Functional Characterization of HOPS Complex in *Leishmania*

Vikas Dhar Dubey

KUSUMA SCHOOL OF BIOLOGICAL SCIENCES

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by

Vikas Dhar Dubey
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Dedicated to my family……

Prof. Amitabha Mukhopadhyay
Ph.D, FNA, FNASc, FASc
J. C. Bose National Fellow
S.S. Bhatnagar & National Bioscience Awardee

Kusuma School of Biological Sciences
INDIAN INSTITUTE OF TECHNOLOGY DELHI
Hauz Khas, New Delhi–110016, India
Tel. : +91-11-2659 1106, Mobile : +91-9810887164
E-mail : amitabha@bioschool.iitd.ac.in
leish.amit@gmail.com

CERTIFICATE

This is to certify that the thesis titled "**Functional Characterization of HOPS Complex in *Leishmania***", being submitted by **Mr. Vikas Dhar Dubey** in the Indian Institute of Technology Delhi for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research carried out by him, which has been prepared under my supervision and guidance in conformity with rules and regulation of the Indian Institute of Technology Delhi, India. The results prescribed in it have not been submitted in part or full to any other University or Institute for the award of any Degree/Diploma.

5th June 2024

New Delhi

Dr. Amitabha Mukhopadhyay

Professor,

Kusuma School of Biological Sciences

Indian Institute of Technology, Delhi

New Delhi-110016, India

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Vikas Dhar Dubey

Abstract

Previously, we have shown that *Leishmania* endocytosed hemoglobin (Hb) by specific receptor (HbR) and traffic to lysosome via early endosomes in Rab5 and Rab7 dependent process. However, the mechanism of transition from endosomes to lysosomes is unknown. Therefore, we have characterized the role of HOPS complex in this process. Here, we have cloned and expressed all components of HOPS complex (LdVps11, LdVps16, LdVps18, LdVps33, LdVps39 and LdVps41) homologue from *Leishmania* to understand their role in endocytosis in *Leishmania*. We have found that RFP-LdVps41 co-localize with GFP-LdVps11, GFP-LdVps16, GFP-LdVps18, GFP-LdVps33 and GFP-LdVps39 in Ld-Rab7 positive and Lysotracker labelled late endosome and lysosome compartments in *Leishmania*. Using *in-vitro* protein interaction, we have found that the LdVps41 specifically interacts with LdVps11, LdVps16, LdVps18, LdVps33 and LdVps39. LdVps11 specifically binds with LdVps16, LdVps18 and LdVps39 but not with LdVps33 while LdVps16 specifically interacts with LdVps18 and LdVps33 but not with LdVps39. Thus, LdVps41 is found to be the central molecule in HOPS complex interacting with various subunits to form HOPS complex in *Leishmania*. Interestingly, we have found that LdVps41 interacts with all other HOPS complex proteins via its C-terminal domain (CTD-LdVps41$^{901-1446}$). In contrast, we have found that N-terminal domain of LdVps41 (NTD-LdVps41$^{1-500}$) specifically binds with GTP form of LdRab7 and LdHbR. These results suggest that LdVps41 possibly acts as tethering molecule between LdHbR and LdRab7 to mediate the transport of Hb from endosomes to the lysosomes in *Leishmania*.

We have overexpressed LdVps41 truncated proteins in *Leishmania* to determine their role in the Hb trafficking in the parasite. We have found that transgenic parasites overexpressing GFP-NTD-LdVps41$^{1-500}$ inhibit the transport of Hb to the lysosomes as it does not interact with other components of HOPS complex. Similarly, trafficking of Hb to the lysosomal compartment is also blocked in GFP-CTD-LdVps41$^{901-1446}$ overexpressed transgenic parasites as this mutant fails to recruit LdRab7 and LdHbR. As Hb is essential for the survival of parasites within macrophages, we have found that transgenic parasites overexpressing GFP-NTD-LdVps41$^{1-500}$ or GFP-CTD-LdVps41$^{901-1446}$ show growth defect in macrophages as parasites fail to acquire heme from lysosomal degradation of Hb. In the present investigation, we have identified and functionally characterized the role HOPS complex in Hb trafficking to lysosomes and have found that HOPS complex function is necessary for the survival of the parasites.

