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ORIGINAL ARTICLE

The Phosphatidylinositol 3-kinase Class III Complex Containing TcVps15 and TcVps34 Participates in Autophagy in *Trypanosoma cruzi*

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Keywords

Autophagy; *Trypanosoma cruzi*; vesicular acidification; Vps15; Vps34.

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ABSTRACT

Autophagy is a degradative process by which eukaryotic cells digest their own components to provide aminoacids that may function as energy source under nutritional stress conditions. There is experimental evidence for autophagy in parasitic protists belonging to the family Trypanosomatidae. However, few proteins implicated in this process have been characterized so far in these parasites. Moreover, it has been shown that autophagy is involved in *Trypanosoma cruzi* differentiation and thus might have a role in pathogenicity. Here, we report the cloning and biochemical characterization of TcVps15. In addition, we demonstrate that TcVps15 interact with the PI3K TcVps34 and that both proteins associate with cellular membranes. Under nutritional stress conditions, TcVps15 and TcVps34 modify their subcellular distribution showing a partial co-localization in autophagosomes with TcAtg8.1 and using an active site TcVps15-mutated version (TcVps15-K219D-HA) we demonstrated that this relocalization depends on the TcVps15 catalytic activity. Overexpression of TcVps15-HA and TcVps15-K219D-HA also leads to increased accumulation of monodansylcadaverine (MDC) in autophagic vacuoles under nutritional stress conditions compared to wild-type cells. In addition, the MDC-specific activity shows to be significantly higher in TcVps15-HA overexpressing cells when compared with TcVps15-K219D-HA. Our results reveal for the first time a role of TcVps15 as a key regulator of TcVps34 enzymatic activity and implicate the TcVps15-Vps34 complex in autophagy in *T. cruzi*, exposing a new key pathway to explore novel chemotherapeutic targets.

AUTOPHAGY is a conserved eukaryotic process of vacuolar/lysosomal-mediated degradation of cytoplasmic proteins and organelles (Mizushima 2011). This process is characterized by the formation of a double membrane structure, the autophagosome. The autophagosome is formed as an isolated membrane that expands outwards from a nucleation site and then closes, engulfing cytoplasmic materials. The autophagosome then fuses with the lysosome, where the materials take contact with and are degraded by acidic hydrolases. The best known role of autophagy is to provide nutrients upon starvation. When amino acid levels in cells become low, autophagy is stimulated to recycle protein containing amino acids and prolongs cell survival. (Kamada et al. 2004; Mizushima et al. 2004; Scott et al. 2004).

Very little is known about autophagy in primitive unicellular organisms such as trypanosomatids. It has been demonstrated that *Trypanosoma cruzi*, the etiological agent of Chagas' disease, which represents a major cause of cardiomyopathy in Latin America where it affects around 8 million of people (Rassi et al. 2010), possess an ATG8 conserved system. Moreover, it has been shown that autophagy is involved in differentiation between developmental stages, a process that is essential for

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parasite cycle maintenance and cell survival (Alvarez et al. 2008a,b).

The phosphorylated derivatives of phosphatidylinositol (PtdIns) are called phosphoinositides and are regarded as important signaling molecules. Their localization pattern at the membrane mark specific subdomains to which they can selectively recruit cytosolic proteins that contain one of several known phosphoinositide interaction motifs. These attracted proteins participate in the first step of downstream signaling cascades, which finally result in a cellular response.

The produced PtdIns3P has been shown to localize to endosomes and specialized domains of multivesicular bodies (Gillooly et al. 2000), phagosomes (Ellson et al. 2001), the midbody (Sagona et al. 2010), peroxisomes (Grunau et al. 2011) and preautophagosomal structures (Axe et al. 2008). The generated PtdIns3P recruits PtdIns3P effector proteins, most of which interact via a FYVE domain or a PX (Phox homology) domain (Lemmon 2008). The subsequent signaling cascades are involved in downstream events such as the down regulation of growth factor receptors, endocytic signaling, cytokinesis as well as autophagy (Funderburk et al. 2010; Nezis et al. 2010). It is important to remark that regarding the autophagic process, the production of PtdIns3P by the class III phosphatidylinositol 3-kinase (PI3K) is essential for autophagosome formation. In addition, Atg18 in yeast and the WIPI proteins in mammals recognize the PtdIns3P and therefore function as effectors recruiting other autophagy-related proteins (ATGs) and triggering the process following steps (Baskaran et al. 2012; Proikas-Cezanne et al. 2015).

