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Two-headed outer- and inner-arm dyneins of *Leishmania sp* bear conserved IQ-like motifs

Aakash Gautam Mukhopadhyay, Chinmoy Sankar Dey *

Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India

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ABSTRACT

Dyneins are high molecular weight microtubule based motor proteins responsible for beating of the flagellum. The flagellum is important for the viability of trypanosomes like *Leishmania*. However, very little is known about dynein and its role in flagellar motility in such trypanosomatid species. Here, we have identified genes in five species of *Leishmania* that code for outer-arm dynein (OAD) heavy chains α and β, and inner-arm dynein (IAD) heavy chains 1α and 1β using BLAST and MSA. Our sequence analysis indicates that unlike the three-headed outer-arm dyneins of *Chlamydomonas* and *Tetrahymena*, the outer-arm dyneins of the genus *Leishmania* are two-headed, lacking the γ chain like that of metazoans. N-terminal sequence analysis revealed a conserved IQ-like calmodulin binding motif in the outer-arm α and inner-arm 1α dynein heavy chain in the five species of *Leishmania* similar to *Chlamydomonas reinhardtii* outer-arm γ. It was predicted that both motifs were incapable of binding calmodulin. Phosphorylation site prediction revealed conserved serine and threonine residues in outer-arm dynein α and inner-arm 1α as putative phosphorylation sites exclusive to *Leishmania* but not in *Trypanosoma brucei* suggesting that regulation of dynein activity might be via phosphorylation of these IQ-like motifs in *Leishmania sp.*

1. Introduction

Axonemal dynein molecule is a complex of two or three heavy chains (HCs) along with numerous intermediate, light-intermediate and light chains [1]. The dynein heavy chains (DHCs) have a relative molecular mass greater than 500 kDa and belong to the AAA+ family of motor proteins. A single HC consists of six AAA+ motor domains. The motor domain of dynein is highly conserved. The motor domain comprises of around 3000 amino acids from the C-terminal end. The sequence of the N-terminal ~1400 amino acids are highly variable and form the "stem" region which is responsible for cargo binding [1–4].

Although dynein was discovered nearly five decades ago, there has been paucity in dynein research, due to its large size and flexibility. Majority of motility related research in trypanosomes have been on *Trypanosoma brucei* due to its RNAi machinery [5–7]. The trypanosome flagellum has been considered to be essential for its viability in the bloodstream [8,9]. In *Leishmania sp*, which lacks RNAi activity, there is hardly any information on axonemal dynein barring one where the distribution of cytoplasmic and axonemal dyneins in various eukaryotes including trypanosomes like *T. brucei* and in one species of *Leishmania*, *L. major* have been demonstrated [10]. The genus *Leishmania* comprises of a group of kinetoplastid protozoan parasites related to the trypanosomatids and are responsible for causing the disease known as Leishmaniasis. Leishmanial motility is essential for it to escape from the blood clot and attach to the sandfly midgut or for it to migrate to the anterior of the sandfly gut after differentiation [11]. However, in *Leishmania* there is hardly any information regarding the biochemical regulation of flagellar motility. In 2005, the sequencing of the complete genome of the 'TriTryps'- *T. brucei*, *T. cruzi* and *L. major* [12–14], of *L. infantum* and *L. braziliensis* in 2007 [15], of *L. donovani* [16] and *L. mexicana* in 2011 [17] have been done. *Leishmania sp* has been an attractive model for studying the assembly and function of flagella in its life cycle [18]. Three dimensional electron microscopy tools have been able to address and elucidate critical flagellar properties of *T. brucei* and *L. mexicana* such as flagellum age, attachment and length [19]. However, there is still extremely less information regarding the axonemal motor protein dynein in *Leishmania species*.

The outer-arm dyneins (OADs) of the biflagellates such as the green alga *Chlamydomonas* and the ciliates such as the protozoa *Tetrahymena*, are three headed bearing three heavy chains namely α, β and γ each having a specific role in flagellar bending. On the other hand, the OADs of metazoans (multicellular animals) are two headed lacking the γ chain [20,21]. The organization of the OADs

* Corresponding author.
E-mail address: csdey@bioschool.iitd.ac.in (C.S. Dey).

