *p53* probe used was a 2.0 kb *Bam*HI cDNA fragment. The Southern blot was carried out as described in Materials and Methods. (**B**) Northern blot analysis of human *p53* mRNA in *Leishmania* cells. RNA (12 μg) was present in each lane from: control untransfected cells, 1S2D; control cells transfected with the pALT-neo plasmid containing no *p53* sequences; cells transfected with the p53-expressing plasmid pLsh-p53. The *p53* probe used was a 2.0 kb *Bam*HI cDNA fragment. Note that only cells transfected with plasmid pLsh-p53 contained *p53* RNA. The Northern blot was carried out as described in Materials and Methods.

in Figure 3, two different anti-p53 monoclonal antibodies, PAb1802 and PAb1801, specifically recognized a full-length p53 of the correct molecular weight in the *Leishmania* cells, as determined by comparing *Leishmania*-derived p53 with the p53 protein present in human fibroblast cells (VA13). There are also truncated p53 proteins present in the *Leishmania* cells and these could represent breakdown products from the full-length p53 protein or internal initiation products. However, since Mab PAb 1802 reacts against an epitope located close to the C-terminal region of p53 (10) and this epitope is present in the smaller band at 45 kDa, this may argue that the major shorter 45 kDa product represents initiation of translation at the second methionine located 40 amino acids from the initiation methionine. There was no detectable p53 protein present in the control cells transfected with the pALT-Neo plasmid and selected in G418. These Western blot data confirm that p53 protein of the same molecular weight as the full-length protein was expressed in the *Leishmania* cells.

Increased p53 protein levels by increasing selection for G418 resistance

Since the pLsh-p53 plasmid contained both the neomycin resistant gene and human *p53* cDNA, we attempted to increase the p53 level in *Leishmania* cells by increasing the concentration of G418 in the culture media. The pLsh-p53-transfected cells originally selected in 8 μg/ml G418 were transferred to G418-free medium for 3 days. These cells were then placed in cultures containing increased amounts of G418 ranging from 0 to 500 μg/ml for 6 days. At the end of the 6 day period the cells were harvested by centrifugation, washed in PBS and lysed as described in Materials and Methods. The protein concentration present in the supernatants derived from the lysates was determined and 10 μg protein from each sample was analysed by

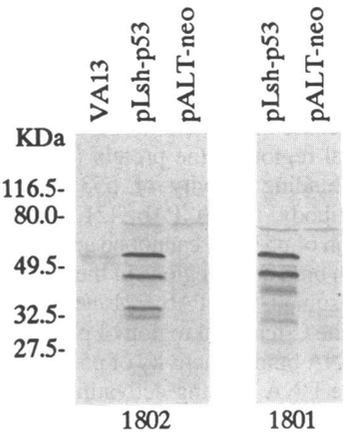

Figure 3. Western blot analysis of human p53 protein in *Leishmania* cells. (Left) The following cell lysates were subjected to Western blot analysis with anti-p53 monoclonal antibody PAb1802: VA13, p53-containing human fibroblast cells; pLsh-p53, *Leishmania* cells transfected with p53-expressing plasmid pLsh-p53; pALT-neo, *Leishmania* cells transfected with the control *Neo* plasmid with no *p53* sequences. (Right) A comparison of p53 reactivity in pLsh-p53 cells using a second monoclonal antibody (PAb1801) against a different epitope on human p53. Note that the two monoclonal antibodies recognize similar p53 proteins which are not present in control cells, confirming the identity of the major bands as p53.

