Chapter 3

Identification & Characterization of a Differentially Regulated Gene (DRG) in Leishmania donovani
3.1 Introduction

*Leishmania* adopts various mechanisms by which it is able to survive in such hostile environments. This is mainly accomplished by profound biochemical and developmental changes during their life cycle. Differential expression of variety of genes is another way to accomplish and it is known to vary significantly during *leishmania* life cycle (Cohen-Freue et al., 2007; Almeida et al., 2004; Holzer et al., 2006; Akopyants et al., 2004; Saxena et al., 2003). To monitor the change in gene expression, several PCR based methods have been developed among others DD-PCR (Liang and Pardee, 1992), subtractive hybridization (Diachenko et al., 1996) and microarray (Schena et al., 1995). The proximal aim of our lab was to identify the molecular markers of the different stages. Differential display PCR (DD-PCR) employed previously in the lab to isolate the transcripts that are specifically expressed at particular stages of the *Leishmania* (personal communication). DD-PCR was performed from the total RNA isolated from the promastigote and amastigote stage of the parasites and 67 differential fragments were isolated and re-amplified for the further analysis. To check their role in virulence these fragments were screened between the virulent and attenuated line of the parasites.

3.2 Results

3.2.1 Identification and isolation of several differentially expressed genes between virulent and attenuated line

In order to screen a large number of cDNA fragments simultaneously, Reverse Northern dot-blot analysis, which is more sensitive than traditional Northern blotting (Lion and Freedman, 1998), was used as an alternative to Northern blot (Zhang et al., 1996). In reverse northern analysis, all of the differentially expressed cDNA fragments and loading control were examined simultaneously cDNA fragments identified in DD-PCR analysis were re-amplified and spotted on the duplicated nylon membrane. *Leishmania* GP63 cDNA fragment and DD-water were used as positive and negative controls respectively. Membranes were then probed with $^{32}$P labeled cDNA synthesized either from stationary phase virulent or attenuated promastigote RNA. The dot-blots obtained are shown in Fig 3.1. A quantitative analysis was performed in which signal intensities from each
spot was normalized to the background. Then the ratio of the signals from two membranes (Filter “A” & “B”) was calculated. The differential expression was considered as significant when the ratio of the signal from the same spot on two membranes was either greater than 1.5 or less than 0.25 (Annex-I). If the ratio is greater than 1.5 then that spot was considered as virulent specific cDNA and if the ratio is less than 0.25 then it was considered as attenuated specific cDNA fragment.

Since the screening of the cDNA fragments was performed to identify the virulent specific cDNA fragments so therefore one would expect the more signal in the membrane probed with $^{32}$P labeled virulent cDNA and indeed I have found some of the spots showing more intensity in filter probe with $^{32}$P labeled cDNA from virulent RNA. But interestingly I have found several fragments for which intensity of signal is more in membrane probed with $^{32}$P labeled attenuated cDNA than that of virulent cDNA (Fig 3.1).

![Filter A and Filter B](image)

**Figure 3.1 Reverse northern analysis of differentially expressed cDNA fragments** obtained from DD-PCR between promastigote and amastigote stage of *L. donovani*. 100 ng of each fragment were spotted on the Hybond membrane in duplicate and membranes were probed with $^{32}$P labelled total cDNA synthesized from RNA isolated from virulent (Filter A) and attenuated (Filter B) stationary phase promastigote culture.

Summary of the reverse northern experiment is as follow:

**Summary of the Reverse Northern**

- Number of fragments spotted on membrane: 70
- Numbers of positive fragments: 35
Number of virulent specific 6
Number of attenuated specific 9
Number of constitutively expressed 8
Number of fragment with weak signal 12
Number of negative fragments 35

These virulent specific, attenuated specific fragments were re-amplified for further screening by northern blot. Among the 15 virulent and attenuated specific fragments when used as probes on northern blots containing 5 ug of total RNA, 8 fragments gave detectable signals. Based on expression profile examined by northern blot (discussed in next section), an attenuated specific fragment (F106), was chosen for the subsequent study.

3.2.2 Northern blot analysis of F106, an attenuated specific fragment

F106 (~650 bp) specifically hybridized to two distinct transcripts of sizes ~1.8 kb and ~1.6 kb, when used as a probe for northern analysis (Fig 3.2). Both transcripts showed differential regulation at different stages. The expression of ~1.8 kb transcript increases when attenuated line of Leishmania moves from log phase to stationary phase. This increase in expression was missing in virulent line. However shorter transcript showed no change in expression either in virulent or attenuated line (Fig 3.2). Since northern blot analysis shows differential regulation of transcripts at different stages of parasite; we termed this gene as LdDRG (Differentially Regulated Gene). This cDNA fragment (F106) was cloned in pMOS blunt end cloning vector and sequenced. Comparison of nucleotide sequence using BLAST-N and BLAST-P showed that F106 contain an ORF which showed very high homology to a hypothetical protein (LmjF31.0900) in L. major. The nucleotide sequence and deduced amino acid sequence of F106 is shown in Fig 3.3.
Figure 3.2 Differential expression pattern of an attenuated specific fragment (F106) in *L. donovani*. 5 μg of total RNAs from log and stationary phase culture of both virulent and attenuated line were separated in an agarose/formaldehyde gel, transferred onto nylon membrane and hybridized with $^{32}$P labelled F106. Top panel: F106 purified fragment (0.01ng) and gDNA of *L. donovani* (1ng) were used as a positive control and bluescript plasmid (0.1 ng) as a negative control. These were spotted on nylon membrane and hybridized in same bottle. Arrow indicates the size of the 23S rRNA of *L. donovani*. A ethidium bromide stained agarose gel showing the ribosomal RNA in each of the RNA samples used.

