Chapter-4
Discussion
*Leishmania* are protozoan parasites from the family Trypanosomatididae that cause Leishmaniasis. This disease represents a major public health risk in many tropical and subtropical regions of the world. Latest reports show that this disease affects an estimated 12 million peoples around 88 countries, with approximately 2 million new cases appearing every year ([www.who.int/ctd/html/leis.html](http://www.who.int/ctd/html/leis.html)). Some latest reports of the spread of Visceral Leishmaniasis had come up in Sudan where around 1,00,000 lives seemed affected (Seaman et al, 1996). Hence, there is an immediate need to develop newer methods to combat this disease as most of the existing drugs are either ineffective against this parasite due to drug resistance or are unaffordable to the patients due to high prices. To develop more effective and less toxic newer anti-*Leishmania* drugs, it is required to explore the molecular biology of the parasite in order to identify the parasite proteins that are essential for its survival.

The nucleus of any organism is an indispensable organelle as it carries and protects the genetic material of any cell which has the basic information hidden in it in form of nucleotide sequences which are later translated into protein molecules. A number of diseases are linked to the expression or non expression of various genes (that is, their expression at a wrong time or non expression at desired time). The expression of most of these genes is controlled by various chromatin remodeling complexes in the ways that they can regulate transcription by preventing RNA polymerase from unnecessarily accessing the promoter regions of genes which are not needed by the cell, as well as by changing the position of the nucleosome to allow access to promoter of any gene to get expressed at any particular stage of parasite's life cycle. The proteins present in these chromatin remodeling complexes play a key role in the functioning of these complexes. In light of the above information, it may be mentioned that the study of these proteins that are present in the nucleus and are a part of any chromatin remodeling complex is of immense importance and such proteins may serve as a
drug target for control and eradication of the diseases like leishmaniasis. The present study, for the first time, shows the presence of actin-related protein in *Leishmania* and its localization in the nucleus as well as its interaction with chromatin, suggesting its possible role in chromatin remodeling or remodifying processes.

ARP2/3 complex is both an essential and ubiquitous component of the actin filament network. This complex is a seven subunit protein complex whose composition appears to be highly conserved throughout the evolution. This complex contains two actin-related proteins, viz. ARP2 and ARP3 together with 5 small subunits, and was first identified in *Acanthamoeba*, using profilin affinity chromatography (Machesky et al, 1994). These subunits (called Arcs for ARP2/3 complex) include p40-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc (the molecular mass of each of these components varies slightly among species). The ARP2/3 complex exhibits three types of interactions with actin: (1) it binds to the sides of actin filaments, (2) it caps the slow growing pointed ends of actin filaments, and (3) it promotes the nucleation of actin monomers (Mullins et al 1997, 1998a). The high affinity pointed end binding activity of ARP2/3 complex could also lead to enhanced actin assembly where this complex binds and stabilizes the pointed ends of actin filaments generated by other mechanisms such as severing of existing filaments (Bailly et al, 1999). The pointed end capping activity of ARP2/3 complex together with a cellular concentration of 2μM suggests that in vivo most of the actin filament pointed ends are capped by ARP2/3 complex. The ARP2/3 complex is attached to the actin filaments at 70° angle in lamellipodia of keratocytes and fibroblasts, suggesting that it organizes filaments into networks *in vivo*.

WASp-related proteins have long been implicated in regulation of actin cytoskeleton. Scar1, a member of the WASp (Wiskott-Aldrich Syndrome protein) related protein family, also activates actin nucleation by ARP2/3 complex *in vitro*. The C termini of Scar1 and N-WASp which includes domains with homology to cofilin flanked by acidic regions is important for stimulation of the actin nucleating activity of this complex. WASp related proteins help to link signal
transduction pathways to stimulated actin assembly via small GTP-binding proteins. In assays using purified components the actin nucleation activity of ARP2/3 complex is highly stimulated by including both GTP-charged Cdc42 and phosphatidylinositol 4,5-bisphosphate (PIP₂) in the assay mixture. The binding of PIP₂ and Cdc42 to N-WASp may help its localization to the membrane and promote interactions with ARP2/3 complex thereby activating actin assembly. Unlike ORF LmjF21.0230 (Leishmania ARP), ORF LmjF19.1200 (ARP2) and ORF LmjF15.1360 (ARP3), were predominantly localized in the cytoplasm, but not with the actin network, suggesting that unlike in other eukaryotic cells, ARP2/ARP3 in Leishmania perhaps plays no role in actin filament nucleation and branching. Since Leishmania actin does not appear to exist in form of branched filaments, ARP2 and ARP3 may play some other important role rather than assisting actin.