Previously in our laboratory, we have identified the first PI3K in *T. cruzi*, named TcVps34, with similarity with its yeast counterpart, Vps34p. We have also demonstrated that this enzyme plays a prominent role in vital processes for the parasite survival such as osmoregulation, vesicular acidification, and vesicular trafficking (Schoijet et al. 2008). In *Trypanosoma brucei*, this enzyme showed to be essential by RNAi experiments, having a role in coordinating segregation of the Golgi complex at cell division (Hall et al. 2006).

To accomplish its full enzymatic and biological activity, Vps34 associates with further regulatory proteins. The core components are the serine/threonine protein kinase Vps15 and the multivalent adaptor protein Beclin 1 (Funderburk et al. 2010; Kang et al. 2011). To date, the role of Vps15 in the regulation of Vps34 has not been extensively studied. In vitro, the human Vps15 (hVps15) leads to a 2-fold activation of hVps34 lipid kinase activity (Panaretou et al. 1997). Yeast Vps15p is required for the activity of Vps34p, as deletion of *Vps15* leads to a loss of PtdIns3P production and a disruption of vesicular trafficking (Stack et al. 1995). Interestingly, an increase in PtdIns3P was detected in *Vps15*-null yeast strains that overexpress Vps34p, but this does not lead to a restoration of Vps34p-dependent vesicular trafficking (Stack et al. 1995). Thus, while Vps34p activity may not be strictly dependent on Vps15p, its function in vesicular trafficking is Vps15p dependent. In addition and based on results from yeast and flies (Kihara et al. 2001; Lindmo et al. 2008), hVps15 would be expected to play a role in hVps34-dependent autophagy, but this has not been examined in mammalian cells neither in protozoa.

In this study, we have examined the role of TcVps15 in the regulation of TcVp34 in *T. cruzi*. We describe the cloning and expression of TcVps15 and show that both proteins interact by a two-hybrid assay. Moreover, functional studies reveal that both TcVps34 and TcVps15 participate in autophagy in *T. cruzi*. In addition, we found that TcVps15-K219D (a catalytic domain mutant version of TcVps15)-overexpressing cells, showed reduced protein kinase activity when compared with the overexpression of the wild-type protein, are less efficient modulating the lipid kinase activity of TcVps34 and displayed lower autophagy levels than those observed when wild-type TcVps15 is overexpressed. These experiments provide new information on the role of TcVps15–TcVps34 in autophagy in *T. cruzi*, a mechanisms that has been showed to be essential for the parasite survival.

MATERIAL AND METHODS

Chemicals and reagents

All radiochemicals used in this work were purchased from PerkinElmer Life Sciences, and restriction endonucleases were from New England Biolabs, Beverly, MA. Bacto-tryptose, yeast nitrogen base, and liver infusion were from Difco (Detroit, MI). All other reagents were purchased from Sigma (St. Louis, MO).

Cell cultures

Trypanosoma cruzi epimastigote forms (CL Brener strain) were cultured at 28 °C for 7 d in LIT medium [5 g/l liver infusion, 5 g/l bacto-tryptose, 68 mM NaCl, 5.3 mM KCl, 22 mM Na_2PO_4, 0.2% (W/V) glucose, 0.002% (W/V) hemin] supplemented with 10% (V/V) newborn calf serum, 100,000 units/l penicillin and 100 mg/l streptomycin. Cell viability was assessed by direct microscopic examination.

For starvation induction, mid-log parasites (2×10^7 cells/ml) were washed twice with PBS (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2), resuspended in the same buffer at equal cell concentration, and incubated for 16 h at 28 °C.

Cell extracts and membrane fraction preparations

For *T. cruzi* extracts, 10^8 epimastigotes were harvested by centrifugation at 1,500 *g* for 10 min and washed two times with phosphate-buffered saline (PBS). Cell pellets were then resuspended in lysis buffer (50 mM HEPES buffer, pH 7.3, 0.01 mg/ml leupeptin, 25 U/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride and 14 mM 2-mercaptoethanol), and lysed by six cycles of freezing in liquid N_2 and thawing at 4 °C. The total extracts were further centrifuged for 1 h at 100,000 *g* to obtain P100 and S100 fractions.