Aatatgtagatgctctccagcctctcactgtccagtttgccctacgccttttcagcaagcc -60

ATG TCC GTG ATG CAG CTG CTG ATT GTG GCC CTG GCC GGC TGC CTG TCG GTC GGC GCG GCA 60
MSVMQLILVAAACLSVAA20
AAG TCC AAG GAG GAG TTC CCC CTG GTG GTG TGC TCC GCC ATC AGC GTG GTT GGC GTG 120
KSKKEEFPPLVWVCLGIVTVGVV40
ATG GCC GCA CTG TAC GTT GCC TAC AAG CCG CCT GAT GAG GTG CAG CTG CCC GGC TCC GTG 180
IAALYVAKRPDEELHILPGSV60
GTG ATG AGC GCG GTG GAG AGC GAC CCT GCC AAC AGC AGC GCC GGG GAA CCC ATT TAG 240
VMTAVESDPNGKSCANAEPI -79
tgaagagatgcgtgaggtgcctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctgtgctgcctgtgtgtctgtgcctgtgtgtctgtgaagatgcgtgaggtgcctgctgctg

Figure 3.3 Nucleotide and predicted amino acid sequences of an attenuated specific fragment (F106). The nucleotide sequence is numbered from putative start codon on the top. The predicted amino acid sequence is numbered on the bottom and given in single letter code.
3.2.3 Genomic Organization of DRG

3.2.3.1 Southern blot analysis of DRG gene

Northern blot analysis of F106 indicated the presence of two DRGs in *L. donovani*. To examine the genomic organization and copy number of the LdDRG, genomic DNA from *L. donovani* was digested with several enzymes. These enzymes were selected on the basis of restriction map of LdDRG ORF. Digested genomic DNA was separated by agarose gel electrophoresis and then blotted onto nylon membrane. Southern hybridization was carried out using the $^{32}$p-labeled LdDRG ORF as a probe (Fig 3.4). Under stringent conditions, Except EcoRI all enzymes that did not cut within the LdDRG ORF (e.g. Xhol, HindIII) gave two bands of hybridization with this $^{32}$p labeled probe. This result is in consistent with the northern analysis that there are two DRG related sequence in *L. donovani* genome. Single hybridization band of 8kb in the lane containing the genomic DNA digested with EcoRI suggest that these LdDRGs are located in single chromosomal locus.

![Southern blot analysis of LdDRG gene locus](image)

**Figure 3.4 Southern blot analysis of LdDRG gene locus.** 10 µg of *L. donovani* gDNA was digested with restriction enzymes that lack a cleavage site in LdDRG ORF (EcoRI, Xhol, HindIII and XmnI) and with restriction enzymes that cut once (SmaI and SacI), separated on 1 % agarose gel, transferred to nylon membrane and hybridized with $^{32}$P labeled LdDRG1 ORF. DNA molecular size markers are shown on the left.

An enzyme that cuts once within the LdDRG ORF (eg SmaI) gave three bands, while SacII that also cuts once at extreme 3' end of LdDRG ORF gave two bands of hybridization with this probe. On the basis of hybridization patterns restriction map
of LdDRG locus has been generated (Fig 3.5). Restriction map suggest that the LdDRG is present as a two copy gene (DRG1 & DRG2) and the distance between these two copies is ~4.3kb in L. donovani. A schematic of the genomic organization of DRG1 and DRG2 in Leishmania donovani is shown in Fig 3.5. Moreover hybridization patterns in SacII and XmnI lane suggest that these ORFs are flanked by distinct sequences. Our cDNA clone obtained from DD-PCR analysis shows more sequence similarity to DRG1 ORF and some part of 3'UTR of DRG1 (Fig 3.5).

![Genomic Organization of LdDRG](image)

**Figure 3.5** A schematic of genomic organization and restriction map of LdDRG locus determined by southern blot analysis. Restriction sites were indicated arbitrary (not to scale). Two ORFs DRG1 and DRG2 were separated by ~4.3 kb and both ORFs have distinct 5' and 3' flanking sequences.

### 3.2.3.2 Sequence analysis of DRG locus

To analyze the genomic organization in more detail, primers were designed on the basis of L. infantum genome sequence available at [www.genedb.org](http://www.genedb.org). Primers and used in this study are listed in Annex-II and position of the different primers on the LdDRG locus are also shown in Annex-III at the end of material and methods section. Different fragment were amplified from gDNA of L. donovani and sequencing was carried out for and sequence obtained was then compared with the sequence of other Leishmania DRG locus. We analyzed the level of synten (conservation of gene
order) among these organisms by looking at homologous chromosomal segments. A diagrammatic representation of comparison of genomic organization, gene order and sequence of different *Leishmania* species is shown in Fig 3.6.

![Diagram showing genomic organization of LdDRG locus](image)

**Figure 3.6 A schematic of genomic organization of LdDRG locus in different species of Leishmania.** Comparison of region with conserved synteny containing orthologs of DRGs in *L. donovani, L. infantum and L. major* is shown. For each species ORFs of DRG1 and DRG2 were show as red boxes, conserved but different 5' upstream region and 3'downstream regions of DRG1 and DRG2 are shown in similar color. Distance between DRG1 and DRG2 gene in *L. infantum* and *L. major* were calculated on the basis of sequence information available on GeneDB database.

All *Leishmania* species examined carry single copy of DRG1 and DRG2, in tandem, per haploid genome (Table 3.1). Till date in *L. major* sequence data base, DRG2 is not annotated as an ORF. Still the gene order for DRG genes is conserved in all *Leishmania* species except the length of intergenic region between these DRG1 and DRG2 ORF. As southern blot analysis demonstrated that two ORFs of DRG is separated by ~4.3kb, Sanger sequence database suggest that these two ORFs are apart by ~16kb in *L. infantum* and ~42kb in *L. major*. Genes predicted in this intergenic region (~42 kb) of *L. major* are predicted at some other location in *L. infantum* but on the same chromosome suggesting a genomic rearrangement. Genes predicted in this intergenic region of *L. major* and *L. infantum* are shown in Fig 3.7.
Table 3.1 Orthologs of DRG genes in other *Leishmania* species. The annotation of DRG orthologs in genome sequences of other *Leishmania* are provided, as well as the chromosomal distance separating each pair. In the Sanger database, *DRG1* homolog in *L. major* is not yet annotated as an ORF; hence, we have provided the chromosomal position.