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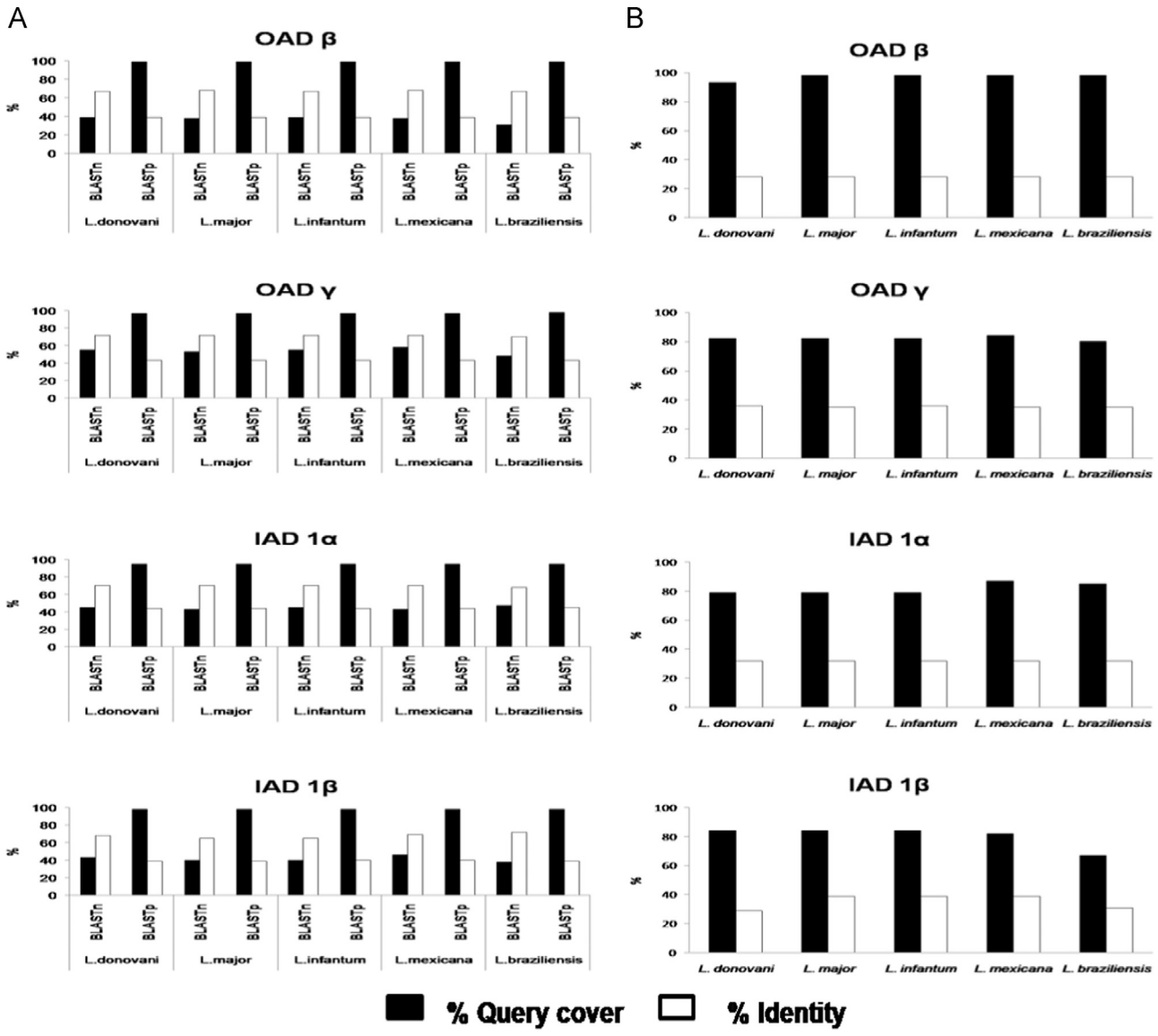

Fig. 1. *Leishmania* possesses orthologs to *C. reinhardtii* OAD β, γ and IAD 1α, 1β. A. BLASTn and BLASTp results of *C. reinhardtii* OADs and IADs against selected leishmanial DHCs. B. BLASTp results of N-terminal of *C. reinhardtii* OADs and IADs against N-terminal of selected leishmanial DHCs. For gene ID's of the leishmanial OAD and IAD sequences refer Table 1. DHC: dynein heavy chain; OAD: outer-arm dynein; IAD: inner-arm dynein.

in the flagellated protozoa *Leishmania* is yet to be studied. The OADs can be divided into two families: OAD α ancestral group-comprising the innermost of the outer arms including *Tetrahymena* OAD α, metazoan OAD α and *Chlamydomonas* OAD γ (ortholog of OAD α of other animals) and OAD β ancestral group-comprising *Tetrahymena* OAD β and OAD γ (ortholog of *Chlamydomonas* OAD α) and metazoan OAD β [10].