4.1. Phylogeny

The actin-related proteins (ARPs) superfamily came into light in the early 1990s with the discovery of several novel proteins whose primary sequences suggested similarity, but not identity to conventional actin. Since then, several families of actin-related proteins have been defined with each ARP assigned to a family on the basis of its predicted amino acid sequence and its extent of homology to conventional actin. The actin-related proteins are members of a highly diverse and ancient superfamily, the Actin Superfamily. This superfamily includes, actins, ARPs, hsp/hsc70s, divergent forms of hsp/hsc70s (which can be described as hsp70-related proteins), sugar kinases of both prokaryotes and eukaryotes, a variety of bacterial ATP-binding proteins, such as FtsA, StbA, MreB, and certain phosphatases. The budding yeast Saccharomyces cerevisiae whose entire genome sequence is known has been used to classify and analyze the functions of ARPs. This organism shows the presence of 10 ARPs (ARP1-ARP10) that
have been classified according to their similarity to yeast actin, where ARP1 is the most similar and ARP10 is the least similar. Three major classes of ARPs, ARP1, ARP2 and ARP3 have been named and classified according to the level of their amino acid sequence identity (ARP1-50%, ARP2-45% and ARP3-35%) with yeast actin. ARPs closely related to ARPs 1-3 of S. cerevisiae have been characterized in various organisms including vertebrates. A growing number of more distantly related ARPs exist in a variety of cells which no longer phylogenetically correlate with actin. As the reports of the presence, localization and functional analysis of novel ARPs in various organisms are increasing rapidly, a new method of classification had immerged, where these ARPs are classified into various subfamilies based on their similarities to various ARPs present in budding yeast. On the basis of the above classification, it may be assumed that Leishmania ARP may belong to ARP6 subfamily because it shows maximum similarity to ARP6 of yeast. Analysis of the complete genome sequence of Arabidopsis shows the presence of nine highly divergent ARP genes that fall into eight ancient classes. Surprisingly, yeast ARP1 and ARP10 classes are completely absent in Arabidopsis, rice and possibly other plants, where the loss of these ARPs is predicted to correlate with the loss of motile sperms in pre-angiosperms.

Cytosolic ARPs, are those that are found in the cytoplasm and are involved in cytoskeletal functions, like vesicle motility, formation of branched actin filaments, etc. Apart from ARP1, ARP2, ARP3 and ARP10 that are found in the cytoplasm, rest of the more diverged ARPs like ARP4, ARP5, ARP6, ARP7, ARP8 and ARP9 are found to be localized in the nucleus of a variety of cell types and termed as nuclear ARPs. A common feature of actin and actin-related proteins is the presence of Actin Fold, a tertiary structure that can tolerate enormous sequence diversity and consists of the core domain and the ATP-binding pocket.
Most ARPs are predicted to be larger than actin. The presence of large insertions that create surface loops in many ARPs or deletions that remove an entire subdomain provide the structural basis for novel biochemical properties of actin-related proteins that are distinct from those of conventional actins. In case of budding yeast, the largest insertion is found in subdomain 4 in ARP5p and represents 223 amino acids out of a total of 755 whereas the greatest number of insertions are found in ARP8p (881 amino acids). In case of *Leishmania* ARP, six long insertions are seen at various positions in the molecule whereas in *Leishmania* ARP2, only two small amino acid sequence insertions and two deletions are present. Further *Leishmania* ARP3 possesses five small insertions at different positions.

### 4.2. Divergence

Unlike ARP1, ARP2 and ARP3, the nuclear ARPs show a wide range of divergence when compared to their conventional actins. A number of insertions and deletions have been reported in nuclear ARPs present in various organisms. In budding yeast 881 amino acid sequence insertion has been reported in ARP8 and 223 amino acids in ARP5, whereas in case of ORF LmjF21.0230 (*Leishmania* ARP), six long insertions are present at various positions in the whole molecule when compared to *Leishmania* actin. The presence of large amino acid sequence insertions in these molecules create surface loops which likely provide the structural basis for novel biochemical properties of these molecules. The presence of these insertions may help the molecule to interact with histone proteins present in the nucleosomes, which in turn may regulate the positioning of nucleosomes to provide or hinder access of RNA polymerases to promoter regions of various genes. These amino acid sequence insertions may
also provide regions for other ARP molecules to interact with each other or with actin. From studies carried out in a number of organisms it has been well established that most of the ARPs, whether cytoplasmic or nuclear function in association with other ARPs or actin. Apart from various amino acid sequence insertions *Leishmania* ARP shows only 13% amino acid sequence identity when compared to *Leishmania* actin. When all *Leishmania* ARPs and *Leishmania* actin were analysed using Phylip package, it was found that *Leishmania* ARP is the least similar to all other *Leishmania* ARPs, but it showed the maximum similarity to ARP6, suggesting that this protein may belong to ARP6 subfamily.