<table>
<thead>
<tr>
<th></th>
<th><em>L. major</em></th>
<th><em>L. infantum</em></th>
<th><em>L. braziliensis</em></th>
<th><em>L. donovani</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DRG1</strong></td>
<td>Not annotated</td>
<td>LmjF31.001.2.30050601.V5.2 371431-371252</td>
<td>LinJ31_V1.0960</td>
<td>LbM31_V2.1120 1D DRG1</td>
</tr>
<tr>
<td><strong>DRG2</strong></td>
<td>LmjF31.09000</td>
<td>LinJ31_V1.0930</td>
<td>LinJ31_V1.0930</td>
<td>LbM31_V2.1050 1D DRG2</td>
</tr>
<tr>
<td>Distance between <strong>DRG1</strong> &amp; <strong>DRG2</strong></td>
<td>-37 kb</td>
<td>-15.9 kb</td>
<td>-63.9 kb</td>
<td>-4.3 kb</td>
</tr>
</tbody>
</table>

Figure 3.7 Comparison of the genomic region between the **DRG1** and **DRG2** in *L. major* and *L. infantum*. Total seven genes were predicted in *L. major* whereas only two genes were predicted in *L. infantum* between DRG1 and DRG2. Identity of the genes are listed in Annex-IV.
From southern analysis and sequencing of DRG locus in \textit{L. donovani}, it is unlikely that an additional ORF can be accommodated in this short intergenic region. Since \textit{L. donovani} genome sequence is not available but one can presume that this difference in genome architecture is due to some genomic rearrangements of DRG locus.

In \textit{Leishmania}, transcriptional control of gene expression is superseded by post transcriptional regulatory events, mediated through sequences within 3'UTRs of genes (Clayton CE, 2002; Haile S and Papadopoulou B, 2007; Brittingham et al., 2001; Murray et al., 2007). Therefore in our bioinformatic analyses, we paid particular attention to the flanking sequences of DRGs across \textit{Leishmania} species. In every case, 5' and 3' flanking sequences of a given DRG1 show minimal homology to the flanking sequences of the DRG2 of the same \textit{Leishmania} species (Table 3.2).

<table>
<thead>
<tr>
<th></th>
<th>\textit{Ld} DRG1</th>
<th>\textit{Lm} DRG1</th>
<th>\textit{Lin} DRG1</th>
<th>\textit{Lbr} DRG1</th>
<th>\textit{Ld} DRG2</th>
<th>\textit{Lm} DRG2</th>
<th>\textit{Lin} DRG2</th>
<th>\textit{Lbr} DRG2</th>
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<tr>
<td>\textit{Ld} DRG1</td>
<td>100</td>
<td>85</td>
<td>82</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Lm} DRG1</td>
<td>87</td>
<td>100</td>
<td>82</td>
<td>14</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>\textit{Lin} DRG1</td>
<td>91</td>
<td>88</td>
<td>100</td>
<td>23</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Lbr} DRG1</td>
<td>22</td>
<td>19</td>
<td>19</td>
<td>100</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Ld} DRG2</td>
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<td>1</td>
<td>3</td>
<td>2</td>
<td>100</td>
<td>87</td>
<td>93</td>
<td>62</td>
</tr>
<tr>
<td>\textit{Lm} DRG2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>87</td>
<td>100</td>
<td>84</td>
<td>62</td>
</tr>
<tr>
<td>\textit{Lin} DRG2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>99</td>
<td>86</td>
<td>100</td>
<td>64</td>
</tr>
<tr>
<td>\textit{Lbr} DRG2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3.2 Conservation of 5' and 3' flanking sequences of DRG1 and DRG2 genes. The alignment scores (100 = maximum) for the comparison of up to 2 kb of sequence 5' to the Start codon and 3' to the Stop codon are provided for DRG1 and DRG2 genes from multiple \textit{Leishmania} species. The boxed region contains scores for alignment of 3' sequences and unboxed region represents the 5' sequences.

However, the 5' and 3' flanking sequences of both DRGs are highly conserved when compared to the respective homologs in other \textit{Leishmania}. Sequence alignment of \textit{Ld}DRG1-3'UTR Vs \textit{Ld}DRG2-3'UTR and \textit{Ld}DRG1-3'UTR Vs \textit{Lm}DRG1-3'UTR is shown in \textit{Annex IV}. Thus the differential pattern of DRG expression in \textit{L. donovani} is highly likely to be maintained in other \textit{Leishmania} species.
3.2.4 Cloning of full length of DRG1 and DRG2 cDNA

To clone the full-length transcript including coding and UTR sequences of the DRG1 and DRG2 cDNA, 5' and 3' RACE-PCR was performed on total RNA from the stationary phase culture. In Leishmania, trans-splicing of a capped, 39-nt splice leader (SL) sequence onto the 5' end of the each RNA and coupled polyadenylation at the 3' end of the upstream transcript give rise to a mature mRNA (Clayton CE, 2002; Palenchar et al., 2006; Myler et al., 1999). Hence in Leishmania, we can use this 39 nt splice leader (SL) sequence to perform the 5'RACE-PCR to obtain 5'UTR of the genes. SL primer and gene specific primer for DRG1 and DRG2 ORF were used to amplify the both ORF with their 5'UTR. Cloning and sequencing of these PCR product revealed that DRG1 and DRG2 transcripts have 180 bp and 169 bp of 5'UTR respectively.