The IQ-motif, {(F/I/L/V)Qxxx(R/K)Gxxx(R/K)} is a consensus sequence that binds proteins belonging to the calmodulin (CaM)-family in eukaryotes in a calcium-independent manner. IQ-motifs also bind CaM in a calcium-dependent manner in some cases (IQGAPs, myosin I). It has been previously reported that OAD γ in *Chlamydomonas* bears two IQ-motifs at its N-terminal region which allows LC4 (CaM-family protein) bind and alter the interactions between the DHCs and microtubules in response to calcium [22]. The IQ-like motif {(F/I/L/V)Qxxx(R/K)xxx} is similar to the IQ-motif but allows a broader range of calcium-dependent protein binding to the sequence [23]. IQ-motifs of neuromodulin and neurogranin are targets of phosphorylation by protein kinase C (PKC) [23]. There are several reports establishing the role of calcium in regulating (dynein associated) flagellar motility [1,22,24,25]. Protein kinase C has been reported to be associated with the regulation of ciliary and flagellar motility [26,27]. There are a total of 199 eukaryotic protein kinases present in *L. major* [28]. PKC plays an important role in regulation of the parasite–macrophage interaction [29]. A mitogen activated protein kinase has been shown to play a pivotal role in flagellar biosynthesis and maintenance [30]. The fact that protein kinases and phosphorylation play an important role in flagellar motility is already established. However, the role of protein kinases in regulation of flagellar motility in *Leishmania* is yet to be explored.

Hence, our major aim was to identify and characterize various orthologs of OAD and IAD in five *Leishmania* species: *L. donovani*, *L. major*, *L. infantum*, *L. mexicana* and *L. braziliensis* using bioinformatic tools.