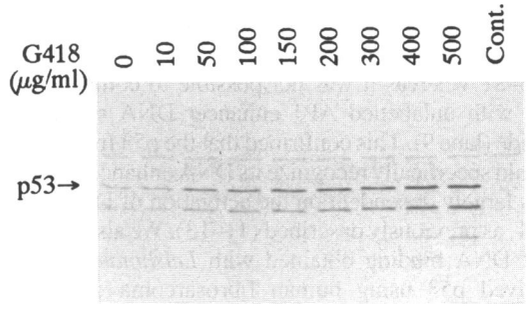

Figure 4. Induction of increased p53 protein levels by selection in higher concentrations of G418. Cells selected in 8 μg/ml G418 were placed in medium minus G418 for 72 h and then placed back into medium containing the indicated amounts of G418 for 6 days. Equal amounts of protein were then isolated from each culture and subjected to Western blot analysis as described in Materials and Methods. Note that no p53 protein was present in the control lane (Cont.), which contained protein derived from control transfected and selected *Leishmania* cells.

Western blot analysis with anti-p53 monoclonal antibody PAb1802. As shown in Figure 4, there was an increase in p53 production associated with increasing amounts of G418 in the culture medium. These data demonstrate that increasing the selective pressure for the neomycin resistance gene product derived from plasmid pLsh-p53 also resulted in an increased level of *p53* gene product from this plasmid and that it is therefore possible to increase the relative amount of recombinant protein production in this system. However, we have also observed that it was difficult to go beyond 500 μg/ml, as this becomes very toxic to the cells. Perhaps it will be possible to go beyond the 500 μg/ml G418 concentration with a more gradual increase in G418 concentration over a longer period of time and this requires further investigation.

Comparison of p53 protein levels synthesized in *E.coli* and *Leishmania*

The most convenient method for producing recombinant protein is to do so using prokaryotic expression systems involving bacteria. The most attractive feature of such prokaryotic systems is their simplicity and high yield. As outlined in the above experiments, the use of the *Leishmania* system is as simple as the use of bacterial systems in so far as both systems use a single episomal replicating plasmid which contains the gene of interest as well as the antibiotic selection gene. Furthermore, the transfection and selection procedure for *Leishmania* is also simple and does not require cloning of individual colonies. One issue is, however, whether *Leishmania* cells can produce comparable levels of recombinant protein to bacterial systems and therefore we compared these two systems directly.

Previously we have expressed human p53 as a fusion protein with β-galactosidase in *E.coli* using a pUR-291 plasmid which contained the strong *lacZ* promoter (9). A fusion protein with β-galactosidase was made in order to stabilize the p53 protein in *E.coli* and this fusion protein was then used in antigenic studies of the p53 protein. Using the same p53 expression plasmid we compared the level of p53–β-galactosidase fusion protein synthesized in *E.coli* DH5α (cultured overnight in the presence of 50 μg/ml ampicillin and 100 μg/ml IPTG to induce expression from the *lacZ* promoter) to the amount of p53 synthesized in *Leishmania* selected in 500 μg/ml G418 as described above. *Escherichia coli* and *Leishmania* cells were resuspended in lysis buffer as described in Materials and Methods and then sonicated. The protein concentration in the resulting extracts was measured and equal amounts of total protein were then subjected to Western blot analysis with monoclonal antibody PAb 1801. As shown in Figure 5, there were comparable amounts of p53 protein detected in the *Leishmania* and *E.coli* lysates. At the lowest concentration of protein analysed, 1.5 μg, the signal for full-length p53 was somewhat weaker from the *Leishmania* cells, yet this was still of the same order of magnitude as that observed from the *E.coli* cells. It was not possible to distinguish p53 by staining total protein in *Leishmania* because of the abundant amount of endogenous protein of similar molecular weight to p53. These data do, however, indicate that *Leishmania* cells do produce significant levels of recombinant protein when compared with *E.coli.*

Phosphorylation of p53 in *Leishmania* cells

The inability of bacteria to carry out post-translational modifications, such as glycosylation and phosphorylation, is a major drawback, since many eukaryotic proteins require such modifications to be biologically active. It has been reported that p53 is a substrate for casein kinase II and there is evidence that phosphorylation is required for p53 DNA binding activity (12). Since p53 is a phosphoprotein, it was of interest to determine whether *Leishmania* cells could phosphorylate human p53. *Leishmania* cells containing plasmid pLsh-p53 were cultured in the presence of ^{32}P and then the p53 was subjected to immunoprecipitation analysis followed by autoradiography. As shown in Figure 6, both the full-length and the truncated p53 protein species were phosphorylated in the *Leishmania* cells under these conditions. These data demonstrate that *Leishmania* cells are able to carry out phosphorylation of recombinant proteins.