3'UTR of DRG1 and DRG2 was amplified by using 3'RACE kit (Ambion). 3'UTR outer RACE primer and gene specific primer amplified the specific 3'UTRs. These amplified products were cloned and then sequenced to determine the polyadenylation site. Full length sequence of DRG1 and DRG2 cDNAs are shown in Annex V with ORF in bold and capital letter and 5' Splice leader acceptor site and polyadenylation site marked.

3.2.5 Expression pattern and regulation of DRG transcripts

3.2.5.1 Northern blot analysis of DRG1 and DRG2 expression

Northern blot analyses were performed to examine the expression pattern of LdDRG1 and LdDRG2 mRNA during the different developmental stages of the parasite. RNA from promastigotes (both logarithmic and stationary phase) and axenic amastigotes either from virulent and attenuated lines were separated on agarose/formaldehyde gel, blotted on nylon membrane then hybridized with p32 labeled LdDRG1 ORF probe. This probe specifically hybridized with two distinct mRNA of ~1.8kb and ~1.6kb. Results show that both of the transcripts show differential regulation at different stages of parasite (Fig 3.8).
Figure 3.8 Differential expression pattern of DRG1 and DRG2 gene in different developmental stages of virulent and attenuated line of *L. donovani*. 5 μg of total RNAs were isolated from different developmental stages of virulent and attenuated line of log (V3 and A3) and stationary phase (V7 and A7) promastigote and axenic amastigotes (Vir Amas. And Att Amas.) cultures and separated in an agarose/formaldehyde gel, transferred onto nylon membrane and hybridized with 32P probes: P1: common DRG ORF, P2: DRG1 3’UTR, DRG1 specific probe, P3: DRG2 3’UTR DRG2 specific probe. Position of the three probes were shown in schematic (Top panel). Sizes of the transcripts are shown on the right. Equal loading were verified by staining the RNA with ethidium bromide in the agarose gel prior to norther blot (last Lower panel).

From southern blot analyses it was clear that *LdDRG* is two copy gene and these two ORFs have distinct 3’UTR. Hence, 32p labeled probe specific to each 3’UTR were hybridized to the blotted membrane. Probe specific to 3’UTR of *LdDRG1* specifically hybridized to the ~1.8 kb transcript. Densitometric analysis shows that this longer transcript has very little increase (~1.2 fold) in the intensity from the logarithmic to the stationary phase in virulent RNA but this increase is more (~2 fold) in attenuated RNA (Fig 3.8). This transcript is also detectable in axenic amastigote generated from both virulent and attenuated line.

In contrast, northern hybridization using probe specific to 3’UTR of *LdDRG2* revealed a single band of ~1.6 kb only in promastigote stage of parasite that increased the intensity (~2.4 fold in virulent RNA and ~3fold in attenuated RNA) from the logarithmic to stationary phase (Densitometric analysis). This 1.6kb transcript is not detectable in amastigote stage of the parasite (Fig 3.8). While *DRG2*
is highly expressed, its expression seems to be restricted to promastigotes. DRG1 expression is lower but detected in all stages. To quantify these differences accurately, levels of specific DRG transcripts were determined by quantitative RT-PCR.

3.2.5.2 Quantitative Real Time PCR analysis of DRG transcripts

Primers were designed to differentiate between two transcripts. Primers used in this study are listed in Annex-I at the end of material and methods. Levels of mRNA determined by quantitative RT-PCR were normalized to GAPDH. Results were shown as fold difference in stationary phase and in amastigote as compared to logarithmic phase promastigote of virulent cultures. Results obtained by quantitative real time RT-PCR were shown in Fig 3.9.

![Graph showing fold difference for DRG1 and DRG2](image)

**Figure 3.9 QRT-PCR analysis of DRG1 and DRG2 in different developmental stages of virulent and attenuated line of L. donovani.** The relative expression of each gene in various stages is presented as fold difference over expression in Log phase cells. virulent and attenuated line of log (V3 and A3) and stationary phase (V7 and A7) promastigote and axenic amastigotes (VA and AA).

As virulent *Leishmania* culture move from log phase to a stationary phase, DRG2 showed an up regulation in expression by 4.5 fold whereas DRG1 showed only 1.2 fold up regulation in expression. In contrast, in attenuated line both DRG1 and DRG2 showed an up regulation in expression (DRG1 by 3.2 folds and DRG2 by 5.4 fold) from log phase to stationary phase. When promastigotes of either virulent or attenuated *Leishmania* line are shifted to amastigote conditions, both DRG transcripts are down regulated vis a vis promastigotes but the DRG2 transcript is
down regulated to far greater extent than that of *DRG1* transcript (6.6 fold versus 1.8 fold). These results are consistent with northern blot based observations of expression pattern of *DRG1* and *DRG2*. Table below summarizes the expression pattern of *DRG1* and *DRG2* in different stages or line of the parasite:

<table>
<thead>
<tr>
<th>Stages or line</th>
<th>Differential Expression</th>
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<tbody>
<tr>
<td>1. Log Vs Stationary</td>
<td><em>DRG2</em> is up regulated in virulent line</td>
</tr>
<tr>
<td></td>
<td><em>DRG1</em> is up regulated in attenuated line</td>
</tr>
<tr>
<td>2. Promastigote Vs Amastigote</td>
<td><em>DRG2</em> is promastigote specific</td>
</tr>
<tr>
<td></td>
<td><em>DRG1</em> is present in both stages</td>
</tr>
<tr>
<td>3. Virulent Vs Attenuated line</td>
<td>only <em>DRG1</em> is up regulated in attenuated line</td>
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</table>

Therefore, results taken together demonstrate that *DRG1* and *DRG2* show a differential regulation at different stages of parasite's life cycle.