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For TcVps15 and TcVps34 membrane extraction, P100 fractions were prepared and this pellets were independently washed with lysis buffer containing 1M NaCl, 0.1% (w/v) sodium cholate, 0.5% (w/v) sodium cholate, or 8 M urea at 4 °C for 30 min. After each wash, pellets were centrifuged for 40 min at 100,000 *g* and P100 and S100 were loaded into SDS-polyacrylamide gels.

Cloning of TcVps15 gene and oligonucleotide-directed mutagenesis

The gene sequence corresponding to the *Saccharomyces cerevisiae* Vps15 (ID 852394) was used to screen *T. cruzi* sequences in the GeneDB database using the WU-Blast2 algorithm. As result of this search, a high-scored target was detected: TcCLB.510901.120 (previously known as: Tc00.1047053510901.120). Based on this sequence, two oligonucleotides carrying hemi- restriction sites (Vps15-Fw-pENTR-KpnI 5′GGTACCATGGGGAATCAGCTGGCAGCA GCA3′ and Vps15-Rv-pENTR-XhoI 5′CTCGAGTTACTCTG AATTAAGCCACACCGT3′) were designed. PCR amplifications were carried out using 600–800 ng of *T. cruzi* genomic DNA, 100 ng of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2 units of Pfu Ultra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA). DNA fragment was gel-purified by Axy-Prep DNA Gel Extraction Kit (Axygen, Biosciences, Union City, CA) and cloned into pGEM-T Easy vector (Promega, Madison, WI).

For *TcVps15* catalytic domain mutation generation, *TcVps15-K219D*, lysine 219 within the catalytic domain was mutagenized to an aspartate, using as template the wild-type gene cloned in pGEM-T Easy vector according to protocols from the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). Point mutation was confirmed by DNA sequencing (Macrogen, DNA sequencing service, Seoul, Korea).

Sequence analysis

The search in *T. cruzi* genome database (http://www.genedb.org) was performed with Wu-Blast2. Sequence identity and similarity were analyzed with the EMBOSS Needle Pairwise Sequence Alignment tool with default parameters settings (Matrix: BLOSUM62, gap open penalty: 10 and gap extend penalty: 0.5). Protein domains were determined using SMART (URL http://smart.embl-heidelberg.de/), and PROSITE (URL http://us.expasy.org/prosite/) software. The protein regions used for the Vps15 catalytic domain analysis were: *T. cruzi*: residues 51 to 379; *Homo Sapiens*: 25 to 320; *S. cerevisiae*; 26 to 304; *Leishmania major*: 10 to 259, and *T. brucei*: 55 to 379.

Expression and purification of recombinant TcVps15 in *Escherichia coli*

Full-length *TcVps15* gene was amplified using the following primers carrying hemi-restriction sites: (Vps15-Fw-pENTR-KpnI 5′GGTACCATGGGGAATCAGCTGGCAGCAGC A3′ and Vps15-Rv-pENTR-XhoI 5′CTCGAGTTACTCTGA ATTAAGCCACACCGT3′), cloned into pGEM-T Easy plasmid (Promega) and subcloned into the Gateway® pENTR3C vector (Invitrogen, Carlsbad, CA).

To express the N-terminal GST-tagged *TcVps15* in *E. coli*, a recombination reaction was performed, according to the manufacturer instructions, to transfer the *TcVps15* coding sequence from pENTR3C into the pDEST15 expression vector (Invitrogen). The construct (pDEST15-*TcVps15*) was transformed into *BL21(DE3) pLysS* host (*E. coli B, F^-, dem, ompT, hsdS, (rb^-, m_B^-), galλ(DE3), [pLysS, cam^r]*) and the recombinant protein was induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 2 h. Cells were harvested by centrifugation and resuspended in lysis buffer [10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% (V/V) Triton X-100, 10 mM 2-mercaptoethanol] containing a protease inhibitor cocktail (Complete mini, Roche, Mannheim, Germany). The protein extract was prepared by sonication, centrifuged, and resuspended in the same lysis buffer. This fraction was purified using a Glutathione sepharose matrix (GE Health Care Lifesciences, Piscataway, NJ), washed with TEN buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl) and eluted with elution buffer (10 mM reduced glutathione, 50 mM Tris–HCl pH 8.0).

TcVps15-K219D was also subcloned into the Gateway® pENTR3C vector and expressed in the same way as described above.