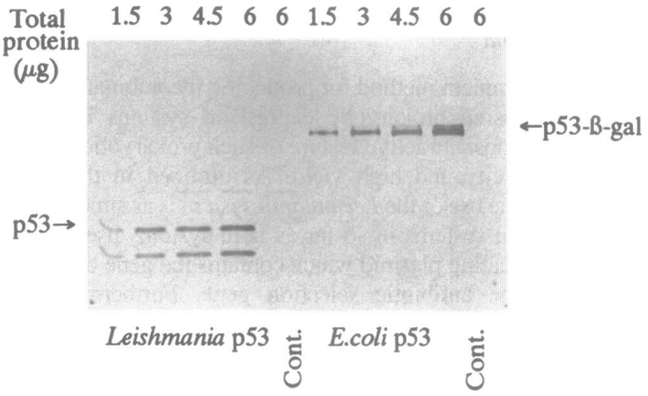

Figure 5. Comparison of the level of p53 synthesized in *Leishmania* and *E.coli*. *Leishmania* cells selected in 500 μg/ml G418 expressing p53 protein and *E.coli* DH5α expressing p53–β-galactosidase fusion protein were harvested and the indicated amount of protein loaded onto each lane and subjected to Western blot analysis as described in Materials and Methods. Note that no p53 protein was present in the control lanes (Cont.), which contained 6.0 μg protein derived from control *Leishmania* and *E.coli* cells.

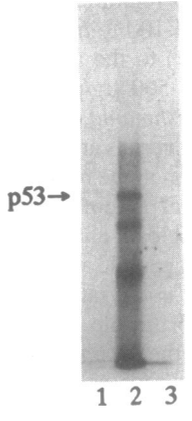

Figure 6. Phosphorylation of p53 in *Leishmania* cells. p53-expressing *Leishmania* cells (lane 2) and control *Leishmania* cells (lane 3) were cultured in the presence of ^{32}P-labelled inorganic phosphate and subjected to immunoprecipitation analysis and autoradiography as described in Materials and Methods. Lane 1 contains ^{35}S-labelled p53 protein synthesized in an *in vitro* transcription/translation system used here as a control to show the migration of authentic p53.