### 3.2.6 Differential Regulation of DRG transcripts

In *Leishmania* and trypanosomes, the regulation of gene expression occurs at the post transcriptional level. These processes include differential processing of polycistronic transcripts, altered rate of mRNA decay and protein translation (Clayton C and Shapira M, 2007; Haile S and Papadopoulou B, 2007). Therefore I have examined whether *de novo* protein synthesis is required for change in steady state level of both *DRG* transcripts in different developmental stage. The virulent and attenuated cultures from different stages (logarithmic or stationary phase promastigotes and amastigotes) were treated with protein inhibitor cycloheximide (0.5ug/ml) up to 12hr. This concentration was previously determined in the lab to inhibit the protein synthesis by more the 90%. Level of *DRG* transcripts was measured by quantitative RT-PCR and normalized to 18s rRNA. The stability of 18s rRNA has been shown to be not affected by cycloheximide treatment (Brittingham et al., 2001). Results are shown as fold difference in treated samples as compared to untreated sample.

At logarithmic stage, the stability of *DRG1* transcript induced by almost to the same extent in both attenuated and virulent cultures on cycloheximide treatment
(Fig 3.10). Whereas the stability of DRG2 transcripts increased by 3.6 fold in attenuated culture as compared to 2.2 fold in virulent culture after 12 hr of cycloheximide treatment. A similar increase in the stability of DRG1 and DRG2 mRNA was observed when the stationary phase promastigote of attenuated and virulent cells were treated with cycloheximide (Fig 3.10). In first 30 min after the treatment with cycloheximide, the abundance of both mRNA decreases by 3- to 5-fold but after that slight increase in the abundance of DRG1 and DRG2 mRNA was observed. This later increase in abundance of DRG2 mRNA is more in the case of attenuated culture indicating differential regulation.

The major difference in the abundance of DRG1 and DRG2 mRNA was observed when the axenic amastigote culture from virulent and attenuated were treated with cycloheximide (Fig 3.10). DRG1 mRNA was induced by ~2 fold in virulent amastigote as compare to ~5 fold in attenuated amastigote. The stability of DRG2 mRNA was increased by ~5 fold in virulent amastigote and ~9 fold in attenuated amastigote culture treated with protein translation inhibition.

Differences in the steady state level of DRG1 and DRG2 mRNA could be because of differential stabilities of these RNA. The differential stabilities of these mRNA at different stages are remains to be studied. However observations with cycloheximide treatment suggest that DRG1 and DRG2 transcripts are labile and are regulated by two different mechanisms in attenuated and virulent culture.
Figure 3.10 Effect of Cycloheximide on steady state levels of DRG1 and DRG2 transcripts in different developmental stages of virulent and attenuated line of L. donovani. Cells were treated with Cycloheximide for up to 12 hours. Levels of DRG1 and DRG2 transcripts were measured by QRT-PCR. The observed fold change in expression of each gene at the indicated time point is provided normalized to the expression of the gene in the corresponding untreated sample. The data represents an average of 3 biological replicates.
3.2.7 DRG protein are highly conserved across *Leishmania* genus

Sequence analysis of the DRGs revealed that the ORFs of *DRG1* and *DRG2* genes were >98% identical in different species except at the C-terminus. We compared DRG protein sequences in other *Leishmania* species. All available *Leishmania* DRGs encode proteins that are extremely conserved. Overall, the divergence between DRG proteins is mainly restricted to the last 6 amino acids (Fig 3.11). Across species, DRG1 homologs terminate in aa sequence SxxEPI while the DRG2 homologs terminate in xxxExV (Fig 3.11). The LbrDRGs exhibit the most divergence particularly because LbrDRG1 has a 110 aa addition at the 5'end. After this point, even the LbrDRG sequences differ at only nine amino acids.

We have identified DRG related sequences in other pathogenic Kinetoplastids as well. Not unexpectedly, the extent of homology decreases as one compares *Leishmania* sequences to other Kinetoplastids. However, across all the Kinetoplastid species we have examined, a 36 aa region is conserved in all species (Fig 3.12).

3.2.8 Bioinformatic analysis of DRG protein

Sequence analyses of *LdDRG* showed that the composition of *LdDRG* ORF was GC-rich which is consistent with the overall GC content of the *Leishmania* genome (Ivens et al., 2005). Both *LdDRG* ORFs encode a polypeptide of 79aa with a calculated molecular mass of 8.4 kDa and a pI of 8.3.

A search against all available non redundant protein databases (pfam, SWISS-PORT, UniProt and PROSITE server at www.expasy.org) showed that DRGs are novel protein with no similarity to any known protein families. The LdDRG deduced protein sequence was analyzed using TMPred and TMHMM server at www.expasy.org. Those analyses indicated that this protein has two hydrophobic transmembrane domains: TM-1 (5-20aa) and TM-2 (25-42aa) (Fig 3.11 & 3.13). Based on the von Heijne algorithm the N-terminal transmembrane domain (5-20aa) constitutes a putative signal peptide. WOLF-PSORT server at www.expasy.org predicts a cleavage site between the two transmembrane domains (Fig 3.13).
<table>
<thead>
<tr>
<th></th>
<th>Ld_DRG1</th>
<th>Lmj_DRG1</th>
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<td>SSVENV</td>
<td>NSEEYV</td>
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**Figure 3.11 ClustalW alignments of DRG protein sequences from multiple *Leishmania* species** (Ld, *L. donovani*, Lin, *L. infantum*, Lmj, *L. major*, Lbr, *L. braziliensis*). The numbers on the right indicate aa positions. Colored regions indicate the predicted trans-membrane domains (TM1 and TM2). Highlight of conserved changes at the carboxyl terminus of the DRG proteins. Identity across all sequences are indicated with (*), conserved substitutions by (:) and semi-conserved changes by (.)
Figure 3.12 ClustalW alignment of DRG1 protein sequence and its homologs from other Kinetoplastids. Conserved 36 aa region was underlined. Colored region indicate the second predicted transmembrane domain. Identity across all sequences are indicated with (*), conserved substitutions by (.), and semi-conserved changes by (>).
Figure 3.13 Bioinformatic analysis of DRG protein. A schematic showing the predicted two transmembrane domains of DRG protein; result of TMPred and PHDHTM server showing the domain position, H denotes transmembrane domains; cleavage site between two transmembrane domain predicted by SignalP algorithm.

