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The present study aimed to identify and characterize *Leishmania donovani* specific Ubiquitin related modifier-1 (LdUrm1); a post translational modifier and its associated conjugation pathway by employing the basic and advanced molecular techniques. The study contributes towards understanding the mechanism of *Leishmania* pathogenicity and virulence. The specific objectives of the study involved:

1. **Bioinformatics analysis to identify the structural and functional homology of protein components of Urm1-Uba4 conjugation pathway in *Leishmania donovani*.**

   Bioinformatics analysis had been carried out to identify the homologues of Urm1 and its E1-like enzyme Uba4p using the corresponding gene sequences from *S. cerevisiae* as query. Phylogenetic analysis was performed to understand the evolutionary relationship amongst the homologues of the above said genes from various species of *Leishmania* and *Trypanosoma* with respect to yeast and human. Subsequently homology model for *Leishmania* specific Urm1 was generated using Swiss Model Workspace (An automated homology modelling tool available online). The quality assessment was carried out using Ramachandran plot and PROCHECK etc. The putative E1-like enzyme, Uba4 from *Leishmania* was analyzed for the sequence identity, phylogenetic analysis and InterproScan analysis performed to search for conserved domains.

2. **Evaluation of the expression profiles of LdUrm1 and LdUba4 in *Leishmania* both at RNA and protein level.**

   Gene expression profiles generated at both RNA and protein level are believed to be important from functional point of view. They are the direct tools to estimate the utility of a gene both at the transcriptional and translational fronts. Therefore, RNA preparations were made both from *Leishmania* promastigote cultures and patient samples (blood and bone marrow aspirates). Subsequently, Reverse transcriptase PCR was performed with the primers designed for *Leishmania* specific Urm1 and Uba4 genes and their relative abundance at RNA level was predicted using appropriate endogenous controls. Likewise, subsequent to generation of polyclonal antibodies against recombinant Urm1 and Uba4 proteins of *Leishmania*, the respective expression analysis was performed for both the above stated proteins by Western Blotting.
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   (i) PCR amplification, cloning and sequence determination:

   Using the primers designed for URM1 and UBA4, the corresponding genes were amplified from *Leishmania* genome, cloned into TA cloning vector pGEMT Easy and the sequences were determined. A comparative analysis with other trypanosomatids sequences available on database was done. The gene sequence were further subcloned into expression vectors like pET28a(+), pPROEXHTC, pGEX4T after the restriction digestion with the enzymes corresponding to vectors used for expression of recombinant proteins. These vectors have characteristic N or C terminal His/GST tag that assisted to identify the positive clones downstream.

   ii) Selection of positive clones expressing recombinant proteins:

   Subsequent to gene sequencing and cloning into expression vectors, clones were checked for their correct orientation into the vector backbone by restriction digestion. The positive clones thus identified were used to express the corresponding proteins under defined/ controlled laboratory conditions using IPTG induction method.

   iii) Purification of recombinant LdUrm1 and LdUba4 proteins:

   Recombinant proteins were purified using either Ni-NTA metal affinity chromatography or gel exclusion chromatography for 6XHIS tag and GST tag containing proteins respectively. Purified recombinant protein preparations were assessed for their purity on Western Blots using anti-Histidine and Anti-GST monoclonal antibodies.

4. Raising polyclonal antibodies against both LdUrm1 and LdUba4 proteins in rabbit.

   Polyclonal antibodies were raised against both the recombinant proteins in rabbits. At day zero before immunization pre immune serum was collected, which served as negative control. Rabbits were immunized with proteins along with an adjuvant to raise immune responses against the proteins. First and second bleed were collected and sensitivity of the polyclonal antibodies raised eventually was assessed by Western blotting before undertaking any further studies.
5. Characterization of LdUrm1 and LdUba4 proteins.

i) Identify the intracellular location of LdUrm1 and LdUba4 proteins:

Once the polyclonal antibodies raised against the LdUrm1 and LdUba4, their specificity to the recombinant proteins was checked by Western blotting. Pre-immune serum was used as the negative control. The intracellular localization of the proteins was predicted subsequently to identify if they are organelle specific or cytoplasmic in location employing immunofluorescence assay.

ii) Adenylation and activation of LdUrm1 by LdUba4:

To establish Uba4 as the activation enzyme for LdUrm1, in vitro activity assay (Molybdenum blue assay) was performed. The protocol for this assay was adapted from a work by Berndsen and Wolberger, 2011. Immunoprecipitation methods were employed to show activation of LdUrm1 in the presence of ATP.

iii) Evaluation of the importance of a C-terminal diglycine motif for LdUrm1:

To establish the importance of C-terminal di-glycine motif of LdUrm1 form specific interaction with its target proteins and its E1 enzyme i.e. LdUba4, a non-conjugatable form of the gene with the deleted C-terminal di-glycine motif was prepared. The mutant constructs were cloned into Leishmania specific expression vector pKSNEO. The plasmid containing insert in correct orientations were transfected into the wild type parasites by electroporation and the effect of mutation was studied by analysing the effect on the growth pattern of the parasite.

6. Identification of the protein targets of LdUrm1 in Leishmania by mass spectroscopic methods.

Using antibody specific to LdUrm1, a pull down was performed to immunoprecipitate the LdUrm1 substrateproteins from L. donovani lysate preparations. Western blot analysis was performed using α-LdUrm1 antibody to discriminate the non-specifically pulled down proteins onto gel. The corresponding bands identified on the blot were cut from the gel and further analyzed by MALDI-TOF/MS tools to identify the putative LdUrm1 targets in L. donovani to identify the probable role of this ubiquitous gene in Leishmania pathogenesis.
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7. Conclusion and Thesis writing.

Data analysis was done and Results were accounted. On the basis of the results of the research work carried out for my thesis, a manuscript entitled “Ubiquitin related modifier-1 (LdUrm1): an early endosome associated ubiquitin like conjugation in Leishmania donovani” was drafted and communicated to Molecular Microbiology, a peer reviewed journal of Wiley Blackwell’s. The article has got published with following citation:


Scopes and Limitations of the study

Due to a large spectrum of drug resistance and emergence of kala-azar as an opportunistic infection in HIV infected persons, the incidence of the disease is increasing at an alarming proportion throughout the world. Chemotherapy and vector control are the only effective measures for combating the disease, which is nearly always fatal, if left untreated. Past efforts for development of vaccines have clearly shown the need for a better understanding of the mechanism of Leishmania pathogenesis. In such situation basic studies underlining the biology of Leishmania may provide new leads for translational outcome. Studies outlined contributes towards our understanding of the mechanism of post-translational modification in Leishmania and upgrades our knowledge on development of disease Leishmaniasis. The proposed study to our knowledge is the first demonstration of Leishmania specific ubiquitin related modifier-1 (LdUrm1), its conjugation pathway and its target proteins in L. donovani. Moreover, the identification of LdUrm1 mediated protein modification pathways in Leishmania, with its distinct subset of diverse substrate proteins highlighting their role in parasite biology and may be evaluated in future as potential drug targets.

The present study is a preliminary step to highlight the mechanism of post-translational modification procedure in L. donovani mediated by Ubls. It generates a comprehensive data set outlining the number of proteins and the interacting partners important for the cellular machinery of this parasitic protozoan, however subsequent studies with extensive experimental evidences are further required to identify and establish suitable drug targets for future implementations.