Involvement of *Leishmania donovani* major surface glycoprotein gp63 in promastigote multiplication

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The major surface glycoprotein gp63 of the kinetoplastid protozoal parasite *Leishmania* is implicated as a ligand mediating uptake of the parasite into, and survival within, the host macrophage. By expressing gp63 antisense RNA from an episomal vector in *L. donovani* promastigotes, gp63-deficient transfectants were obtained. Reduction of the gp63 level resulted in increased generation times, altered cell morphology, accumulation of cells in the G2/M phase of the cell cycle, and increased numbers of binucleate cells with one or two kinetoplasts. Growth was stimulated, and the number of binucleate cells reduced, by addition to the culture of a bacterially expressed fusion protein containing the fibronectin-like SRYD motif and the zinc-binding (metalloprotease) domain of gp63. These observations support an additional role of gp63 in promastigote multiplication; the fibronectin-like properties of gp63 may be important in this process.

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1. Introduction

The major surface glycoprotein gp63 is a zinc-requiring ectoprotease which is anchored on the surface of kinetoplastid protozoal parasites of the genus *Leishmania*. It is encoded by multiple genes which are clustered on a 650 kb chromosome (Button *et al* 1989; Ghosh *et al* 1998). In *L. major* and *L. chagasi*, there is evidence for differential expression of these genes during the life cycle: some are highly expressed in log-phase promastigotes, while others are expressed in stationary phase promastigotes, and in amastigotes (Ramamoorthy *et al* 1992). The molecular determinants of protease activity, N-glycosylation, glycophosphatidylinositol – anchoring, and membrane targeting have been identified on the protein by site – directed mutagenesis (McGwire and Chang 1996).

Early experiments with gp63-deficient variants of *L. amazonensis*, obtained after long-term culture, suggested a role of gp63 in the binding and survival of promastigotes within host macrophages (Liu and Chang 1992). Macrophage-binding could be mediated in two ways: (i) indirectly, through the complement component C3bi, which, after being generated from C3b by gp63 protease, attaches to the complement receptor on macrophage (Brittingham *et al* 1995); here C3bi acts as a bridge between gp63-bearing promastigotes and the macrophage; and (ii) gp63 binds directly to an integrin receptor on the macrophage. gp63 has been shown to have fibronectin-like properties: it contains the SRYD motif which is antigenically similar to the RGDS region of fibronectin (Soteriadou *et al* 1992), and gp63 on promastigotes mediates binding to mammalian cells expressing $\alpha 4/\beta 1$ fibronectin receptors

Keywords. Antisense; cytokinesis; gp63; *Leishmania*

Abbreviations used: AS, Antisense; FACS, fluorescence activated cell sorter; CR, coding region; GP, glycoprotein; GST, glutathione S-transferase; HEPES, hydroxyethyl piperazine ethanesulphonic acid; PARP, procyclic acidic repetitive protein; IR, intergenic region.

(Brittingham *et al* 1999). *L. amazonensis* gp63-deficient promastigotes generated by antisense RNA expression showed reduced macrophage binding and intramacrophage survival (Chen *et al* 2000). In contrast, deletion of 6 of the 7 genes of *L. major* did not affect infection, or intracellular survival within, macrophages *in vitro*, infectivity in mice, or parasite development within sandfly vectors (Joshi *et al* 1998). The gp63 deletion mutant is more sensitive to complement-mediated lysis, leading to the suggestion that gp63, through proteolytic action on C3b, has a protective effect against complement attack in the mammalian host (Joshi *et al* 1998). The differences in the results of gp63 depletion in the two species may reflect differences either in experimental protocol, or in genuine inter-species variability caused, for example, by the different numbers and types of gp63 genes. No information is yet available on the effect of gp63 depletion in viscerotropic species such as *L. donovani*. Moreover, the possibility that this conserved and abundant surface protein plays any role in the normal physiology of the protozoan quite apart from its interaction with the host, has not yet been given serious consideration.

In this study, we have used the antisense approach to generate gp63-deficient *L. donovani*. The transformants show multiple abnormalities in cell growth and division, which are partially reversed by administration of a bacterially-expressed fragment of gp63. These experiments reveal an unexpected role of gp63 in the cell cycle of *L. donovani*.

2. Materials and methods

2.1 *Cell culture*

Promastigotes of *L. donovani* strain Ag83 were passaged in Schneider's Drosophila medium or M199 containing 10% fetal bovine serum (FBS), 100 μM adenine, 3·6 μg/ml biopterin, 20 μg/ml hemin at 22°C. Hygromycin–resistant variants were selected and maintained in the presence of 16–20 μg/ml hygromycin B.