The alignment of ORF LmjF19.1200 (ARP2) with *Leishmania* actin, shows an identity of 24%, whereas the identity is 29% with yeast ARP2. Similarly ORF LmjF15.1360 (ARP3) is 29% identical to *Leishmania* actin and 30% to yeast ARP3.

### 4.3. Localization

ARPs present in budding yeast were analysed for their similarity to Yeast actin, it was seen that ARPs showing higher sequence identity as well as more conserved amino acid sequences, as compared to Yeast actin are localized in the cytoplasm. On the contrary, the diverged ARPs are found mainly in the nucleus. In the same way, when *Leishmania* ARP, a highly diverged protein, was analysed by immunofluorescence microscopy using anti-*Leishmania* ARP antibodies, it was seen that this protein is localized mainly in the nucleus of *Leishmania* promastigotes. The nuclear localization of *Leishmania* ARP is further revealed by overexpressing this protein as its GFP conjugate and demonstrating that GFP-*Leishmania* ARP conjugate, like native ARP is predominantly localized
in the nucleus. Nuclear localization of *Leishmania* ARP was further confirmed by chromatin immunoprecipitation (ChIP) assay.

The nuclear localization of this ARP is of immense importance from physiological as well as pathogenic point of view, as this protein may play a crucial role in pathogenesis of this organism which would come to light in subsequent studies. The presence of high amounts of ARP in the promastigotes as well as its association with chromatin further suggests that this protein may be involved in chromatin condensation during cell division process. Three types of mitosis have been reported in eukaryotes- 1) Open: that occurs in higher eukaryotes, where at the onset of prophase, nuclear envelope disassembles and reforms at the telophase stage, 2) Closed: where nuclear envelope partly disintegrates at the prophase stage, and 3) semi-closed: where nuclear envelope disintegrates from poles only at the metaphase stage (Kiseleva et al, 2001). Although, the formation of intra-nuclear spindle fiber has earlier been reported in *Leishmania* (Urena, 1986, 1988), little is known about the type of the mitosis process that *Leishmania* follows. It is quite likely that nuclear ARP in *Leishmania* may play a significant role in the control of mitosis.