Based on its overall hydrophobicity, presence of two transmembrane domains suggests that the LdDRG proteins represents a membrane associated protein or insoluble protein.
3.2.9 Expression of Recombinant DRG1 Protein

For the expression of DRG1 in *E. coli*, the *DRG1* gene was cloned in pET28a+ expression vector. The vector is an *E. coli* expression vector containing the T7 promoter, which is not recognized by *E. coli* RNA polymerase, and therefore virtually no expression occurs until a source of T7 RNA polymerase is provided. It allows the expression of the recombinant protein with N-terminal or C-terminal fusion with 6X-His tag. This His-tag will be used for purification of recombinant DRG1 protein.

For that coding sequence of DRG1 was PCR amplified from genomic DNA of *L. donovani*. The amplified 245bp PCR product was cloned into TOPO-TA PCR2.1 vector and sequence verified. Then *DRG1* ORF was sub cloned into pET28a+ vector. Positive clones were first selected by PCR and reconfirmed by restriction digestion and then sequence verified for the right frame. A positive clone was then chosen for expression studies. First pET28a+DRG1 was transformed into *E. coli* BL-21 (λ-DE3) and induced with IPTG for the expression of recombinant protein. I optimized expression conditions by testing several different IPTG concentrations (0.5-2.0 mM), induction durations (1-4 h) and growth temperature (18°C & 37°C). Best conditions for induction were 1 mM IPTG for 4 hr of induction duration at 37°C. These standardized conditions were used for batch induction and purification of 6X-His-DRG1 protein.

To devise an appropriate purification strategy, I proceeded to examine the relative distribution of the expressed recombinant protein in the soluble and insoluble fractions. Both the supernatant and the pellet of the cell lysate after sonication were examined to detect the recombinant protein. As shown in (Fig 3.14) almost 90% of the expressed recombinant protein was present in the insoluble fraction. Several unsuccessful attempts were made to get the recombinant DRG1 protein in soluble fraction like induction with minimum amount of IPTG, growing the cells at low temperature (18°C) and using different bacterial host cells etc. This is consistent with DRG being a part of insoluble fraction because of the presence of two transmembrane domains.
Figure 3.14 SDS-PAGE analysis of crude lysate of 6X-His-DRG1 expressed in recombinant *E. coli* BL21 containing pET28a-6X-His-DRG1 vectors. After 3 hrs of IPTG induction, cells grown at 37°C were collected by centrifugation. Crude protein samples were fractioned into Whole cell lysate (WCL), soluble fraction and insoluble fraction. Protein band on SDS-PAGE gel were visualized by Coomassie blue staining. Protein size markers were shown on left.

Finally the protein was purified in denaturating condition (**Fig 3.15**). The yield of purified protein was excellent (2-3 mg of purified protein per 100 ml of culture).

Figure 3.15 SDS-PAGE analysis of purification of 6X-His-DRG1 in denaturating condition from the soluble fraction of recombinant *E. coli* BL21 that contain over expressed 6X-His-DRG1. After binding to Ni-NTA slurry, unbound fraction was also analyzed on SDS-PAGE to ensure complete binding of recombinant protein to resin. Wash 1-4 and then purified protein was eluted in five fractions (Elute 1-5). Protein bands on SDS-PAGE gel were visualized by Coomassie blue staining. Protein size markers were shown on left.
Recombinant 6X-His-DRG1 purified protein was excised from SDS-PAGE in denatured form for raising antibodies. Antibodies against recombinant DRG1 (purified from SDS-polyacrylamide gels) were raised in mouse and the antisera were analyzed by using dot-blot assay to determine the antibody titer, after the second booster dose. The titer was very good and able to pick up 10 ng of purified recombinant protein on western blot with 1:5000 dilutions (Fig 3.15).

3.2.10 Specificity and Sensitivity of DRG1 antibody

The antiserum were able to recognize the recombinant protein but was unable to detect the endogenous protein on western blot analysis when using 10 μg of total protein lysate from Leishmania (Fig 3.16).

![Figure 3.16 Western blot of whole cell lysates of E. coli](image)

**Figure 3.16 Western blot of whole cell lysates of E. coli** transformed with either pET28a vector (Lane 1) or pET28a-6X-His-DRG1 (uninduced and induced culture; Lane 2 and 3) and 10 ng of purified recombinant 6X-His-DRG1 protein. Also 10 μg of whole cell lysates from stationary phase promastigote (Lane 5 and 6) and axenic amastigote (lane 7 and 8) of attenuated and virulent line of L. donovani respectively was also probe with polyclonal anti-DRG1 antibody. Molecular size protein markers are shown on the left.

Since recombinant 6 X-His-DRG1 protein was used to generate the antibody, it was possible that DRG1 sequence was not very immunogenic and antibody has been generated for only the epitopes of 6X-His tag. To check that I thought of blocking most of the 6X-His epitopes with anti-META-1 antibody which is generated in our lab against recombinant 6X-His-META1 protein. 50 ng of purified recombinant DRG1 and META1 proteins were loaded in alternate lane and separated on SDS-PAGE and then transferred onto nitrocellulose membrane. This filter was then cut into four filters (A-D) in such a way that each filter contains one lane of each protein. Each filter was then incubated separately with different antibodies.
Schematic of sequential incubations (I-V) and dilution of the different antibodies are shown in Fig 3.17 (Top panel).