2.2 *Molecular cloning of antisense construct*

A 3·0 kb genomic *Xba*1 fragment, containing the gp63 coding sequence plus intergenic region on the 5′-side, was cloned into pBluescript vector to yield pGP63-2 (figure 1A). This plasmid contains 3 *Sal*I sites: (i) at the 5′ end of the coding region; (ii) about 400 bp downstream; and (iii) within the polylinker at the 3′ end. By partial digestion with *Sal*I, followed by religation, clone pGP63-2′ was selected in which the 1·45 kb internal *Sal*I fragment was ligated in the inverse orientation to the remainder of the gp63 sequence (figure 1B). This plasmid was digested with *Sac*I, and the 2 kb fragment containing the gp63 intergenic region, 400 bp of the coding region (sense orientation), followed by 590 bp of the C–terminal region (antisense orientation) was cloned into the *Sac*I site of plasmid pRH2, an episomal vector (figure 1C), to yield the antisense plasmid pGP63-AS2 (figure 1D). Standard recombinant DNA procedures (Maniatis *et al* 1989) were used.

2.3 *Transfection*

Electroporation was carried out according to the method of Beverley (Kapler *et al* 1990). Log-phase *L. donovani* strain Ag83 promastigotes were washed, suspended in HEPES–buffered saline (HBS) at 1×10^8 cells/ml, 20 μg/ml supercoiled plasmid DNA was added, and 0·4 ml of the mixture was electroporated in a BioRad Gene Pulser apparatus set at 500 μF (capacitance) and 450 V (voltage) (time constant about 4 ms) using a 0·2 cm-gap cuvette. The electroporated cells were kept on ice for 10 min. Culture medium (10 ml) without antibiotics was added and the cells incubated overnight at 22°C. The cells were then passaged 3–4 times in medium containing 20 μg/ml hygromycin B before cloning by limiting dilution in 96-well microtiter plates.

2.4 *Blot hybridization*

Genomic DNA was prepared from promastigotes, digested with restriction enzymes, electrophoresed, transferred to a nylon membrane (Hybond N, Amersham Pharmacia Biotech, UK) and hybridized with [^{32}P]-labelled probes, according to standard procedures (Maniatis *et al* 1989). Total RNA was prepared by guanidine isothiocyanate extraction (Chomczynski and Sacchi 1987), and contaminating DNA was removed by digestion with RNase–free RQ1 DNase (Promega). Glyoxal–denatured RNA was electrophoresed and blotted as described (Maniatis *et al* 1989).

2.1 *Western blotting*

Cells (3×10^7) were suspended in phosphate–buffered saline (PBS) and lysed by sonication. The clarified extract (20 μl) was subjected to SDS–10% PAGE and blotted to nitrocellulose. The blot was blocked, incubated with 1 : 200 dilution of polyclonal antiserum against *L. amazonensis* gp63, washed and developed with [^{125}I]-labelled protein A, as described by Sanyal *et al* (1994).

2.6 *Microscopic techniques*

Promastigotes were immobilized on polylysine-coated glass coverslips as described by Adhya *et al* (1997), before

Giemsa staining and visualization at 1000X magnification. Rosettes in live cell cultures were counted in a hemocytometer. Photomicrographs were scanned in a BioRad GST10 densitometer using Quantity One software.

2.7 *Flow cytometry*

Washed promastigotes (5×10^6) were fixed in 40% ethanol overnight at 4°C, pelleted down, suspended in 500 µl 38 mM Na-citrate, digested with 0·5 mg/ml RNase A for 45 min at 37°C, spun down again, and resuspended in 500 µl 38 mM Na-citrate containing 69 µM ethidium bromide, incubated for 30 min at room temperature. The DNA content was analysed in a flow cytometer (FACS Calibur, Beckton-Dickinson) using Cell Quart software, as described by Mitra *et al* (2000).

2.8 *Expression and purification of gp63 fusion protein*

A 400-bp *Bam*HI fragment containing codons 156–306 of the gp63 coding region (Webb *et al* 1991; see figure 5A) was fused in frame to the glutathione S–transferase (GST) gene in vector pGEX-4T-1 (Pharmacia). The recombinant plasmid pGEX-gp63 or control vector was introduced into *Escherichia coli* strain BL-21, and a single colony of the transformant was grown in 2xYTA medium containing 100 µg/ml ampicillin. Log-phase cultures were induced with 1 mM isopropyl thio *b*-galactoside (IPTG) for 5 h, cells pelleted, washed with PBS, and lysed by sonication. Fusion protein was purified from the clarified extract by affinity chromatography on Glutathione Sepharose 4B (Pharmacia), as per the manufacturer's instructions. Before addition to cell culture, protein preparations were sterilized by centrifugation filtration using Spin-X filter units (Costar).