2. Materials and methods

2.1. Data source and sequences

All data used in this study was sourced from National Center for

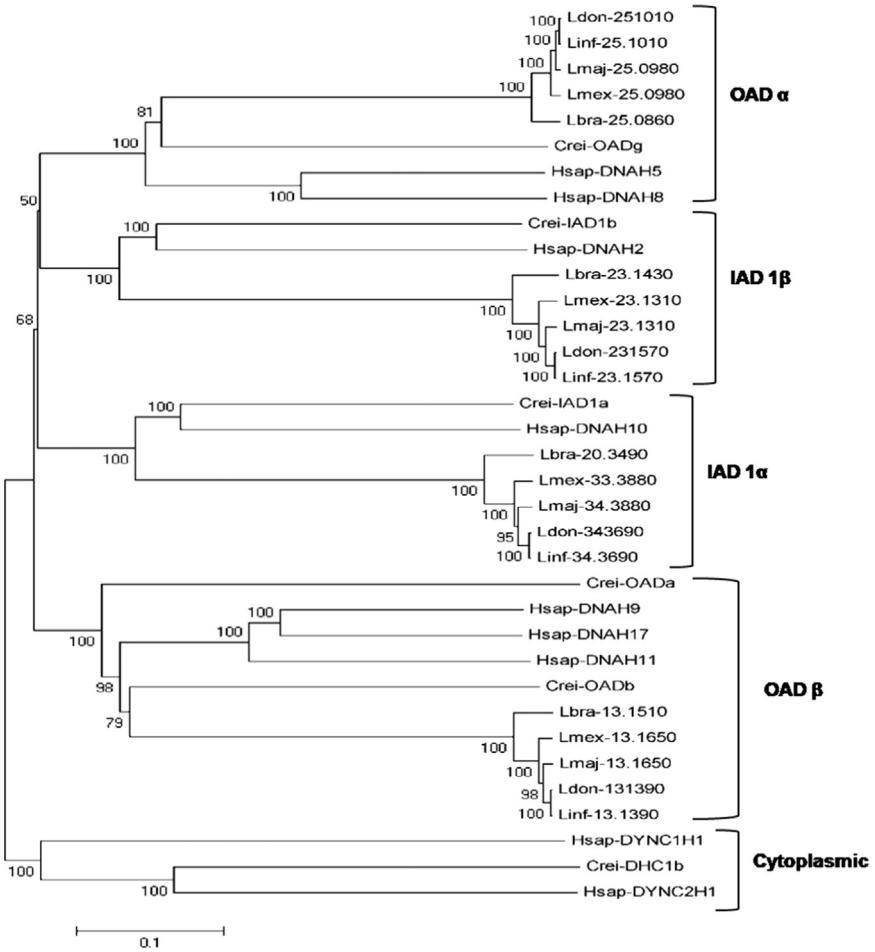

Fig. 2. Phylogenetic tree showing the relationships between OAD and IAD families. OADs and IADs of *Chlamydomonas* and *Homo sapiens* (metazoan) were aligned with leishmanial dynein heavy chains (DHCs) and the phylogenetic tree was recreated using neighbor-joining with 500 number of bootstrap replicates. Prefixes: Crei, *Chlamydomonas reinhardtii*; Hsap, *Homo* sapiens; Ldon, *L. donovani*; Lmaj, *L. major*; Linf, *L. infantum*; Lmex, *L. mexicana*; Lbra, *L. braziliensis*. OAD: outer-arm dynein; IAD: inner-arm dynein. Scale- 0.1 substitutions per site. Accession nos. – Crei-OADa: XP_001695733; Crei-OADb: XP_001695126; Crei-OADg: XP_001702026; Crei-IAD1a: XP_001703170; Crei-IAD1b: XP_001692717; Crei-DHC1b: XP_001696428; Ldon-251010: XP_003861431; Ldon-131390: XP_003859350; Ldon-343690: XP_003864528; Ldon-231570: XP_003861050; Lmaj-25.0980: XP_001683852; Lmaj-13.1650: XP_001681909; Lmaj-34.3880: XP_001686494; Lmaj-23.1310: XP_001683477; Linf-25.1010: XP_001466130; Linf-13.1390: XP_003392353; Linf-34.3690: XP_001468726; Linf-23.1570: XP_001465830; Lmex-25.0980: XP_003876152; Lmex-13.1650: XP_003873366; Lmex-33.3880: XP_003878948; Lmex-23.1310: XP_003875773; Lbra-25.0860: XP_001565591; Lbra-13.1510: XP_001563291; Lbra-20.3490: XP_001564629; Lbra-23.1430: XP_001565215; Hsap-DYNC1H1: NP_001367; Hsap-DYNC2H1: NP_001073932; Hsap-DNAH9: NP_001363; Hsap-DNAH11: NP_001264044; Hsap-DNAH17: NP_775899; Hsap-DNAH5: NP_001360; Hsap-DNAH8: NP_001193856; Hsap-DNAH10: NP_997320; Hsap-DNAH2: NP_065928.

Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The *C. reinhardtii* sequences obtained were: OAD α gene 5721278, OAD β gene 5720753, OAD γ gene 5727568, IAD 1α gene 5728710 and IAD 1β gene 5718404. *T. brucei* sequences obtained were: OAD α (dynein heavy chain XP_843689.1), OAD β (dynein heavy chain XP_828407.1), IAD 1α gene (dynein heavy chain XP_844237.1) and IAD 1β gene (dynein heavy chain XP_847156.1).

2.2. BLAST

Nucleotide and protein BLASTs were performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the default parameters.

2.3. Multiple sequence alignment

Sequences were aligned using ClustalW. Phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA) v5.05 with 500 bootstrap replicates.

2.4. IQ-motif and calmodulin binding domain identification

The presence of any CaM-family protein binding domain in the protein sequences of *Leishmania species* outer-arm and inner-arm dynein heavy chains were identified using the calmodulin target database (http://calcium.uhnres.utoronto.ca/) and by analysis of consensus sequences.

2.5. Phosphorylation site and secondary structure prediction

The presence of any protein phosphorylation sites in the IQ-motifs of the outer-arm and inner-arm dynein heavy chains of *Leishmania species* were predicted using GPS 2.1.2 Server (http://gps.biocuckoo.org/). Secondary structures of the N-terminal region of DHCs were predicted using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/).

3. Results

3.1. Leishmania species possesses orthologs to C. reinhardtii OAD β, γ and IAD 1α, 1β

There is a wealth of information that exists on the characterization of axonemal OADs and IADs in *Chlamydomonas* [31].

Table 1
Analysis of the presence of IQ-like motifs, CaM-binding ability and phosphorylation sites of the IQ-like motifs in the N-terminal region of OADs and IADs.