सारांश

इससे पहले, हमने दिखाया है कि विशिष्ट रिसेप्टर (एच. बी. आर.) द्वारा लीशमैनिया एंडोसाइटोस्ड हीमोग्लोबिन (एच. बी.) को एलडी-रैब5 और एलडी-रैब7 आश्रित प्रक्रिया में प्रारंभिक एंडोसोम के माध्यम से लाइसोसोम तक स्थानांतरित करता है। हालाँकि, एंडोसोम से लाइसोसोम में स्थानान्तरण की क्रियाविधि अज्ञात है। इसलिए हमने इस प्रक्रिया में हॉप्स कॉम्प्लेक्स की भूमिका का वर्णन किया है। यहां हमने लिश्मेनिया में एंडोसाइटोसिस प्रक्रिया में भूमिका निर्वहन करने वाले सभी घटकों की भूमिका को समझने के लिए लिश्मेनिया से हॉप्स कॉम्प्लेक्स (एलडीवीपीएस11, एलडीवीपीएस16, एलडीवीपीएस18, एलडीवीपीएस33, एलडीवीपीएस39, एलडीवीपीएस41) के सजातीय सभी घटकों को क्लोन और व्यक्त किया है। हमने पाया है कि आरएफपी-एलडीवीपीएस41 जीएफपी-एलडीवीपीएस11, जीएफपी-एलडीवीपीएस16, जीएफपी-एलडीवीपीएस18, जीएफपी-एलडीवीपीएस33 और जीएफपी-एलडीवीपीएस39 के साथ एलडी-रैब7 पॉजिटिव और लाइसोट्रैकर चिह्नित लेट एंडोसोम और लाइसोसोम कक्षों में लीशमैनिया में सह-स्थानीयकृत है। इन-विट्रो प्रोटीन इंटरैक्शन का उपयोग करते हुए, हमने पाया है कि एलडीवीपीएस41 विशेष रूप से एलडीवीपीएस11, एलडीवीपीएस16, एलडीवीपीएस18, एलडीवीपीएस33 और एलडीवीपीएस39 के साथ अंतःक्रिया करता है। एलडीवीपीएस11 विशेष रूप से एलडीवीपीएस16, एलडीवीपीएस18 और एलडीवीपीएस39 के साथ जुड़ता है, लेकिन एलडीवीपीएस33 के साथ नहीं, जबकि एलडीवीपीएस16 विशेष रूप से एलडीवीपीएस18 और एलडीवीपीएस33 के साथ अंतःक्रिया करता है, लेकिन एलडीवीपीएस39 के साथ नहीं। इस कारणवश, एलडीवीपीएस41 को हॉप्स कॉम्प्लेक्स का केंद्रीय अणु पाया गया है। जो विभिन्न उपइकाइयों के साथ अंतःक्रिया करके लिश्मेनिया में हॉप्स काम्प्लेक्स का निर्माण करता है। हमने अपनी शोध में यह रोचक निष्कर्ष भी प्राप्त किया कि एलडीवीपीएस41 अपने C-टर्मिनल डोमेन (सीटीडी-एलडीवीपीएस41$^{901-1446}$) के माध्यम से अन्य सभी HOPS कॉम्प्लेक्स प्रोटीन के साथ अंतःक्रिया करता है। इसके विपरीत, हमने पाया है कि एलडीवीपीएस41 (एनटीडी-एलडीवीपीएस41$^{1-500}$) का एन-टर्मिनलडोमेन विशेष रूप से एलडीएचबीआर और एलडी-रैब7 के GTP फॉर्म से जुड़ता है। प्रेक्षित परिणाम यह प्रस्तावित करते हैं कि एलडीवीपीएस41 संभवतः लिश्मेनिया में एंडोसोम से लाइसोसोम तक Hb के स्थानांतरण में मध्यस्थता करने के लिए एलडीएचबीआर और एलडी-रैब7 के बीच बंधन अणु के रूप में कार्य करता है। हमने लिश्मेनिया में एलडीवीपीएस41 खंडित किए गए प्रोटीन को ओवरएक्सप्रेस किया जिससे लिश्मेनिया में एचबी की ट्रैफ़िकिंग में उनकी भूमिका का निर्धारण हो सके। तदनुसार यह भी पाया गया है कि जीएफपी-एनटीडी-एलडीवीपीएस41$^{1-500}$ को ओवरएक्सप्रेस करने वाले ट्रांसजेनिक लिश्मेनिया लाइसोसोम में Hb के परिवहन को रोकते हैं क्योंकि वे HOPS कॉम्प्लेक्स के अन्य घटकों के साथ अंतःक्रिया नहीं करते हैं। तदनुरुप, जीएफपी-सीटीडी-एलडीवीपीएस41$^{901-1446}$ ओवरएक्सप्रेस्ड ट्रांसजेनिक