Functional analysis of the p53 protein synthesized in *Leishmania*

The major biochemical activity of p53 is its ability to act as a transcriptional transactivator and this is accomplished through p53 binding to specific enhancer DNA sequences. Furthermore, p53 must be in the wild-type conformation for it to bind its enhancer DNA sequence, since p53 proteins with point mutations lose their ability to bind DNA and activate transcription. Previous results presented in this study demonstrated that the p53 protein synthesized in *Leishmania* was of the correct molecular weight, was soluble in mild detergent and was phosphorylated. It was therefore of interest to determine whether *Leishmania*-derived recombinant p53 retained the ability to fold into the wild-type conformation, as determined by its ability to specifically bind to its enhancer DNA sequence under conditions previously described for p53 DNA binding. Previous studies have shown that the DNA binding activity of p53 derived from mammalian cells is very weak, because of a potential negative regulatory element in the C-terminal region of the protein (11–13). However, the specific DNA binding activity of p53 can be activated by monoclonal antibody (Mab) PAb 421, which binds to the C-terminal region of p53, but cannot be activated by monoclonal antibodies which bind other regions of the p53 protein (11,12). It is currently presumed that PAb 421 neutralizes the negative element within the C-terminal region of p53 (11–13). Therefore, to analyse the DNA binding activity of p53, DNA binding assays must include the DNA binding activating Mab PAb 421. This provides a very specific means by which to demonstrate specific p53 DNA binding. As shown in Figure 7, the lysate derived from the pLsh-p53 plasmid-containing cells demonstrated poor DNA binding activity (lane 1). However, when Mab PAb 421 was added to the p53-containing lysate it stimulated p53 binding to its target DNA (lane 2), whereas a control anti-p53 Mab PAb 1620 (which binds to the central region of p53) did not stimulate p53 binding to its target DNA (lane 10). Note that the p53 protein–PAb 421 antibody–DNA complex runs close to the top of the gel just under the well, but can be clearly seen as a large band. In contrast, there was no binding activity in lysates of control *Leishmania* cells which did not contain p53 (lanes 3), even when Mab PAb 421 was present (lane 4). It was possible to compete away p53 binding to the labelled enhancer DNA sequence with increasing excess amounts of unlabelled p53 enhancer DNA (lanes 6–8), whereas it was not possible to compete p53 DNA binding with unlabelled AP1 enhancer DNA sequence oligonucleotide (lane 9). This confirmed that the p53 from *Leishmania* cells could specifically recognize its DNA enhancer sequence and this was largely dependent on the activation of DNA binding by PAb 421, as previously described (11–13). We also compared the level of DNA binding obtained with *Leishmania*- and human cell-derived p53 using human fibrosarcoma cells (HT1080) which contain high levels of wild-type p53 (13). As shown in Fig. 7, lane 14, there was only a small amount of p53 binding activity stimulated by Mab PAb 421 observed in the human cell-derived lysate as compared with the p53 binding activity derived from *Leishmania* cells (lanes 2 and 5). Note that in human HT1080 cells there is a lower molecular weight band which occurs in the presence or absence of Mab PAb 421 and this is consistent with previous such analyses with these cells. The nature of this non-specific binding from HT1080 cell lysates is not known and is not p53 dependent. Taken together, these data reveal that the p53 synthesized in *Leishmania* cells was biochemically active with respect to recognizing its target DNA sequence and behaved exactly as did p53 derived from human cells with respect to its DNA binding activity being stimulated by Mab PAb 421. Clearly there was much more active p53 in the engineered *Leishmania* cells than in the human cells.

Cellular location of human p53 in *Leishmania* cells

The transfection and selection procedure used involved mass culturing of pooled G418-resistant cells and individual clones were not selected, as described in Materials and Methods. Therefore, It was necessary to determine what percentage of the transfected and selected cells expressed the human p53 protein.