3. Results

3.1 *Generation of gp63-deficient cell lines*

About 12 copies of gp63 genes are present on a single 650 kb chromosome of Indian strains of *L. donovani*

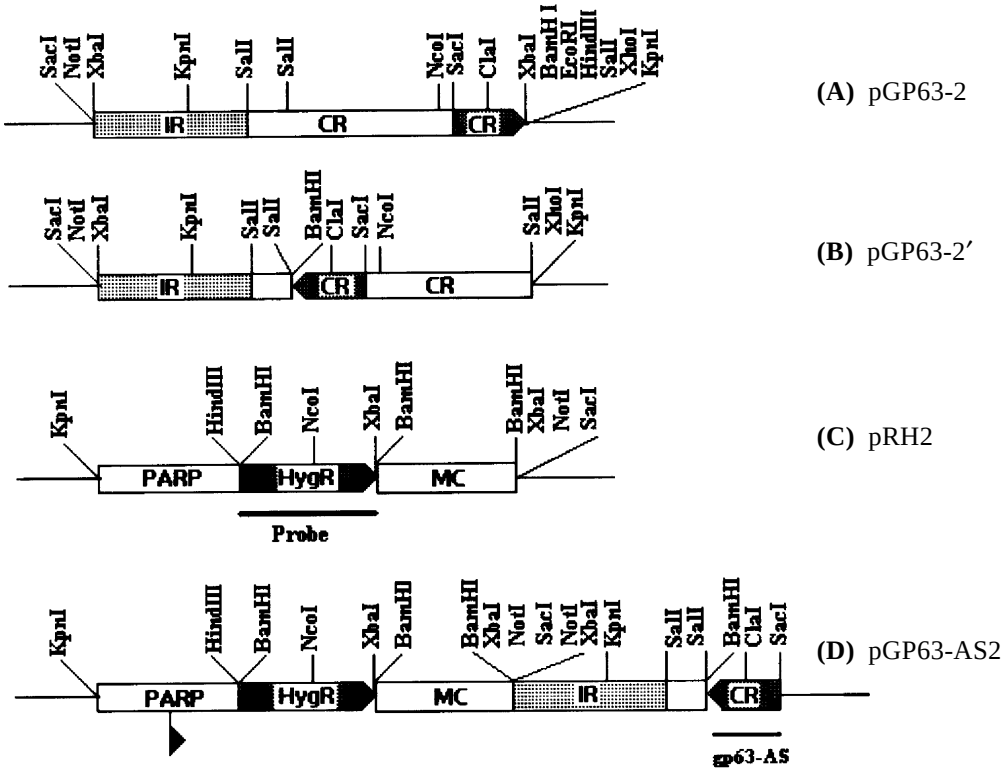

Figure 1. Construction of gp63 antisense vector pGP63-AS2 (7·95 kb). (**A**) Map of pGP63-2 (6·0 kb), consisting of 3·0 kb gp63 gene with intergenic region (IR); coding region (CR) (shaded region used as antisense). (**B**) Map of pGP63-2′; coding region inverted. (**C**) Map of pRH2; PARP, procyclin promoter; HygB, hygromycin-resistant gene; MC, minicircle sequence. (**D**) Map of pGP63-AS2; antisense construct consists of pRH2 backbone and *L. donovani* gp63 major repeat IR, gp63 coding sequence (400 bp, sense orientation) CR (open box) and, C-terminal region of gp63 (590 bp, antisense orientation) CR (shaded arrow). For details of construction, see § 2.

(Ghosh *et al* 1998; S Pandey, P Chakraborti, R Sharma, S Bandyopadhyay, D Sarkar and S Adhya, unpublished data). Both tandemly repeated and dispersed copies are present. This makes it technically difficult to generate null mutants by homologous recombination. We therefore took the alternative approach of expressing antisense RNA from an episomal vector introduced into *L. donovani* promastigotes (figure 1). We present here the data for one such antisense transfectant clone, GP63-AS2-7. The properties of the other clones were qualitatively similar.

Southern blot analysis of genomic DNA from the antisense transformant, as well as from the vector-transfected control, showed the presence of plasmid DNA of the expected size (figure 2A). The amount of vector sequence per unit of genomic DNA was roughly equivalent in the two strains (the apparent difference in the signal intensities in the figure are due to different amount of DNA loaded).