Kinase activity assays

Protein kinase activity was determined as previously measured for the hVps15 (Panaretou et al. 1997). Briefly, between 30 and 70 μg of the purified recombinant protein were incubated in 35 with 15 μl of a phosphorylation mixture containing 5 μCi of [γ-^{32}P]ATP, 10 μM ATP, 25 mM Tris-HCl pH 8.0, 20 mM $MnCl_2$, 1 mM DTT, 500 μM NaF, 400 μM Na_3VO_4, and 0.5 mg/ml of the histone H2As (Histone from calf thymus, Sigma) or 0.2 mg/ml of the myelin basic protein (MBP, Sigma) as substrates. After 30 min of incubation at 30 °C, the kinase reaction was stopped by spotting 40 μl of the reaction onto 2 × 2 cm squares of phosphocellulose P-81 paper. [γ-^{32}P]ATP was separated from the labeled substrate by washing the P-81 filter papers tree times for 5 min with 75 mM H_3PO_4. The papers squares were dried, and the radioactivity incorporated was determined in a liquid scintillation counter.

For the determination of kinetic parameters, at least three independent assays were performed with 30 μg of protein and 0.01–250 μM ATP. The different ions were used to a final concentration of 20 mM in the assay.

Alternatively, after a standard in vitro kinase assay, the phosphorylated products were resolved on 15% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and exposed to Kodak X-Omat films (Sigma-Aldrich), or signal was scanned with a Storm 820 Phosphorimager (Pharmacia Biotech, Piscataway, NJ).

PI3K activity assays using *T. cruzi* epimastigote cell extracts were carried out as described before (Schoijet et al. 2008). Briefly, 50 μg of total protein was assayed in

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75 μl reactions containing 20 mM Tris–HCl, pH 7.4, 4 mM $MgCl_2$, 100 mM NaCl, 2 mg/ml sonicated phosphatidylinositol, 40 μM ATP, and 25 μCi/ml [^{32}P]ATP. The mixtures were incubated at 30 °C for 15 min, and reactions were terminated by the addition of 150 μl of 1 M HCl. Lipids were extracted first with $CHCl_3/CH_3OH$ (1:1) plus 10 mM EDTA, and the resulting organic phase was then extracted with CH_3OH/HCl (100:1) plus 10 mM EDTA. The obtained organic phase was dried with nitrogen gas, resuspended in $CHCl_3/CH_3OH$ (1:1), and radiolabeled phosphoinositides were measured in a liquid scintillation counter.

Parasite transfection

The full-length *TcVps15* gene was amplified using the primers Vps15-Fw-pribo-HindIII 5′AAGCTTATGGGGAATCAG CTGGCAGCA3′ and Vps15-Rv-priboHA-XhoI 5′CTCGAGT TAAGCGTAATCTGGAACATCGTATGGGTACTCTGAATTAA-GCCACACCGT3′. The 4,026-bp PCR product, with an hemaglutinin tag at its C-terminal, was then cloned into pGEM-T Easy plasmid and subcloned into the pRIBOTEX plasmid, which integrates into the ribosomal locus and therefore presents higher levels of expression than the episomal pTEX plasmid (Martinez-Calvillo et al. 1997). *Trypanosoma cruzi* epimastigotes of CL Brener strain were transfected with the pRIBOTEX-TcVps15-HA construct as described previously (Vazquez and Levin 1999). Stable cell lines were achieved after 60 d of treatment with 500 μg/ml G418 (Gibco BRL, Carlsbad, CA) and the transgenic condition was confirmed by Southern and western blot analyses.

A catalytic site mutant, where the lysine 219 present at the active site was replaced by an aspartic acid was also subcloned into pRIBOTEX taggued to hemaglutinin (TcVps15-K219D-HA) with the same primers, using as template the gene cloned into pGEM-T Easy vector.

Southern, northern, and western blot analyses

Genomic DNA was purified as described by Pereira et al. (2000). Total cellular RNA was isolated from 10^8 epimastigotes in the exponential growth phase using TRIzol reagent, as described by the manufacturer (Invitrogen). Southern and northern blot analyses were performed as described by Alonso et al. (2007). The products were revealed with a specific 2,152-bp *TcVps15* probe obtained by digestion of the gene cloned into pRIBOTEX with HindIII and SacI. All probes were labeled with [α-^{32}P]-dCTP using the Prime-a-Gene kit (Promega) following the manufacturer's instructions.