Organism	Gene ID	IQ-like motif				CaM Binding	Phosphorylation site (conserved IQ-like motif)
		OAD α	OAD β	IAD 1α	IAD 1β		
L. donovani	**OADα:** LDBPK_251010 **OADβ:** LDBPK_131390 **IAD1α:** LDBPK_343690 **IAD1β:** LDBPK_231570	**866IQSNLKAVS** 892VQLQERHIR	1076LQEFDRAIT	**1039FQRWMRGTC** 1296VQEVSKFLD	982VQKVRKIHE	–	Ser-868, 874 of OAD α; Thr-1046 of IAD 1α
L. major	**OADα:** LMJF_25_0980 **OADβ:** LMJF_13_1650 **IAD1α:** LMJF_34_3880 **IAD1β:** LMJF_23_1310	**849IQSNLKAVS** 875VQLQERHIR	1076LQEFDRAIT	**1039FQRWMRGTC** 1295VQEVSKFLD	983VQKVRKIHE	–	Ser-851, 857 of OAD α; Thr-1046 of IAD 1α
L. infantum	**OADα:** LINJ_25_1010 **OADβ:** LINJ_13_1390 **IAD1α:** LINJ_34_3690 **IAD1β:** LINJ_23_1570	**866IQSNLKVVS** 892VQLQERHIR	1076LQEFDRAIT	**1039FQRWMRGTC** 1296VQEVSKFLD	982VQKVRKIHE	–	Ser-868, 874 of OAD α; Thr-1046 of IAD 1α
L. mexicana	**OADα:**LMXM_25_0980 **OADβ:**LMXM_13_1650 **IAD1α:**LMXM_33_3880 **IAD1β:**LMXM_23_1310	**866IQSNLKAVS**	1078LQEFDRAIT	**1038FQRWMRGTC** 1295VQEVSKFLD	–	–	Ser-868, 874 of OAD α; Thr-1045 of IAD 1α
L. braziliensis	**OADα:**LBRM_25_0860 **OADβ:**LBRM_13_1510 **IAD1α:**LBRM_20_3490 **IAD1β:**LBRM_23_1430	**872IQSNLKAVS** 898VQLQERHIR	–	**1037FQRWMRGTC**	–	Unclassified motif at 1178 of IAD 1β	Ser-874, 880 of OAD α; Thr-1044 of IAD 1α
T. brucei	**OADα:**Tb927.3.930 **OADβ:**Tb11.02.0760 **IAD1α:**Tb927.4.870 **IAD1β:**Tb927.8.3250	814IQVNLKLIA 840VQVQEKHIR 1212LQYGFKKGL	1072LQDFDKAIT 1194IQAAIRMLK	1135LQEGNRLRK	921LQAIHRRLV 976LQVVERMLP 1220IQALTKEPQ	Unclassified motif at 603 of OAD α, 1029 of OAD β	–

"–" indicates absence. IQ-like motifs conserved in all five species of *Leishmania* are highlighted in bold.