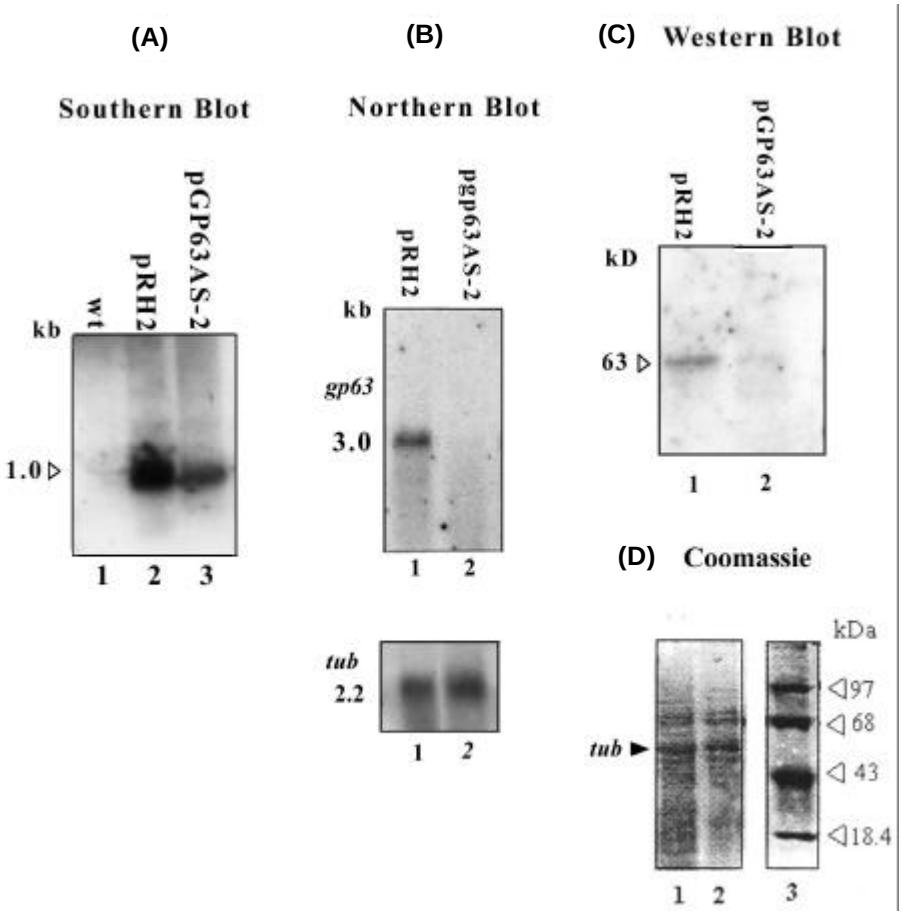

Figure 2. Confirmation of antisense clone by Southern, Northern and Western blot analysis. (**A**) Genomic DNA from wild-type (lane 1, 5 μg), uncloned pRH2 transformant (lane 2, 5 μg) or pGP63-AS2 transformant clone 7 (lane 3, 3 μg), double-digested with *Hind*III and *Xba*I. The blot was hybridized with a 1·0 kb *Bam*HI Hyg B fragment from pRH2 (lower bar in figure 1C). (**B**) *Upper panel*: Depletion of gp63 in antisense transformant was confirmed by Northern blot hybridization of total RNA (5 μg) from pRH2 control (lane 1), and pGP63-AS2 transformant clone 7 (lane 2). The probe was a [^{32}P]-labelled oligonucleotide complementary to positions 901–918 of the *gp63* gene (Webb *et al* 1991). *Lower panel*: Control hybridization of pRH2 (lane 1) and pGP63-AS2 (lane 2) transformants with a *b*-tubulin coding probe (Ghosh *et al* 1998). (**C**) Western blot analysis of pRH2 control and the antisense clone 7. Total protein extracts equivalent to 6×10^6 cells were electrophoresed on SDS-10% PAGE and blotted to nitrocellulose. The probe used was polyclonal antibody against *L. amazonensis* gp63. Lane 1, pRH2 transformant and lane 2, pGP63-AS2 transformant, clone 7. (**D**) Coomassie stain of total protein from pRH2 (lane 1) and pGP63-AS2 (lane 2) transformants. Arrowhead shows tubulin band (55 kDa). Lane 3, molecular weight markers.

3.2 *Depletion of gp63 mRNA and protein in the transfected lines*

Total RNA from transfectants was analysed for gp63 mRNA by Northern blot hybridization, using an antisense oligonucleotide complementary to the gp63 coding sequence, as probe. Compared to the wild type strain, or vector-transfected control, gp63 mRNA was reduced by more than 85% in antisense-transfected clone 7. Control hybridizations showed that *b*-tubulin and other unrelated mRNAs are expressed normally in the vector controls and the antisense transfectants (figure 2B).