For western blot analysis, proteins were solved in 8% (w/v) SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970) and electrotransferred to Hybond-C membranes (Amersham Pharmacia Biotech). The membranes were blocked with 5% (w/v) nonfat milk suspension in TBS-Tween for 2 h and detected with a rat anti-HA (Roche) and a goat anti-GST antibodies (GE Healthcare Life Sciences). The transferred membranes were then incubated for 2 h with a 1:4,000 dilution of rabbit anti-GST (Sigma, Aldrich) or a 1:4,000 dilution of rat anti-HA high affinity monoclonal antibody (Roche Applied Science). Detection was carried out by incubating with a 1:5,000 dilution of a goat anti-rabbit or goat anti-rat conjugated to peroxidase (Perkin Elmer and Sigma, Aldrich, respectively). The latter were developed with the ECL Plus™ Western blotting detection system (PerkinElmer Life Sciences).

Yeast two-hybrid assay

The ProQuest™ Yeast Two-Hybrid Gateway® System was used for the yeast two hybrid experiments, as indicated by the manufacturers (Invitrogen). The ORFs of *TcVps15* and the PI3K *TcVps34* were transferred from the entry vector pENTR3C to the pPC97 (DNA-Binding Domain [BD]) and pDEST22 (Activation Domain [AD]) fusion vectors. Mav203 yeast cells were first transformed with the BD fusion plasmids using the lithium acetate method (Sambrook and Russell 2001). Transformants were selected on plates with synthetic complete medium (SC) containing 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% (w/v) ammonium sulfate and 2% (w/v) glucose, lacking Leu. Thereafter, cells carrying each BD plasmid were transformed with the corresponding AD plasmids. Double transformants were selected on plates lacking Leu and Trp. The identity and integrity of the BD and AD fusion products was confirmed by colony PCR on yeast colonies.

Activation of *His3* gene induced by interaction pairs was studied analyzing the ability of yeast expressing the corresponding GAL4 fusion proteins to grow on plates lacking histidine (SC $Leu^-Trp^-His^-$), in the presence of up to 50 mM of 3-amino-1,2,3-trizole (3AT) and incubated at 30 °C for up to 48 h.

For the negative selection assay, selected colonies were plated separately in Leu^-Trp^-plates in the presence of 5-fluororotic acid (5FOA 0.2%) and incubated at 30 °C for up to 48 h. ProQuest's negative Control A (empty DB and AD) and positive Control E (DB full-length GAL4 protein) were used as standard controls.

Monodansylcadaverine (MDC) labeling assay

The assay was performed according to Munafo and Colombo (Munafo and Colombo 2001). Briefly, after autophagy induction by serum deprivation, parasites were incubated with 0.05 mM MDC in PBS at 28 °C for 16 h. After incubation, cells were washed three times with PBS and immediately analyzed in an Olympus BX41 fluorescence microscope. To quantify the labeling of MDC, cells were washed twice in PBS and lysed in 10 mM Tris–HCl pH 8.0 containing 0.1% Triton X-100. MDC stain was measured by fluorescent photometry (excitation wavelength 380 nm, emission filter 525 nm). To normalize the measurements, fluorescence of total DNA was quantified previously staining with 0.2 μM ethidium bromide (excitation wavelength of 530 nm and an emission filter of 590 nm). Results were expressed as specific activity (percentage respect to the control).

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Analysis of acidic organelles with acridine orange

In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red. The intensity of the red fluorescence is proportional to the degree of acidity. Therefore, the volume of the cellular acidic compartment can be quantified (Paglin et al. 2001; Traganos and Darzynkiewicz 1994). Cells were loaded with 0.1 μM acridine orange for 10 min. Green (510–530 nm) and red (650 nm) fluorescence emission from 1×10^4 cells illuminated with blue (488 nm) excitation light was measured by flow cytometry analysis (FACS Aria, Becton Dickinson, Sumter, SC).

For fluorescence analysis of acidic organelles, control or starved cells were loaded with 3 μM acridine orange for 10 min, washed once with PBS, adhered to poly-L-lysine-coated coverslips and the staining was then immediately evaluated by microscopy using an Olympus BX41 fluorescence microscope.