<table>
<thead>
<tr>
<th>Filter/Incubation</th>
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<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
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<td>anti-META1 1:5k</td>
<td>anti-META1 1:5k</td>
<td>anti-META1 1:5k</td>
<td>anti-DRG1 1:500</td>
</tr>
<tr>
<td></td>
<td>anti-mouse FITC 1:1k</td>
<td>anti-mouse FITC 1:1k</td>
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<tr>
<td>II</td>
<td></td>
<td></td>
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<tr>
<td>III</td>
<td>anti-DRG1 1:500</td>
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<tr>
<td>IV</td>
<td>anti-mouse biotinylated 1:2k</td>
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</tr>
<tr>
<td>V</td>
<td>avidin conjugated with HRPO 1:4k</td>
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<td>avidin conjugated with HRPO 1:4k</td>
<td>avidin conjugated with HRPO 1:4k</td>
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Figure 3.17 Specificity of polyclonal antibody generated against the recombinant 6X-His-DRG1. Top panel: a schematic of different antibodies used in the western blot. Name and dilution used for each antibody are given. Each antibody were incubated minimum for 1 hr. Bottom Panel: Western blot of purified protein of recombinant 6X-His-DRG1 and 6X-His-Meta1, filter A-D were incubated with different series of antibodies shown in the schematic and all filter were developed at the same time with same exposure time. Protein size markers are shown on the left.

Filter A & B was incubated with anti-META1 antibodies in 1st incubation which will bind to 6X-His-META1 protein and only His epitopes of 6X-His-DRG1 protein. IIrd incubation with anti-mouse FITC antibodies should block all the epitopes of anti-META1 antibodies leaving DRG1 epitopes free. After that only filter A was incubated with anti-DRG1 antibodies. Filter C & D were used as a control for anti-META1 and anti-DRG1 antibodies. The IVth and Vth incubation were done.
simultaneously for all filters with anti-mouse biotinylated and avidin conjugated with HRPO respectively to enhance the signals for better detection.

In filter C & D each antibody specifically binds to their respective proteins. Some cross reactivity was also observed that is because of presence of common His epitopes in both proteins. In filter B no or very less signal was expected because anti-mouse FITC antibodies should block all binding sites for anti-mouse biotinylated antibody but signals were observed in both lane of filter B. But only a little decrease in intensities of both bands was observed in filter B than that of filter C & D. That may be either because of improper blocking of anti-META1 antibodies binding site by anti-mouse FITC antibodies or higher dilution of anti-mouse FITC antibodies. A very high Increase in the intensity in the DRG lane was observed in filter A where this filter was incubated with anti-DRG1 antibodies after blocking. This proved that polyclonal antibody has been generated for all epitopes of the recombinant 6X-His-DRG1 protein (Fig3.17).

Once I was sure that antiseras is raised against all to recombinant 6X-His-DRG1 protein, then only possibility because of it is not able to pick the endogenous protein, remain was the availability of DRG epitopes in the protein lysate. For that I have made the protein lysate from different stages of parasite or lysate made in various biochemical conditions. I have tried following conditions to increase the availability of DRG epitopes:

To increase the availability of DRG epitopes:
1. increase the protein availability: More amount of protein (up to 100 µg)
2. Change the antigen/antibody ratio: Lower dilution of Ab (1:500):
3. Changes the kind of epitopes: lysate were made in denaturating condition (in 8M urea)
4. Changes the cytological availability: Soluble Vs insoluble fraction 100 µg each
5. Stage specific availability of DRG epitopes: lysate from Different stages of parasite; Promastigote Vs Amastigote and Log vs Stationary phase
6. Effect of host factors: in-vitro Vs in-vivo (Infected tissue samples)
7. Tested culture media in the case DRG protein is being secreted
8. Tested whether DRG is labile protein (lysate in presence of different lysosomal protease inhibitor or lysate made from the cells grown in presence of proteasomal inhibitor MG132)

In all above biochemical condition, I was not able to detect the endogenous protein by polyclonal antibody generated. This was very striking that antibody is able to detect the recombinant protein but not the endogenous. It left several unanswered questions and challenges in front of me to study this protein.
3.3 Discussion

We have used differential display PCR to identify the stage specific genes between promastigote and amastigotes stages of the parasite. In the present study, I describe here the identification and characterization of a novel pair of paralogs, *DRG1* and *DRG2* in *Leishmania donovani*. The proteins encoded by these paralogs of *DRG* are highly conserved, differing only at 3 amino acids of the last 6 amino acids.

**DRG paralogs are differentially expressed**

Northern blot analyses and QRT-PCR analysis revealed the differential expression of *LdDRG1* and *LdDRG2* mRNA in *L. donovani*. *LdDRG1* transcripts showed a up regulation of 1.2 fold in virulent and 3.2 fold in attenuated promastigotes from logarithmic phase to stationary phase and its expression was also detected in amastigotes whereas *LdDRG2* mRNA shows 4-5 fold increase in expression from in both virulent and attenuated promastigote from logarithmic phase to stationary phase and but *DRG2* expression was highly down regulated in amastigotes (Fig 3.8 & 3.9). Since the *Leishmania* genome is shown to be constitutively expressed (Sexena et al. 2007; Akopyants et al. 2004; Almeida et al. 2004; Leifso et al. 2006), very few genes showed differential expression in different stages. A study revealed that only ~3.5% of total genes shows differential regulation between promastigote and lesion derived amastigote mRNA in *L. mexicana* (Holzer et al. 2006). Also promoter for RNA polymerase II enzymes have not been identified in *Leishmania*, transcription initiates at 5’ end of gene clusters and undergo polycistronic transcription (Martinez-Calvillo et al., 2003; Martinez-Calvillo et al., 2004). Mature RNA is produced by 5’ trans-splicing and 3’ polyadenylation that occurs co-transcriptionally. Therefore differential stage-specific RNA expression is regulated by post transcriptional and processing events leading to differential mRNA stability (Clayton, 2002; Kelly et al., 2001; McNicoll et al., 2006; Palenčár and Bellofatto, 2006; Murray et al., 2007). In the absence of transcriptional control of mRNA abundance, sequences within 3’UTR play an important role in regulation the differential expression of the genes in *leishmania* (McNicoll et al., 2006). Since both *LdDRG* genes have distinct 3’UTR, these were expected to be differentially expressed.
This is consistent with our findings that the two DRGs are expressed differentially and therefore I expect them to be regulated by different mechanism.