Western blot analysis showed that the gp63 protein level in antisense transfectant clone 7 was reduced to less than 10% that of the wild type strain (figure 2C). The overall pattern of protein synthesis was not affected and the level of specific proteins, such as tubulin, were unaltered (figure 2D), indicating a specific effect on gp63 expression.

3.3 *Effect of depletion of gp63 on growth rate*

The gp63-deficient clones grew poorly on conventional liquid or biphasic medium. In medium 199, the doubling time of the antisense transfectant was 120 h, compared to 24–26 h for wild type or vector control (table 1). In Schneider's medium, clone 7 grew better (doubling time 75 h), but still poorly compared to wild type (table 2).

3.4 *Effect of gp63 depletion on cell morphology*

Antisense transfectant clone 7 had remarkably altered cell morphology. Most of the cells were shorter and more rounded, with an apparently enlarged nucleus. A significant number of large binucleate cells were also observed. Many of these latter cells were biflagellate and bikinetoplastid, indicating the completion of nuclear, flagellar and mitochondrial division, but no cleavage (cytokinesis) (figure 3). Whereas wild type or vector-transfected controls had about 2–3% of binucleate cells, the corresponding number in clone 7 was more than 10% (table 1).

3.5 *Accumulation in the G2/M phase of the cell cycle*

Ethidium bromide-stained cells were subjected to flow cytometry in order to assess their relative DNA contents. In wild type and vector transfected controls, about 60% of the cells had a diploid content of DNA (i.e. were in the G1 phase), while about 30% cells had double this amount (i.e. in the G2/M phase) (figure 4, table 1). In the antisense transfectant clone 7, on the other hand, the proportion of G2/M cells was significantly higher (45%) while the G1 phase cells were correspondingly reduced in number (47%). This indicates that gp63 depletion leads to accumulation of cells in the G2 and/or M phases of the cell cycle.

3.6 *Reversal of phenotype by a gp63 fragment*

It was necessary to determine if the gross abnormalities in growth and cellular morphology observed in the antisense transfectants were caused primarily by a deficiency of cellular gp63 or secondarily by some other defect. Consequently, a fragment of gp63 from codon 156 to codon 306 of the *L. donovani* sequence (figure 5A) was fused

Table 1. Effect of gp63 depletion on cell division.

Strain	Generation time (h)	Cells (%)[a]		Binucleate cells (%)[b]	Rosettes	
		G1	G2/M		Total cells (%)	No. of cells/rosette
Wild type	24–26	60·8	27·4	2·1	1·2	10–12
pRH2 transfectant	24–26	62·7	29·7	2·4	1·02	10–12
pGP63-AS2 transfectant	120	46·7	45·5	11	0·77	3–4

[a]Determined by flow cytometric analysis of ethidium-bromide stained cells (figure 4); the G1 and G2/M cells correspond to the peaks M2 and M3 respectively.
[b]Determined by Giemsa staining of fixed cells (figure 3).

Table 2. Effect of gp63 fragment on growth of gp63-deficient cells.

Strain	Addition[a]	Generation time (h)	Binucleate cells (%)[b]
GP63-AS2-7	GST	75	18·5
GP63-AS2-7	GST-gp63	35	4·1

[a]The fusion protein was added at 6·67 μg/ml.
[b]By Giemsa staining.

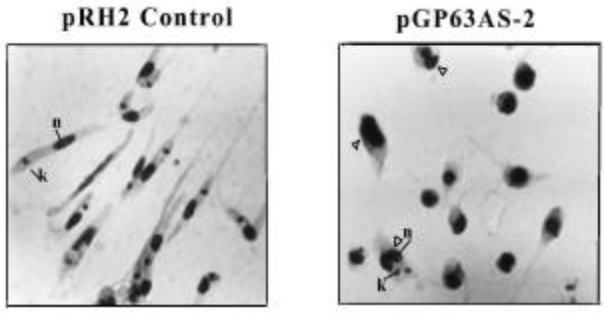

Figure 3. Giemsa stained photomicrographs of pRH2 transformant (left) or pGP63-AS2 transformant clone 7 (right). n, nucleus; k, kinetoplast. Binucleate cells are indicated by arrowheads. Magnification, X 1000.