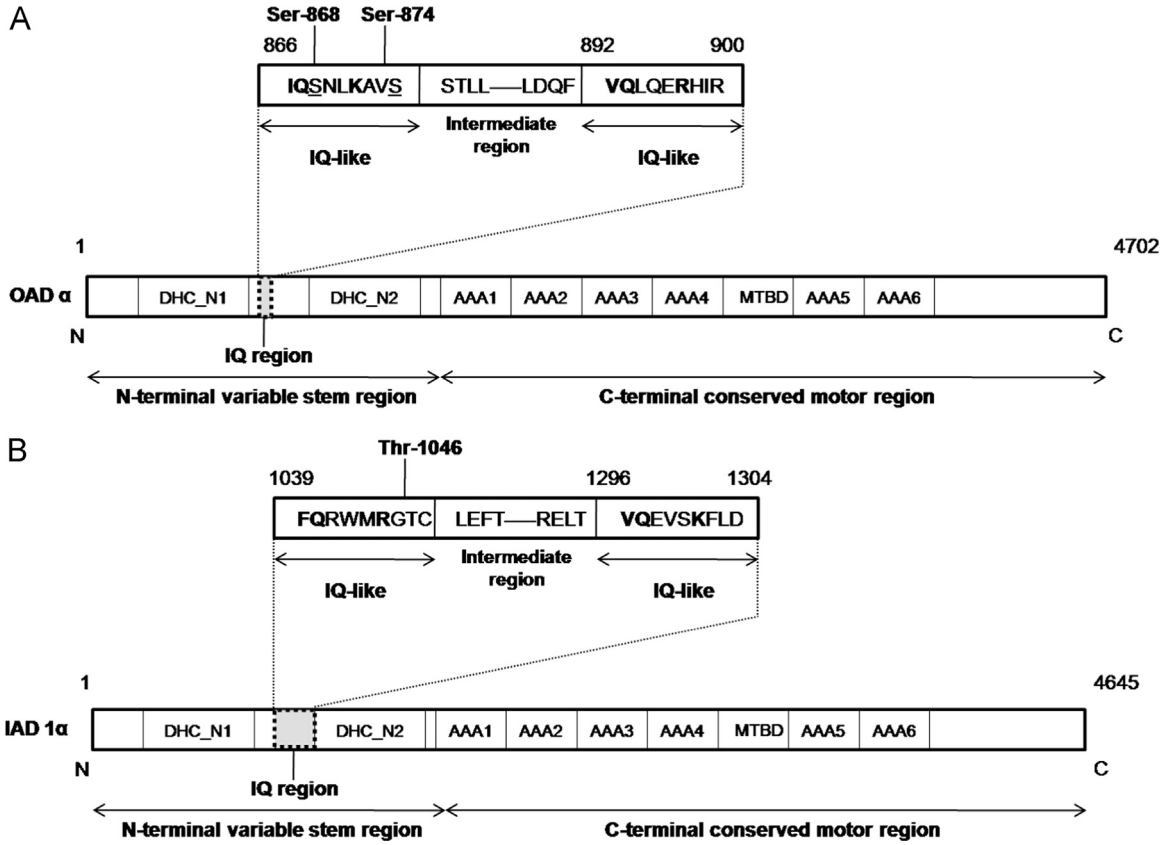

Fig. 3. Diagrammatic representation of map of *L. donovani* OAD α and IAD 1α. The C-terminal conserved motor region and the N-terminal variable stem region are denoted. C-terminal region comprises of six AAA domains, MTBD microtubule binding domain that forms the stalk and a C-terminal unit. N-terminal region comprises of a DHC_N1 domain that takes part in interactions with other heavy chains and a DHC_N2 domain. The putative IQ-region bearing the conserved IQ-like motif that is a site phosphorylation lies in the N-terminal region, in-between DHC_N1 and DHC_N2 domains. A. OAD α of *L. donovani.* Conserved serine residues for phosphorylation labeled. B. IAD 1α of *L. donovani.* Conserved threonine residue for PKC phosphorylation labeled. For species specific differences in the IQ-like motif, refer Table 1. OAD: outer-arm dynein; IAD: inner-arm dynein.

Taxonomically *Chlamydomonas* was the closest organism to *Leishmania* with known OADs and IADs. The OADs and IADs of other organisms that are phylogenetically close by have not been characterized. Therefore, while probing for leishmanial axonemal dyneins we looked through the information available on *Chlamydomonas* OADs and IADs. Also, it has been previously established that *Chlamydomonas* DHC gene classification can be used as a reference for DHC gene classification in other organisms [31]. Nucleotide BLAST (BLASTn) and protein BLAST (BLASTp) was performed taking α, β, γ, 1α or 1β chains of *C. reinhardtii* as query against all possible dynein heavy chain genes in the genome and their corresponding proteins respectively of the five *Leishmania* species. Query cover implies the extent of the query sequence that is aligned with the target sequence. Knowing the fact that at the protein level, the C-terminal motor domains of dynein heavy chains are conserved, we took a query cover of $\geq$ 90% and identity $\geq$ 30% as significant [32]. The four genes and their corresponding proteins (Fig. 1A) of each species of *Leishmania* that satisfied the parameters were selected for further analyses. At the protein level, none of the leishmanial dynein heavy chain sequences exceeded a query cover of 80% against OAD α of *C. reinhardtii.* Data suggests the absence of orthologs to *C. reinhardtii* OAD α in *Leishmania.*

3.2. Two-headed OADs present in Leishmania belonging to OAD α and OAD β family of DHCs

As mentioned previously, the N-terminal region of dynein heavy chains exhibit inter-chain sequence variation. Hence, we decided to align the N-terminal first 1190 amino acids of the four selected leishmanial dynein heavy chain proteins against the 1190 N-terminal amino acids of *C. reinhardtii* OAD α, β, γ and IAD 1α and 1β using BLASTp. Fig. 1B shows the alignment results. Absence of similarity with OAD α of *Chlamydomonas* indicates the presence of two-headed OADs. To study the phylogenetic relationship of OADs and IADs, MSA of the four protein sequences of each of the five species of *Leishmania* with five *Chlamydomonas* OADs and IADs and axonemal dynein heavy chains of *Homo sapiens* (mammalian host of *Leishmania*) were performed using ClustalW and the phylogenetic tree is shown in Fig. 2. The protein sequences have been grouped into the known OAD α, OAD β, IAD 1α and IAD 1β families. The gene IDs of the sequences are mentioned in Table 1. Data indicates intra-chain sequence conservation of OADs.

3.3. Leishmanial OAD α, β and IAD 1β bear IQ-like motifs

In order to determine the presence of IQ or IQ-like motifs in the axonemal DHCs of *Leishmania species* their N-terminal variable region of amino acid positions 800–1300 (region between DHC_N1 and DHC_N2 that binds CaM in *Chlamydomonas*) was analyzed. List of the IQ-like sequences in five species of *Leishmania* are shown in Table 1. To test whether the IQ-like motifs were possible CaM-family protein binding sites the calmodulin target database was searched. The search did not identify any potential CaM binding region in the IQ-like motifs in the OAD α, β, IAD 1α and 1β proteins of *L. donovani*, *L. major*, *L. infantum* and *L. mexicana* identified by us. An unclassified CaM-binding motif was found between the DHC_N1 and DHC_N2 domains of IAD 1β of *L. braziliensis,* however, no IQ or IQ-like motif was present at that region (Table 1). Our data