Immunofluorescence microscopy

For immunofluorescence, cells were fixed with paraformaldehyde in PBS for 15 min. Next, the cells were washed twice in Dulbecco's PBS, pH 7.2, and washed adhered to poly-L-lysine-coated coverslips, and permeabilized for 2 min with 0.3% Triton X-100. Cells were incubated for 10 min with 25 mM ammonium chloride, and washed again with PBS. Cells were blocked for 20 min in 50 mM NH_4Cl and 3% bovine serum albumin in PBS, pH 8.0, and incubated for 1 h with rat anti-HA high affinity monoclonal antibodies (Roche Applied Science) at 1:500 and rabbit anti-ATG8.1 polyclonal antibody at 1:700, kindly donated by Dr. Vanina Alvarez (Alvarez et al. 2008a). Cells were then washed in 3% bovine serum albumin, incubated with secondary antibodies anti-rabbit Alexa 488 at 1:500 and anti-rat Alexa 546 conjugate at 1:500 containing 5 mg/ml DAPI and mounted with Vectashield (Vector Laboratories). Cells were observed in an Olympus BX41 fluorescence microscope.

The number of autophagosomes was calculated with the Image J software as the number of punctate-positive cells divided by the total number of cells in the same field. More than 100 cells were scored for each treatment. Data represent the means of three independent experiments.

RESULTS

Cloning and characterization of the TcVps15 gene

We performed a search through *T. cruzi* genome data bases using the *Vps15* gene (ID 852394) from *S. cerevisiae* as query, which resulted in the identification of an orthologous sequence in this parasite (TcCLB.510 901.120). Two oligonucleotides designed from this sequence: Vps15-Fw-pENTR-KpnI 5′GGTACCATGGGGAA TCAGCTGGCAGCAGCA3′ and Vps15-Rv-pENTR-XhoI 5′CT CGAGTTACTCTGAATTAAGCCACA CCGT3′, were used to amplify the *T. cruzi* gene using genomic DNA as template. After sequencing *TcVps15*, the 4,026-bp open reading frame was identified.

The complete open reading frame of the gene was found to be 4,026-bp long and to encode a polypeptide of 1,341 amino acids. The predicted primary sequence of TcVps15 protein shared 49.4% of identity and 63.4% of similarity with *T. brucei* Vps15 (XP_828984.1), 26.5% of identity and 38% of similarity with the *Leishmania major* ortholog, 18.2% of identity and 32.5 of similarity with *S. cerevisiae* ScVps15p (CBK39172) (Herman et al. 1991b) and 21.4% of identity and 36,6 of similarity with the human VPS15 homolog p150 (NP_055417.1) (Panaretou et al. 1997). When the same analysis was performed comparing the kinase domains, the percentages of identity/similarity were the following 58.2%/69.7% for *T. brucei*, 29.7%/42.3% for *L. major*, 29.8%/44.9% for *S. cerevisiae,* and 29.8%/45.7% for *H. sapiens*.

To extend our analysis, a protein domain architecture analysis of the Vps15 ortholog proteins from Human, *S. cerevisiae* and *T. cruzi* was performed. This study shows a conservation of the kinase and the C-terminal WD40 domains between the three sequences (Fig. S1).

Performing a more detailed analysis, we found that the predicted amino acid sequence of TcVps15 presented an immediate N-terminal potential attachment site for myristic acid (amino acids 1-6, Fig. S2 green line over the sequence). Subsequently is the catalytic domain (amino acids 37-374), shared by the serine/threonine family of protein kinases. The shared sequence DLKTDN (Fig. S2 red line over the sequence) within this region closely resembles the consensus element DIKTEN, which is specific for Ser/Thr protein kinases (Hanks 1987). A lysine residue in this domain (K219, star Fig. S2) is conserved among all known serine/threonine protein kinases while an aspartic-acid residue (D235) and a lysine (K87) are part of the putative ATP-binding site(s) and are also conserved in both serine/threonine and tyrosine protein kinases (Hanks et al. 1988). Alteration of any of these three residues in ScVps15p results in a severe reduction in enzymatic activity and mislocalization of vacuolar enzymes (Herman et al. 1991a). Finally, the C-terminal region possesses three WD repeats (amino acids 951-989, 1,201-1,242, and 1,296-1,338, Fig. S2 blue lines over the sequence) which are thought to mediate protein–protein interactions and have been implicated in the regulation of various cellular functions including cell division, cell fate determination, gene transcription, autophagy, apoptosis, and vesicle fusion (Stirnimann et al. 2010).