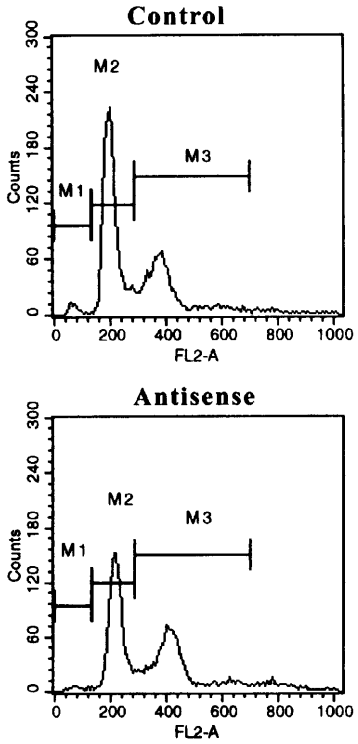

Figure 4. Flow cytometric analysis of ethidium bromide-stained pRH2 transformant (upper) or pGP63-AS2 transformant clone 7 (lower). Cell counts are plotted against fluorescence (FL2-A). The peaks M1, M2 and M3 correspond to cells in G0, G1 and G2/M phases, respectively.

downstream of the GST gene of pGEX expression vector, and expressed as a fusion protein in *E. coli*. The expressed fusion protein (or GST control) was purified by glutathione–sepharose affinity chromatography (figure 5B) and added to a culture of antisense transfectant clone 7. The GST-gp63 fusion protein specifically stimulated the growth of this clone (figure 5C); trypsin treatment of the fusion protein rendered it ineffective (data not shown). The doubling time was reduced from 75 h to about 35 h (table 2). In parallel, the number of binucleated cells was reduced by about 4-fold (table 2) and the cells became less rounded and more elongated, rather like wild type. These results indicate that the gp63 fragment stimulates cells to undergo cytokinesis and that the specific domain of the protein is sufficient for this effect.

4. Discussion

In kinetoplastid protozoa, such as *Leishmania* and *Trypanosoma*, cell division occurs by ordered progression of morphologically visible events including outgrowth of new flagellum, nuclear division, division of the single kinetoplast–mitochondrion, and longitudinal cleavage (Molyneaux and Killick-Kendrick 1987; Sherwin and Gull 1989). Deficiency of gp63 in *L. donovani* results in accumulation of binucleate cells, most of which have two kinetoplasts (figure 3). Flow cytometric analysis showed that the fraction of cells in G2/M phase increases by about 15% compared to vector controls (table 1); of this, about 8% is accounted for by an increase in the binucleate cell count, and the remaining 7% by mononucleate G2 cells. Taken together, the data suggest a defect in cytokinesis, i.e. nuclear as well as cell division.

Dividing *L. donovani* promastigotes form multicellular aggregates (rosettes) in liquid culture, as well as in the midgut of sandfly vectors (Molyneaux and Killick-Kendrick 1987). The number of rosettes observed per 100 cells was about the same (about 1%) in control or antisense transfectants. However, the number of cells per rosette in the antisense transfectant was significantly lower (3–4 compared to greater than 10 for wild type) (table 1). This presumably reflects weaker intercellular interactions in the gp63-depleted clone.

The role of gp63 in cytokinesis is further strengthened by the protein add-back experiment, in which the addition of GST-gp63 fusion protein in the medium specifically stimulated the growth of antisense clone. The exogenous protein also reduces the number of binucleate cells and the cells become normal like wild type from its rounded appearance. All these data indicate that gp63 somehow regulates the process of cytokinesis.

How does gp63 promote cytokinesis? Right now, the question is an open one, but certain possibilities may be considered. The fact that exogenously added, bacterially expressed gp63 fusion protein shows this activity (figure 5) indicates that gp63, which is normally anchored to the plasma membrane, acts as a ligand for intercellular interaction. This may explain the formation of rosettes by dividing *L. donovani* and the reduction in their number and size in gp63–deficient variant (table 1). A ligand role

