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Characterization of DNA Sequences that Confer Complement Resistance in *Leishmania chagasi*

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Abstract

Serial passage of axenically cultured *Leishmania chagasi* promastigotes results in a progressive diminution in resistance to complement-mediated lysis (CML), whereas high CML resistance is seen in infectious metacyclic promastigotes from the sandfly vector as well as metacyclic-like promastigotes within low-passage cultures at stationary growth phase. As we previously reported, in a screen seeking to identify novel genes involved in CML resistance: (1) a genomic cosmid library derived from DNA of CML-resistant *L. chagasi* promastigotes was transfected into high-passage (constitutively CML-sensitive) *L. chagasi* promastigotes; (2) transformants were screened for acquisition of CML-resistance; (3) multiple cosmid-transfectants exhibited partial CML resistance; and (4) the sequence for one of the cosmids (Cosmid 51) was determined. This report extends the analysis of Cosmid 51, and identifies by deletion analysis a subregion of the cosmid insert that is critical to the CML-resistance phenotype of Cosmid 51 transformants. We also report the sequence determination and initial CML-resistance activity of another cosmid that also confers partial resistance to CML.

Keywords

Leishmania; *Leishmania chagasi*; protozoa; complement; immunity

Introduction

Leishmania spp. are the etiological agents of the leishmaniases, a group of human and animal diseases ranging in severity from self-healing cutaneous lesions to potential fatal visceral infections. Transmission occurs when a parasitized blood-feeding sandfly inoculates infectious metacyclic promastigote-form parasites into a mammal or other susceptible vertebrate. Inoculated metacyclic promastigotes are phagocytosed by macrophages and other phagocytic cells of the immune system, cells in which the parasites differentiate into amastigote-form parasites that make up the replicative stage within the mammal.

During the time between inoculation and macrophage phagocytosis, metacyclic promastigotes need to survive exposure to complement, an antimicrobial innate immune defense system present in serum. Complement involves a cascade of serum protein-binding events that can result in formation on the microbe of a membrane attack complex that kills by disrupting the outer membrane. Meta-cyclic promastigotes are thought to survive such

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Conflicts of Interest

The authors declare no conflicts of interest.

complement-mediated lysis (CML) through at least three mechanisms: proteolytic inactivation of the complement cascade, fast release of membrane attack complexes, and a glycocalyx structure that causes membrane attack complex assembly too distal to the parasite membrane. Three highly abundant and developmentally regulated parasite surface macromolecules are thought to be involved in this resistance. Major surface protease (or GP63) is thought to cleave complement C3b into an iC3b-like form able to block the complement cascade while still functioning as an opsinogen able to facilitate parasite phagocytosis into macrophages via receptor CR3.¹ Lipophosphoglycan (LPG) may be involved in ineffective positioning/assembly of the MAC.² Promastigote surface antigen (PSA, or GP46) re-expression in promastigotes having an otherwise low PSA expression level and low CML survival resulted in greatly increased resistance to CML.³ The high CML resistance that is characteristic of metacyclic promastigotes does not apply to the other promastigote stages, including the procyclic, that are present in the insect host and in axenic promastigote cultures.⁴⁻⁷

The promastigote stages (e.g., the insect stages) of *Leishmania* species including *Leishmania chagasi* can be cultured *in vitro* axenically (in the absence of the fly vector). Infectious parasite cultures are established using amastigotes freshly isolated from parasitized, symptomatic animals; the amastigotes quickly differentiate into promastigotes under appropriate conditions.^{8,9} However, serial passage of promastigote cultures results in a progressive diminution in parasite capacity to infect and to resist CML.^{1,10} Based upon these observations, a gene add-back complementation study was undertaken to determine whether high-passage CML-sensitive cells could be used as a genetic background against which to screen for parasites able to resist CML. The aim of the experiment was to identify novel molecules involved in CML resistance, molecules in addition to MSP, PSA, and LPG. The initial results of the experiment, as previously reported, determined that when high-passage parasites were transfected with cosmids containing approximately 35 kb of *L. chagasi* genomic DNA, and then selected with human serum, ≈ 12 different cosmids were identified as conferring partial CML resistance; the sequence and annotation for one of these cosmids, Cosmid 51 (previously referred to as Cosmid 2), was also reported.¹¹ The present report extends this cosmid analysis.