Expression and kinetic characterization of TcVps15 in *Escherichia coli*

Recombinant TcVps15 was subcloned into the pDEST15 expression vector. The expression was confirmed by western blot, revealing a 173 kDa protein representing TcVps15 (147 kDa) tagged with the 26 kDa GST moiety. The cytosolic fraction was used to purify the recombinant protein using a glutathione-Sepharose matrix (Fig. S3). Afterward, the protein kinase activity and the ability of the

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protein to phosphorylate two commercial substrates were investigated. The results demonstrate that TcVps15 is able to phosphorylate both histone H2As (33.46 nmoles/min/mg protein) and myelin basic protein (23.41 nmoles/min/mg protein). The activity of TcVps15 was further characterized, showing a saturable dependence on and a high affinity for ATP and displaying a standard Michaelis–Menten kinetics with a Km value for this substrate of 2.08 ± 0.25 nM (SEM, $N = 4$) and *V*max of 0.1402 ± 0.02 pmol ATP/min.mg protein (Fig. 1A, B, solid line). To study the structural role of TcVps15 in more detail, the lysine 219 within the catalytic domain was replaced to an aspartate. In enzyme-kinetic experiments, the Vps15 mutant protein (TcVps15-K219D) displayed a 5.6-fold increase in Michaelis–Menten constant (11.67 ± 1.0751 nM, SEM, $N = 4$) compared to TcVps15 wild type and no apparent variation in *V*max value (0.1646 ± 0.03 pmol ATP/min/mg protein, $N = 4$) (Fig. 1A, B, punctuate line). In addition, this mutation led to a reduction of 46% ($n = 4$) of in vitro kinase activity (Fig. 1C). Interestingly, the protein kinase activity of TcVps15 displays a cation preference for Mn^{+2} and was blocked by EDTA (Fig. 1D), which is also observed for the Vps34p, Vps15p protein kinase activities (Stack and Emr 1994) and the human homologous to the yeast Vps15p p150 (Panaretou et al. 1997). Finally, to test whether TcVpsl5 is able to undergo autophosphorylation