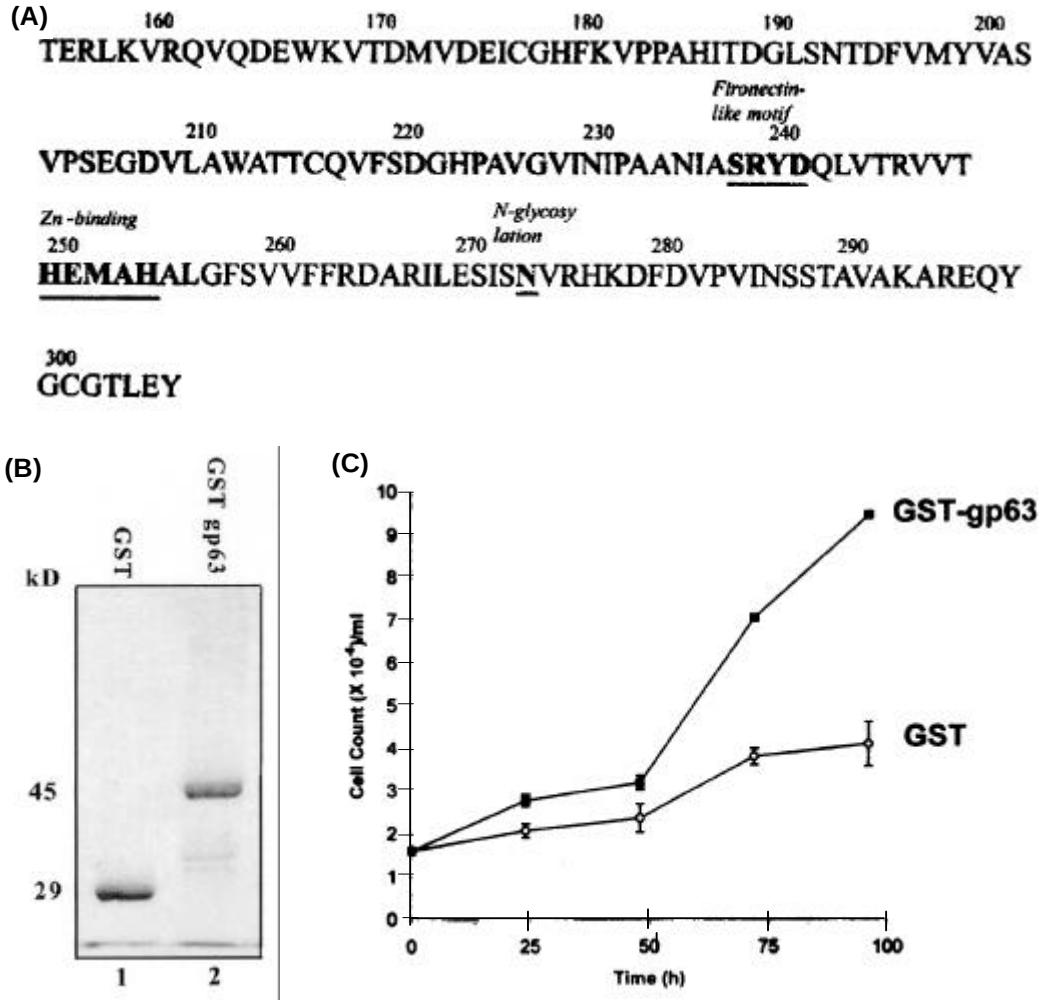

Figure 5. Effect of gp63 fusion protein on growth of gp63-deficient cells. (**A**) Amino acid sequence of the *L. donovani* gp63 expressed as a GST-fusion protein (Webb *et al* 1991; S Pandey, P Chakraborti, R Sharma, S Bandyopadhyay, D Sarkar and S Adhya, unpublished data). Numbers above the sequence indicate positions with respect to the N-terminus. The fibronectin-like motif, zinc-binding domain of metalloprotease activity, and N-glycosylation site (McGwire and Chang 1996) are indicated in bold. (**B**) SDS-10% PAGE analysis of 2 µg of GST (lane 1) or GST-gp63 fusion protein (lane 2) purified by glutathione-sepharose affinity batch adsorption, followed by elution with reduced glutathione. The proteins were stained with Coomassie blue. (**C**) Effect of gp63 fusion protein on growth of pGP63-AS2 transformant clone 7. Cultures of the transformant in Schneider's drosophila medium containing 20% FBS and 16 µg/ml hygromycin were treated with GST (-○-) or GST-gp63 fusion protein (-■-), each at 6·67 µg/ml, and cell counts taken at different times at 22°C.

is further suggested by the fact that the active gp63 fragment contains the SRYD motif which has been shown (Soteriadou *et al* 1992) to be antigenically similar to the conserved RGDS cell attachment motif of fibronectin, an extracellular matrix protein that mediates cell adhesion and stimulates growth and differentiation through interaction with cell–surface integrins, leading to activation of intracellular signalling networks (Giancotti and Ruoslahti 1999; Aplin *et al* 1999). Although the components of the cytokinetic apparatus have been defined in a number of systems (Field *et al* 1999), little is known about the regulation of this process. The exciting possibility that cytokinesis in this protozoal system is linked to the integrin signalling pathway merits further investigation.