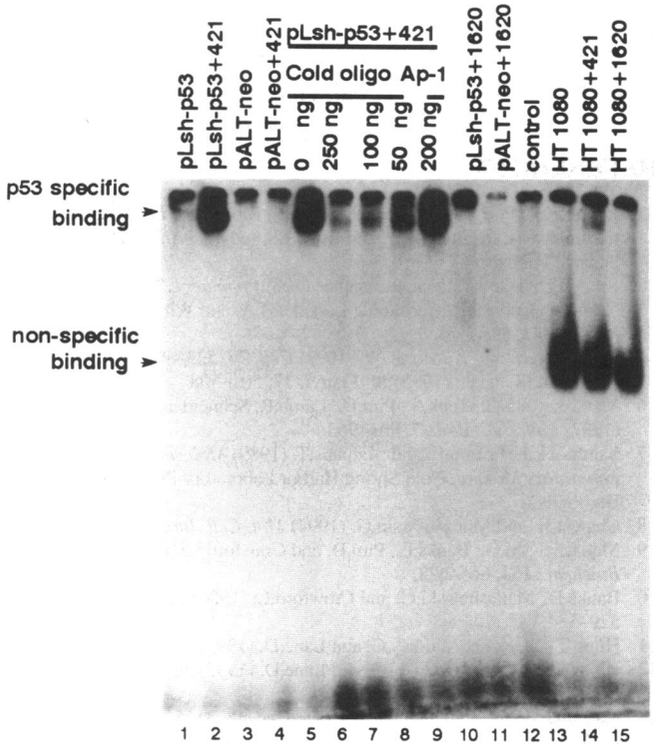

Figure 7. DNA binding activity of p53 synthesized in *Leishmania* and control human fibrosarcoma HT1080 cells. p53 binding activity to its target enhancer sequence was determined from lysates derived from: lane 1, p53 (+) *Leishmania* cells; lane 2, p53 (+) *Leishmania* cells in the presence of Mab PAb 421; lane 3, control p53 (–) *Leishmania* cells; lane 4, control p53 (–) cells in the presence of Mab PAb 421; lane 5, as lane 2; lane 6, as lane 2 plus 250 ng cold competitor p53 enhancer DNA; lane 7, as lane 2 plus 100 ng cold competitor p53 enhancer DNA; lane 8, as lane 2 plus 50 ng cold competitor p53 enhancer DNA; lane 9, as lane 2 plus 200 ng AP1 non-specific control enhancer DNA; lane 10, p53 (+) *Leishmania* cells in the presence of control Mab PAb 1620; lane 11, control p53 (–) *Leishmania* cells in the presence of control Mab PAb 1620; lane 12, labelled p53 enhancer oligonucleotide in the absence of any cell lysate; lane 13, wild-type p53 (+) human HT1080 cell lysate; lane 14, wild-type p53 (+) human HT1080 cell lysate in the presence of Mab PAb 421; lane 15, wild-type p53 (+) human HT1080 cell lysate in the presence of control Mab PAb 1620. Note that the lower band in lanes 13–15 represents non-specific binding as previously demonstrated for HT1080 cells (see 13). Note that for both *Leishmania*- (lanes 2 and 5–9) and human cell-derived p53 (lane 14) Mab PAb 421 specifically stimulated p53 binding to its enhancer sequence as previously described (11–13). This demonstrated that *Leishmania*-derived p53 behaves exactly as does human cell-derived p53.