Experimental Procedures

L. chagasi (strain MHON/BR/00/1669) maintenance in hamster and *in vitro* promastigote cultivation were as described previously.^{8,9,11} Low-passage cells were serially passaged 4 times subsequent to culture initiation with hamster-derived freshly isolated amastigotes. High-passage cells were passaged for > 50 weeks; 1 week \approx 1 passage.

Cosmid construction, manipulation, transfection into *L. chagasi*, and screening for CML resistance of transfectants were as described previously.¹¹ A 3 \times coverage of Cosmid 53 sequence was determined by the Iowa State University DNA Facility utilizing a shotgun strategy, by which 2-kb fragments of randomly sheared cosmid were inserted into a plasmid, and then randomly selected plasmids were sequenced; sequence gaps were filled using specific primer-directed sequencing.

For complement assays, promastigotes from cultures in stationary growth phase were incubated for 30 min at 37°C in PBS supplemented with pooled naïve human serum, then diluted in cold PBS, and viewed microscopically to assess and enumerate survivors (as described).¹¹

Results and Discussion

The Cosmid 53 insert sequence of 40 kb was determined by shotgun sequencing (see above; GenBank accession DQ418548). BLAST comparison to *Leishmania major* and *Leishmania infantum* genomes indicated its correspondence to their chromosome 36. There is very high synteny and sequence identity between these two genomes, and correspondingly between them and that of the *L. chagasi* genomic DNA making up the Cosmid 53 insert. We previously reported the 32-kb sequence (accession AY656839) and chromosome 36 correspondence of the Cosmid 51 insert.¹¹ Although both cosmid inserts correspond to chromosome 36, they have quite separated locations on the chromosome; Cosmid 53 corresponds approximately to *L. infantum* chromosome 36 base 284,000 to 309,000, and Cosmid 53 to base 1,190,000 to 1,230,000. Gene predictions, based upon the *L. infantum* genome annotation, indicate that Cosmid 51 comprises 1 partial (51-A) and 6 complete (51-B to G) genes, and that Cosmid 53 comprises 1 partial (53-M) and 12 complete (53-A to L) genes (Fig. 1). Table 1 lists the detail information on the gene predictions depicted in Figure 1; in the column headed "*L. infantum* V3 Chr 36," V3 refers to genome version 3, and Chr to chromosome. Many of the genes are identified as hypothetical (hp), indicating that the deduced protein sequence is without identifiable homologues within gene databases in the public domain.

Because parasite resistance to CML involves surface interactions with complement proteins, surface localization is a possible characteristic of proteins that directly function in CML resistance. None of the predicted genes in Table 1 encode proteins having a hydrophobic signal peptide that would signal for translation on the rough endoplasmic reticulum, a requisite for almost all surface-located proteins. However, it is also possible for CML resistance to be conferred by genes that indirectly affect the expression of surface macromolecules, or affect other aspects of cell biology including surface membrane turnover. Predicted genes 53J and K encode proteins with possible function in outer-membrane events and in RNA processing; the putative mRNA processing protein is of interest since it could function in modulating expression of surface proteins whose abundance is regulated by RNA-processing events that influence RNA steady-state levels.

To further investigate sequences that function in complement resistance, a set of Cosmid 51 subclones were made by opening the cosmids using insert-specific DNA restriction endonucleases, and then re-ligating the cosmids and determining which resulting sub-clones had "squeezed out" the insert region targeted for removal (Fig. 1). Cosmid 51 subclones were transfected into high-passage complement-sensitive parasites and resulting clonal isolates were tested for CML resistance. As shown in Figure 2, deletion of the *Bam*HI to *Bam*HI insert fragment, which deletes all predicted genes, results in abrogation of almost 70% of the CML resistance activity relative to that of Cosmid 51, while deleting the *Ap*aI to *Ap*aI or *Eco*RV to *Eco*RV fragments had no significant effect on relative CML resistance. The negative controls included non-transformed high-passage (HP) cells and cells transformed with a cosmid from which the entire insert was removed; both controls had extremely low relative CML resistance levels (Fig. 2).

Figure 1 also depicts a similar strategy using deletion analysis for investigating Cosmid 53. However, data are not shown because these studies are still in progress.

These experiments are expanding our understanding of genetic elements and proteins that function in CML resistance. Perhaps as importantly, the studies are needed in order to test the utility of using the gene complementation strategy for identifying CML resistance genes. If the approach proves useful, it can be applied in the study of the CML-resistant phenotype, which is a trait of almost all blood-borne microbes.