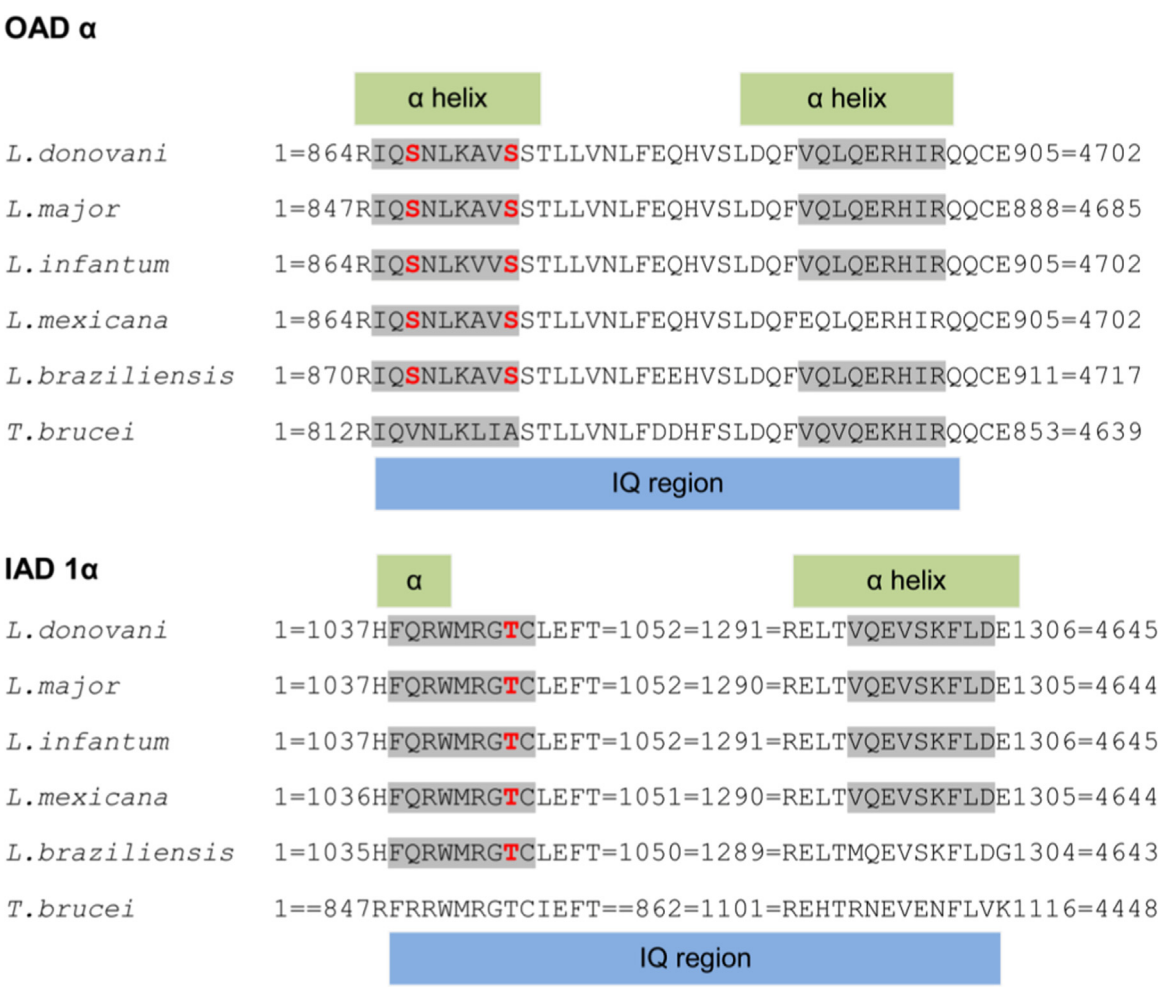

Fig. 4. Sequence alignment. Amino acid sequences of OAD α and IAD 1α of five species of *Leishmania* were aligned with that of *T. brucei*. IQ-like motifs are highlighted in gray. Conserved serine and threonine residues are colored in red. Secondary structural elements (helix) are marked on top. IQ-region formed by the two IQ-like motifs are marked below. Numbers denote sequence positions. '=' indicates intervening sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

indicated that CaM-family proteins do not bind to any of the IQ-like motifs present in OAD α, β and IAD 1α, 1β.