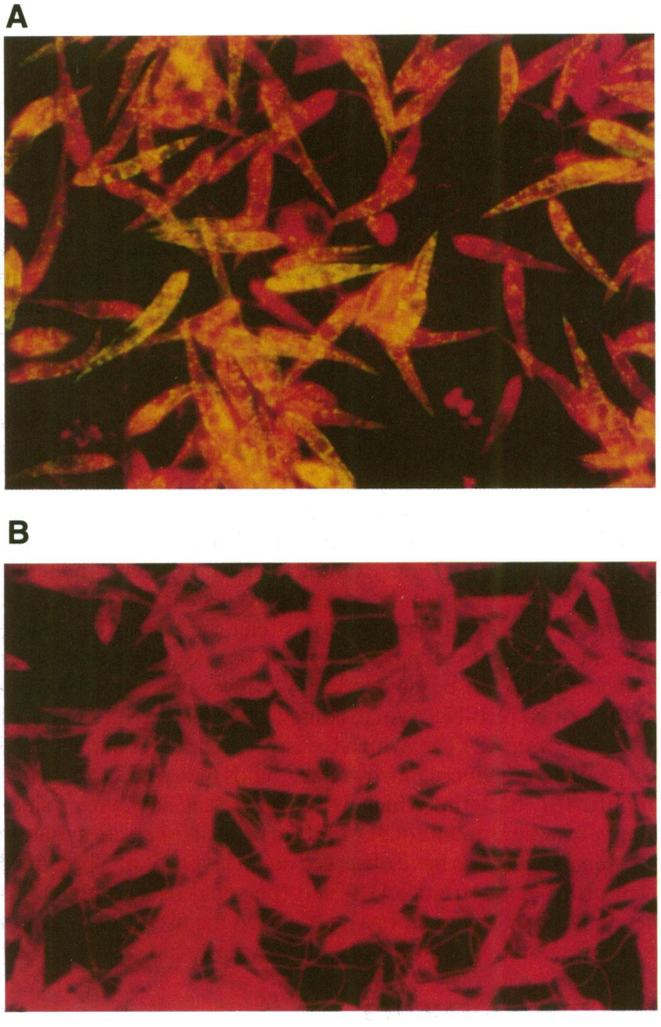

Figure 8. Immunofluorescent localization of p53 in transfected *Leishmania* cells. pLsh-p53- and control pALTneo-transfected cells were stained with a cocktail of anti-p53 monoclonal antibodies PAb 1801, PAb 1802 and PAb 122. (**A**) pLsh-p53-transfected cells. (**B**) Control pALT-neo-transfected cells. Magnification 1000×. Note that the positive green/yellow staining in (A) shows that p53 is present throughout the cytoplasm and no p53 was present in the control transfected cells (B) stained with the same cocktail of anti-p53 antibodies.

As shown in Figure 8, p53 was present in all of the cells at approximately equal levels. This demonstrates that this protocol results in virtually 100% of the cells expressing the recombinant protein. These results also demonstrate that p53 was largely present throughout the cytoplasm of the cell. These analyses, however, do not rule out the possibility that there is also some p53 within the nucleus, as in higher eukaryotic cells. The presence of p53 within the nucleus would also be consistent with the DNA binding assays, which were performed with crude nuclear preparations. However, because of the crude nature of the isolation of nuclei, it was likely that these preparations also contained cytoplasm-derived p53.

DISCUSSION

This study was undertaken to determine whether *Leishmania* represents a potentially useful eukaryotic cell for the development of an expression system for the production of biologically active recombinant proteins. The data obtained in this study show that *Leishmania* does have a number of features which make it an attractive expression system. These include the simplicity of the *Leishmania* system, including its culture requirements, plasmid vector construction and the ease of transfection and selection of these cells. There is no need for a homologous recombination step, as with a number of other eukaryotic expression systems. We also present evidence that *Leishmania* cells produce abundant levels of recombinant protein, which in the case of p53 was phosphorylated and was biologically active with respect to DNA binding. Although we have not investigated the ability of

Leishmania to glycosylate recombinant proteins, it is likely that glycosylation will occur, since *Leishmania* protozoans are rich in glycoproteins. These observations demonstrate that the *Leishmania* system combines many of the advantages of both prokaryotic and eukaryotic expression systems.

Previous studies have expressed foreign proteins in *Leishmania* in order to understand the biology of this protozoan cell. These studies include the examination of drug resistance in *Leishmania*, (for examples 19,20), surface antigen expression in *Leishmania* (21), virulence genes in *Leishmania* (22), gene targetting in *Leishmania* (23) and the biology of *Leishmania* infection on its only host cell, the macrophage (24). These and other studies have established the technology of plasmid transfection, gene targetting through homologous recombination and selection of transfected *Leishmania* cells. The present study has revealed that *Leishmania* can also be considered in the context of a novel means of synthesizing biologically active eukaryotic proteins.

Because of the potential of this expression system, further modifications beyond what is described in this study can and should be pursued. For example, more efficient inducible expression vectors can be developed, leader sequences for secretion of recombinant proteins can be identified, more efficient strains for expression could be identified and better defined media can be developed. We have now constructed a vector termed pLP-Neo which contains the 5′ upstream region of the *L.donovani* A2 gene, which we have previously characterized (8). pLP-Neo produces much greater yields than pALT-Neo when amplified in bacteria, which is an important advantage, since relatively large amounts of plasmid are needed for electroporation into *Leishmania*. We have observed equal levels of p53 produced from pLP-Neo as from pALT-Neo (personal observations). As these and other optimizations are realized the use of *Leishmania* as an expression system may become widespread.

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