**DRG paralogs are differentially regulated in different stages and between virulent and attenuated line**

In the absence of transcriptional control, differential stage-specific RNA expression is regulated by post transcriptional and processing events leading to differential mRNA stability. It is possible that a labile regulatory protein either inhibits or enhances the transcription or causes degradation or stabilizes the target mRNAs, the amount of this regulator could rapidly decrease after its synthesis is inhibited by cycloheximide. This result would result in increase or decrease in the abundance of the targeted mRNA. Therefore I have examined the sensitivity of steady state levels of individual DRGs to cycloheximide. Both DRG1 and DRG2 genes exhibited different responses in virulent and attenuated line of *L. donovani*. In promastigote stage both DRG1 and DRG2 transcripts showed moreover similar responses when the logarithmic and stationary phase cultures either from virulent or attenuated line were treated with cycloheximide except that the stability of DRG2 transcript increases to a greater extent in attenuated logarithmic phase promastigote culture in response to cycloheximide treatment (**Fig 3.10**).

However both DRG1 and DRG2 mRNA showed different stability in response to cycloheximide treatment in amastigote stage. Stability of DRG1 and DRG2 transcripts increases to the greater extent in virulent amastigote than the attenuated amastigote. Here it is important to note that DRG2 transcript is highly down regulated in virulent and attenuated amastigotes (**Fig 3.10**). Thus regulatory mechanisms dependent on de novo protein synthesis may explain the differences in observed levels of expression. Also presence of different labile regulatory factors in the virulent and attenuated line may be possible explanation of differential response of towards the regulation of DRG1 and DRG2.

Sequencing of DRG locus revealed that DRG1 and DRG2 have unique 3’ UTR and 5’ UTR sequences. These distinct sequences in 3’UTR may interact with different regulatory factors hence showing different regulation. Very high degree of sequence identity in flanking sequences of respective DRGs, across *Leishmania* species,
predicts that the pattern of DRG expression would be similar in the different Leishmania species, to that observed in L. donovani. The greatest difference in DRG flanking sequences was observed with L. braziliensis sequences. Interestingly, despite the greater variance of the flanking sequences in L. braziliensis and the presence of additional aa sequence in LbrDRG1, the rest of the protein sequence is virtually identical to the other species. The additional sequence present in LbrDRG1 bears a limited homology to the conjugal transfer protein TrbE1 in bacteria. It is not certain whether divergence observed in L. braziliensis DRGs reflects actual divergence or quality of available sequence information.

**DRGs are highly conserved across Leishmania genus**

We have identified DRG related sequences in other pathogenic Kinetoplastids as well. Not unexpectedly, the extent of homology decreases as one compares Leishmania sequences to other Kinetoplastids. Bioinformatics analyses and different topology prediction programs shows that DRG proteins contain two transmembrane domains. However, across all the Kinetoplastid species we have examined, a 36 aa region encompassing the second predicted trans-membrane domain is conserved in all species (Fig 3.12). Indeed nucleic acid probes designed to detect this sequence do hybridize to genomic DNA from T. brucei and to RNA from procyclic forms of T. brucei. This is indicative that DRG related sequences are present and expressed in other TriTryps. This leads us to hypothesize that DRG protein may have similar functions in other organisms of the family.

Polyclonal antibodies generated against the 6XHis-LdDRG1 recombinant protein are not able to pick the endogenous protein in Leishmania lysate from different stages and conditions where the LdDRG transcripts are expressed in higher amount. Generally expression of mRNA is manifested as the abundance of the protein level but earlier it has been shown that steady state level of a given mRNA in yeast does not necessarily correlate with the amount of the protein encoded by that particular mRNA (Gygi et al. 1999). In L. infantum also, mRNA abundance determined by microarray analysis showed a very weak correlation with protein abundance determined by ICAT analysis (McNicol et al. 2006). This might be the plausible explanation that in spite of very high expression of DRGs RNA, they
produce very low amount of protein or DRG protein are very much modified post translationally so that it is not accessible for the antibodies.

The high degree of conservation and the restriction of substitutions to a very limited region of the protein suggest that the DRG proteins likely play important roles in *Leishmania* biology. The two DRGs can be viewed as natural mutants of each other; if anyone is taken as prototype, the other can be seen as identical at all but 3 positions. The predicted presence of two extremely conserved transmembrane domains suggests that the DRG proteins traverse in and out of membranes, leaving the C terminus available for interactions with other molecules. Furthermore, the positions that are altered are consistent in all *Leishmania* species. Thus, changes at these positions are clearly responsible for alteration in properties and function. It is likely that the substitutions leads to local changes that allow the C termini of the DRGs to interact with unique partners within the cell. Thus DRG1 and DRG2 may interact with a common set of interactors because of their conserved sequences and with a unique set of interactors because of their distinct C termini.

Inability of anti-DRG1 antibodies to detect the endogenous protein by western blot and cellular localization of these paralogs are several such queries that await further studies. Furthermore, the physiological importance of this differential expression of *LdDRG1* and *LdDRG2* remains to be determined. Over expression of DRG1 and DRG2 in *Leishmania* as a fusion protein with GFP or monomeric RFP will certainly help in answering some of these basic questions their localization. These studies can also give me some clues of the function of the DRGs. To address these queries, DRG1-GFP and DRG2-mRFP over expression clones were generated and analyzed.