**4.4. Interaction with chromatin**

To explore the possible interaction of *Leishmania* ARP with chromatin in the nucleus, chromatin immunoprecipitation (ChIP) was performed on normal (wild type) *Leishmania* promastigotes using mono-specific anti-*Leishmania* ARP antibodies. The immunoprecipitated chromatin material was analysed through PCR using set of primers for three different genes, *L. donovani* profilin (Accession No. AF466647), truncated coronin (Accession No. AY995899) product of 621 basepairs (912-1533) and actin (Accession No. AY079087)
genes. The above study brings into light two important aspects of this protein. (1), this protein in normal (wild type) cells is localized in the nucleus, and (2) interacts with chromatin, suggesting its probable role in chromatin remodeling/remodifying processes. In most eukaryotic cells, the nucleus is a membrane enclosed organelle and contains genetic material organized as multiple long linear DNA molecules in complex with a large variety of proteins, such as histones, to form chromosomes. The genes within these chromosomes make up the genome. The function of nucleus is to maintain the integrity of these genes to control the activities of the cell by regulating the gene expression. As the major defining characteristic of eukaryotic cell, the nucleus' evolutionary origin has been the subject of much speculation. Four major theories have been proposed to explain the existence of the nucleus. Firstly, the theory known as 'syntrophic model' proposes that a symbiotic relationship between the archaea and bacteria created the nucleus containing eukaryotic cell. A second model proposes that proto-eukaryotic cells evolved from bacteria without an endosymbiotic stage. This model is based on the existence of modern planctomycetes bacteria that possess a nuclear structure with primitive pores and other compartmentalized membrane structures. The most controversial model, known as viral eukaryogenesis, proposes that the nucleus originated from the infection of a prokaryote by a virus. Finally, a fourth model, termed the exomembrane hypothesis, suggests that the nucleus instead originated from a single ancestral cell that evolved a second exterior cell membrane, the interior membrane enclosing the original cell then became the nuclear membrane and evolved increasingly elaborate pore structures for passage of internally synthesized cellular components, such as ribosomal subunits. During most of the cell cycle the chromosomes are organized into a structure known as chromatin which is a complex of DNA and protein. Nucleosomes are the fundamental repeating subunits of all eukaryotic chromatin. They package DNA into chromosomes inside the cell nucleus and control gene expression, and are made up of DNA and four pairs of proteins called histones (like histones H2A, H2B, H3 and H4), whereas histone H1 is involved in linking DNA between the two nucleosomes.
Nucleosomes appear to serve two major purposes within the cell nucleus. Firstly, they provide the lowest level of compaction which is required to fit dsDNA (on the order of meters) into the cell nucleus. Secondly, they are important in the regulation of transcription by preventing RNA polymerase from unnecessarily accessing the promoter regions of genes which are not needed by the cell. If the requirements of the cell change, enzymes known as remodeling factors can remove or change the position of the nucleosome to allow access. The process of chromatin remodeling may be defined as dynamic structural changes to the chromatin occurring throughout the cell division cycle. These changes range from the local changes necessary for transcriptional regulation to global changes necessary for chromosomal segregation. A number of nuclear ARPs have been reported in different organisms as an integral part of various chromatin remodeling complexes. In case of budding yeast where nuclear ARPs have been extensively studied it was found that three types of chromatin remodeling complexes contain ARPs as their structural and functional units. These are SWI/SNF complex, RSC complex and NuA4 complex. The yeast SWI/SNF complex contains DNA-dependent ATPase, SWI2/SNF2, along with 10 other subunits including the actin-related proteins, ARP7 and ARP9 (Cote et al., 1994, Peterson et al., 1998). Based on sequence homology to the SWI/SNF complex, Cairns et al in 1996 isolated a novel 15-subunit complex termed as RSC, that has DNA-dependent ATPase activity and the ability to remodel chromatin in vitro and contains ARP7 and ARP9 as the key proteins in functioning of this complex. In addition to cytoplasmic actin, both the NuA4 and Ino80 complex contain ARP4 as an essential subunit for the functional integrity of these complexes. Apart from the role in chromatin remodeling, nuclear ARPs now have firmly established roles in other nuclear processes, like assembly of the nuclear envelope, transcription, association of transcription factors with the nuclear matrix, mRNA processing and nuclear export. In case of flowering plants, the knockout of ARP4 protein by RNA interference or T-DNA insertion results in severe defects in overall plant physiology and flower development processes. The increasing reports of the presence and nuclear localization of distant ARPs in a number of organisms is
opening a new dimension for the study of these novel proteins and their possible role in the control of overall physiology of both plants and animals through their interactions with chromatin in the nucleus. The interactions of ARPs with actin in the nucleus is also a new challenge for researchers who are interested in establishing a clear role for these proteins in the overall physiology of plants, animals and particularly the pathogens. Due to our repeated failure to detect actin in chromatin immunoprecipitated by using anti-Leish ARP antibodies, it may be proposed that Leish ARP interacts with chromatin independent of actin and that one or more ARP may be present in Leishmania cells to assist the functioning of this protein.
Summary of the study

Actin-related proteins (ARPs) are evolutionarily a part of actin family of proteins which exhibit moderate sequence similarity to each other as well as actin. Ten ARP subfamilies have been identified in budding yeast and designated as ARP1 to ARP10 depending on their sequence similarity with actin. While ARP1 has the maximum similarity, least similarity has been observed with ARP10. Among these proteins ARP1, ARP2, ARP3 and ARP10 are present in the cytoplasm, whereas ARPs4-9 are primarily localized in the nucleus. Available data on sequencing of *Leishmania major* genome revealed that this organism like other eukaryotic cells also contains several ARPs.

To understand the role of ARPs in *Leishmania* cells, we studied the intracellular distribution of ORF LmjF21.0230 in *Leishmania donovani* promastigotes employing antibodies to this protein. This protein was localized predominantly in the nucleus of *Leishmania* cells, where it interacted with chromatin as revealed by chromatin immunoprecipitation (ChIP) assay. Based on these results, it is suggested that this protein may play an important role in chromatin remodeling or remodifying processes. Phylogenetically ORF LmjF21.0230 (*Leishmania* ARP) was closely related to ARP6 which is known to localize mainly in the nucleus of yeast cells.

Unlike ORF LmjF21.0230 (*Leishmania* ARP), ORF LmjF19.1200 (ARP2) and ORF LmjF15.1360 (ARP3), were predominantly localized in the cytoplasm, but not with the actin network, suggesting that unlike in other eukaryotic cells, ARP2/ARP3 in *Leishmania* perhaps plays no role in actin filament nucleation and branching. Since *Leishmania* actin does not appear to exist in form of branched filaments, ARP2 and ARP3 may play some other important role rather than assisting actin.
Publications from this study