3.4. IQ-like motif of OAD α and IAD 1α bear phosphorylation sites

Sequence analysis of the N-terminal stem region of four *Leishmania species* except *L. mexicana* OAD α and three *Leishmania species* except *L. major* and *L. braziliensis* IAD 1α revealed two IQ-like motifs forming an IQ-region. In order to identify any putative phosphorylation site in the IQ-like motifs, we applied phosphorylation site prediction which predicted two serine residues of the first IQ-like motif of OAD α and a threonine residue of the first IQ-like motif of IAD 1α as putative phosphorylation sites by AGC group (PKC, RSK) and CAMK group (CAMK1, CAMK2) of eukaryotic protein kinases (Table 1) for all five species of *Leishmania*. IQ-like motifs of OAD β and IAD 1β did not bear any potential phosphorylation site. To elucidate whether this conservation is exclusive to only different species of *Leishmania*, the OADs and IADs of the trypanosome *T. brucei* was analyzed for presence of IQ/IQ-like motifs, ability of CaM-binding and phosphorylation. No such conserved serine residues in OAD α and threonine residue in IAD 1α were found (Table 1). Data provides evidence that the IQ-like motifs of OAD α and IAD 1α contains critical serine and threonine residues respectively that are possible targets of phosphorylation and regulation. Fig. 3 depicts a diagrammatic representation of the OAD α and IAD 1α of *L. donovani* highlighting the IQ-like motifs, IQ region and conserved serine/threonine phosphorylation sites.

4. Discussion

Dynein heavy chains are highly conserved proteins throughout evolutionary history. It is primarily the N-terminal region which is poorly conserved amongst axonemal and cytoplasmic forms and allows identification of the same. There is practically no information regarding axonemal DHC in *Leishmania*. Previously it has been reported that between three species of trypanosomes, *Crithidia deanei*, *C. fasciculata* and *L. major* the flagellar beat parameters such as speed, beat frequency, amplitude, wavelength, etc. vary considerably [33]. The most probable explanation to this can be due to the proteins present in the flagellum which regulate flagellar bending. The OADs and IADs, being the major force generator of the flagellar beat may be one of the reasons behind this inter-species variability in flagellar beating.

Present study identified axonemal OAD α, β and IAD 1α, 1β genes and their corresponding proteins in *Leishmania species*. The presence of three-headed OADs in protozoans has been previously reported [34]. However, our results along with results from a comparative genomic analysis on distribution of dyneins in eukaryotes [10] indicate that unlike the biflagellated green algae *Chlamydomonas*, the axoneme of the flagellated protozoa of genus *Leishmania* (by testing five different species) bears two-headed

OADs similar to multicellular animals. This may have taken place by loss of the outermost γ chain in due course of time. The two axonemal OADs are orthologous to OAD β and γ in *Chlamydomonas* and designated here as OAD β and OAD α, respectively in *Leishmania* as they belong to the corresponding dynein heavy chain families in eukaryotes. Whether the γ heavy chain has been lost in *Leishmania* in the process of evolutionary adaptation remains unanswered. Identification of the axonemal outer-arm and inner-arm dynein heavy chains of *Leishmania species* will emphasize further research on the roles of these proteins in regulating flagellar motility.

IQ-like domains were present in OADs and IADs in *Leishmania* but were predicted to be unable to bind CaM-family proteins. One of the reasons could be that as a result of speciation, the IQ-like motifs of the DHCs were retained but lost their ability to bind CaM-family proteins directly. Another possibility might be that besides being a CaM-family protein binding site, IQ-motifs are sites of phosphorylation in response to changes in levels of different second messengers like calcium. Secondary structure prediction of the N-terminal region of OAD α and IAD 1α revealed that both IQ-like motifs are parts of two separate alpha-helices (Fig. 4) in the N-terminal stem region of the DHC. The location renders the motif highly accessible to protein–protein interactions and/or post-translational modifications. Protein phosphorylation site prediction identified conserved serine residues in the first IQ-like motif of OAD α and a conserved threonine residue in the first IQ-like motif of IAD 1α as putative phosphorylation sites by AGC and CAMK protein kinase groups. PKC interacts with calcium resulting in its activation [35]. One of the two catalytic domains in RSK belongs to the calcium/calmodulin-dependent protein kinase family and hence, responds to variations in calcium levels [36]. CAMK (calcium/calmodulin-dependent protein kinase) respond to increase in intracellular Ca^{2+} levels and thereby phosphorylate their corresponding substrates [37]. Phosphorylation site prediction of a large protein like dynein would generally give numerous potential phosphorylation sites. Two conserved IQ-like motifs in OAD α and IAD 1α with conserved serine and threonine residues are targets of phosphorylation in five different species of *Leishmania* but not in the closely related *T. brucei*. This most likely did not happen as a function of chance. It is very much possible that these conserved *Leishmania* specific IQ-like domains instead of allowing CaM binding in response to variations in calcium levels are targets of phosphorylation allowing regulation of flagellar motility. These conserved IQ-like motifs and phosphorylation sites were found to be exclusively present in *Leishmania* but not in the closely related kinetoplastid *T. brucei*. This requires further experimental validation for confirmation. However, the location of the phosphorylation sites amongst different species of *Leishmania* being different indicates the presence of inter-species variability.

Conflict of interest

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at 10.1016/j.bbrep.2015.10.004.

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