लीशमैनिया में भी लाइसोसोमल कक्ष में एचबी की ट्रैफ़िकिंग अवरुद्ध है क्योंकि यह उत्परिवर्ती एलडीएचबीआर और एलडी-रैब7 को रिक्रूट करने में विफल है। चूंकि एचबी मैक्रोफेज के भीतर लीशमैनिया के उत्तरजीविता के लिए आवश्यक है, हमें यह ज्ञात हुआ है कि जीएफपी-एनटीडी-एलडीवीपीएस41$^{1-500}$ अथवा जीएफपी-सीटीडी-एलडीवीपीएस41$^{901-1446}$ को अधिक व्यक्त करते हुए ट्रांसजेनिक लीशमैनिया मैक्रोफेज में संख्या में वृद्धि दोष दिखाते हैं, क्योंकि लीशमैनिया एचबी के लाइसोसोमल खंडन से हीम प्राप्त करने में विफल रहते हैं। वर्तमान अध्ययन में हमने लाइसोसोम में एचबी ट्रैफ़िकिंग में हॉप्स कॉम्प्लेक्स की भूमिका की पहचान की है और कार्यात्मक रूप से इसकी विशेषता बताई है तथा पाया है कि हॉप्स कॉम्प्लेक्स प्रक्रिया लीशमैनिया के अतिजीवन के लिए आवश्यक है।

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Alexa 546-Hb	Alexa Fluor 546 conjugated hemoglobin
ARF	ADP ribosylation factor
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CCV	Clathrin coated vesicles
CCPs	Clathrin coated pits
cDNA	Complementary DNA
C-terminus	Carboxyl-terminus
CORVET	Class C core Vacuole Endosome/Tethering
CHEVI	class C Homologues in Endosome-Vesicle Interaction
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EE	Early endosome
ER	Endoplasmic Reticulum
FCS	Fetal calf serum
FERARI	Factor for Endosome Recycling And Retromer Interactions
GAP	GTPase activating protein
GDF	Guanine nucleotide dissociation factor
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein

GDP	Guanosine diphosphate
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
GP63	Glycoprotein 63
Hb	Hemoglobin
HbR	Hemoglobin receptor
HCl	Hydrochloric acid
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2ethane-sulphonic acid])
HRP	Horse radish peroxidase
HOPS	Homotypic fusion and vacuole Protein Sorting
h	Hour
IPTG	Isopropyl-beta-D-thiogalactopyranoside
KCl	Potassium Chloride
kDa	kilo Dalton
K_2HPO_4	Di potassium hydrogen phosphate
LAMP	Lysosome associated membrane protein
LE	Late endosomes
LPG	Lipophosphoglycan
LPG2-HA	Hemagglutinin tagged Lipophosphoglycan-2
LPS	Lipopolysaccharide
LB	Luria Bertani
M	Molar
M199	Medium 199
$MgCl_2$	Magnesium chloride
miRNA	MicroRNA
M6PR	Mannose-6-phosphate receptor
µg	microgram
µM	micromolar
µl	microliter
mg	milligram

min	minutes
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
NaCl	Sodium Chloride
NaH_2PO_4	Sodium di-hydrogen phosphate
NaOH	Sodium Hydroxide
NEM	N-ethyl maleimide
ng	nanogram
NHS	N-hydroxysuccinimide
Ni-NTA	Nickel-Nitrilotriacetic acid
nM	Nano molar
nm	Nano meter
NSF	NEM sensitive fusion protein
N-terminus	Amino-terminus
$O.D._{600}$	Optical density at 600nm
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Protease Inhibitor cocktail
PI3K	Phosphoinositide-3-kinase
PI3P	Phosphoinositide-3-phosphate
PLC	Phospholipase C
PM	Plasma Membrane
PV	Parasitophorous vacuole
rpm	Revolutions per minute
REP	Rab escort protein

RFP	Red fluorescent protein
RNA	Ribonucleic acids
ROS	Reactive oxygen species
RT	Room Temperature (25°C)
RT-PCR	Reverse Transcription-PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide electrophoresis
SNARE	Soluble NSF Attachment Protein receptor
TGN	trans-golgi network
TNF	Tumor necrosis factor
Tris	Tris-hydroxy methyl- amino methyl
Tx-100	Triton X-100
V	Volt
VAMP	Vesicle associated membrane protein
WT	Wild type