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References

Adhya S, Ghosh T, Das A, Bera S K and Mahapatra S 1997 Role of an RNA-binding protein in import of tRNA into *Leishmania* mitochondria; *J. Biol. Chem.* **272** 21396–21402

Aplin A E, Howe A K and Juliano R L 1999 Cell adhesion molecules, signal transduction and cell growth; *Curr. Opin. Cell. Biol.* **11** 737–744

Brittingham A, Chen G, McGwire B S, Chang K P and Mosser D 1999 Interaction of *Leishmania* gp63 with cellular receptors for fibronectin; *Infect. Immun.* **67** 4477–4484

Brittingham A, Morrison C J, McMaster W R, McGwire B S, Chang K P and Mosser D M 1995 Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis; *J. Immunol.* **155** 3102–3111

Button L L, Russell D G, Klein H L, Medina-Acosta E, Karess R E and McMaster W R 1989 Genes encoding the major surface glycoprotein in *Leishmania* are tandemly linked at a single chromosomal locus and are constitutively transcribed; *Mol. Biochem. Parasitol.* **32** 271–284

Chen D Q, Kolli B K, Yadava N, Lu H G, Gilman-Sachs A, Peterson D A and Chang K P 2000 Episomal expression of specific sense and antisense mRNAs in *Leishmania amazonensis*: Modulation of gp63 level in promastigotes and their infection of macrophages *in vitro*; *Infect. Immun.* **68** 80–86

Chomczynski P and Sacchi N 1987 Single step method of RNA isolation by acid-guanidinium thiocyanate-phenol-chloroform extraction; *Anal. Biochem.* **162** 156–159

Field C, Li R and Oegema K 1999 Cytokinesis in eukaryotes: a mechanistic comparison; *Curr. Opin. Cell Biol.* **11** 68–80

Ghosh S, Mukherjee S and Adhya S 1998 Chromosome profile of *Leishmania donovani*: Interstrain and interspecific variations; *J. Biosci.* **23** 247–254

Giancotti F G and Ruoslahti E 1999 Integrin signaling; *Science* **285** 1028–1032

Joshi P B, Sacks D L, Modi G and McMaster W R 1998 Targeted gene deletion of *Leishmania major* genes encoding developmental stage-specific leishmanolysin (gp63); *Mol. Microbiol.* **27** 519–530

Kapler G M, Coburn C M and Beverley S M 1990 Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression; *Mol. Cell. Biol.* **10** 1084–1094

Liu X and Chang K P 1992 Extrachromosomal genetic complementation of surface metalloproteinase (gp63)-deficient *Leishmania* increases their binding to macrophages; *Proc. Natl. Acad. Sci. USA* **89** 4991–4995

Maniatis T, Fritsch E F and Sambrook J 1989 *Molecular cloning. A laboratory manual*, 2nd edition (New York: Cold Spring Harbour Laboratory Press)

McGwire B S and Chang K P 1996 Post-translational regulation of a *Leishmania* HEXXH metalloprotease (gp63). The effects of site-specific mutagenesis of catalytic, zinc binding, *N*-glycosylation, and glycosyl phosphatidylinositol addition sites on *N*-terminal end cleavage, intracellular stability and extracellular exit; *J. Biol. Chem.* **271** 7903–7909

Mitra B, Saha A, Roy Chowdhury A, Pal C, Mondal S, Mukhopadhyay S, Bandyopadhyay S and Majumdar H K 2000 Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis; *Mol. Med.* **6** 527–541

Molyneaux D H and Killick-Kendrick R 1987 Morphology, ultrastructure and life cycles; in *The Leishmaniases in biology and medicine* (eds) W Peters and R Killick-Kendrick (London: Academic Press) vol. 1, pp 121–176

Ramamoorthy R, Donelson J E, Paetz K E, Maybodi M, Roberts S C and Wilson M E 1992 Three distinct RNAs for the surface protease gp63 are differentially expressed during development of *Leishmania donovani chagasi* promastigotes to an infectious form; *J. Biol. Chem.* **267** 1888–1895

Sanyal T, Ghosh D K and Sarkar D 1994 Immunoblotting identifies an antigen recognized by anti gp63 in the immune complexes of Indian Kala-azar patient sera; *Mol. Cell. Biochem.* **130** 11–17

Sherwin T and Gull K 1989 The cell cycle division of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations; *Philos. Trans. R. Soc. London* **B323** 573–588

Soteriadou K P, Remoundos M S, Katsikas M C, Tzinia A K, Tsikaris V, Sakarellos C and Tzartos S J 1992 The Ser-Arg-Tyr-Asp region of the major surface glycoprotein of *Leishmania* mimics the Arg-Gly-Asp-Ser cell attachment region of fibronectin; *J. Biol. Chem.* **267** 13980–13985

Webb J R, Button L L and McMaster W R 1991 Heterogeneity of the genes encoding the major surface glycoprotein of *Leishmania donovani*; *Mol. Biochem. Parasitol.* **48** 173–184

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