Acknowledgments

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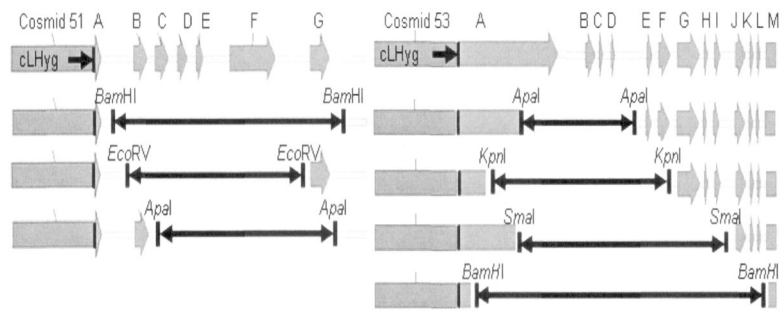


Figure 1. Cosmid 51 and 53 gene organization and subcloning strategy. Rectangles beneath letters indicate predicted genes, short thick arrows indicate orientation of predicted gene transcripts, “cLHyg →” indicates vector backbone, and faint narrow lines indicate regions predicted to be non-protein coding. Double-ended arrows indicate insert regions targeted for deletion in specific subclones.

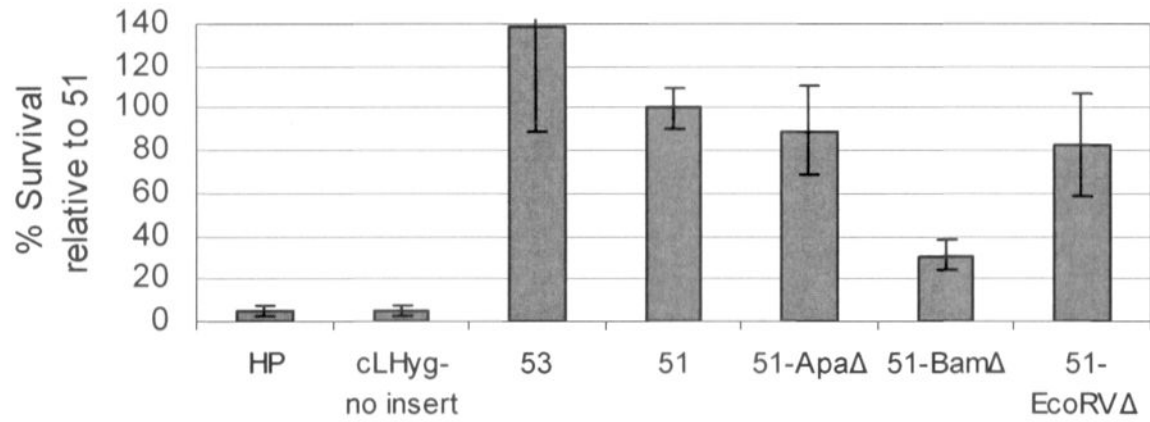


Figure 2.

Complement resistance of cells transfected with Cosmid 53, 51, and 51 subclones. The percentage of cell survival, relative to survival of cells transfected with Cosmid 51, was calculated as the ratio of motile cells present after incubation with, versus without, 12% serum. Error bars indicate the standard error of the mean of at least three experiments.

TABLE 1Cosmid Gene Predictions (Based on the *L. infantum* Genome)

Cosmid gene	<i>L. infantum</i> V3 ^a Chr ^b 36	Predicted gene/protein
51-A	0840	hp ^c
51-B	0850	hp
51-C	0860	hp
51-D	0870	hp
51-E	0880	ADP-ribosylation factor-like protein
51-F	0890	hp
51-G	0900	hp
53-A	3090	hp
53-B	3100	Succinyl-CoA ligase [GDP-forming] beta-chain, putative
53-C	3110	hp
53-D	3120	hp
53-E	3130	hp
53-F	3140	GTP-binding protein, putative
53-G	3150	ATP-dependent RNA helicase, putative
53-H	3160	Glutathione peroxidase, putative
53-I	3170	Exosome complex exonuclease RRP41, putative
53-J	3180	Clathrin coat assembly protein-like protein
53-K	3190	Pre-mRNA branch site protein p14, putative
53-L	3200	hp
53-M	3210	Cullin-like protein

^aV3 = genome version 3.^bChr = chromosome.^cHypothetical protein; many of the genes are identified as hypothetical, indicating that the deduced protein sequence is without identifiable homologues within gene databases in the public domain.