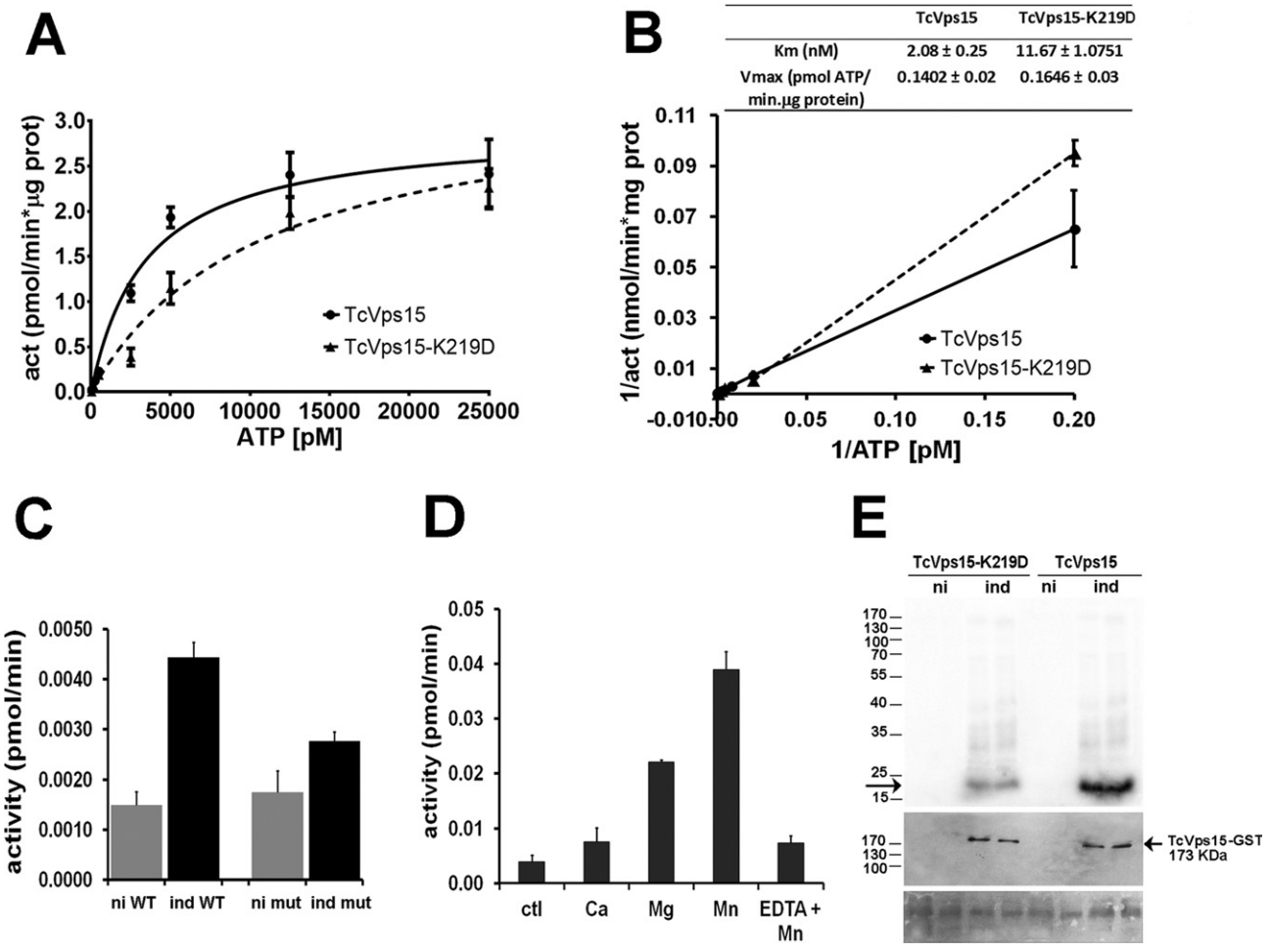

Figure 1 Kinetic parameters and ion dependence of the recombinant TcVps15. Kinase assays were performed with purified recombinant proteins from *Escherichia coli* (25 µg). (**A**) TcVps15 and TcVps15-K219D activity as a function of ATP concentration (solid and punctuate lines, respectively). (**B**) Lineweaver burk plots. The table above shows the *K*m and *V*max values for both proteins (SEM, $N = 4$). (**C**) Kinase activity of TcVps15 and TcVps15-K219D noninduced (non ind) and induced bacteria, using the histone H2As as substrate. Error bars represent SEM ($N = 4$). (**D**) Ion dependence on TcVps15. Chelator EDTA was used at a final concentration of 200 mM and the different ions were added to reach a final concentration of 20 mM. Standard assay conditions were used as a control. (**E**) In vitro kinase assay followed by SDS-PAGE and autoradiography. Protein kinase activity of TcVps15-K219D and TcVps15 was assayed using 5 µCi of [γ-^{32}P]ATP and analyzed by SDS-PAGE followed by autoradiography. Middle panel shows the western blot of the same SDS-PAGE revealed with rat anti-HA antibody which recognize both versions of TcVps15 and bottom panel shows the Ponceau staining of the region corresponding to H2As as substrate loading control. The position of the phosphorylated histone H1 (21.5 kDa) was marked by an arrow. Each condition was evaluated by duplicate.

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in vitro, a kinase assay using the histone H2As as substrate was performed with TcVps15 and TcVps15-K219D recombinant proteins and analyzed by SDS-PAGE followed by autoradiography. A lower level of histone phosphorylation was observed with the mutant version of the protein, similar to that obtained with the in vitro kinase experiments described above. Unlike what happen with Vps15p of *S. cerevisiae*, no apparent autophosphorylation was observed in any case (Fig. 1E).

Interactions among TcVps15 and TcVps34

As it was mentioned above, Vps15 is part of a membrane-associated complex with the phosphatidylinositol 3-kinase Vps34 protein kinase, which is important for protein sorting in prokaryotic and eukaryotic cells. To elucidate the interacting relationship among TcVps15 and TcVps34 we designed a yeast two-hybrid experiment using the Gateway® System (Fig. 2A). Both *T. cruzi* proteins were subcloned into the corresponding vectors to obtain the DNA-binding domain (BD) and activation domain (AD) fusions and combinations of the constructs were transformed into *S. cerevisiae* MaV203 cells. We performed both forward and reverse Y2H analysis. In forward analysis, growth is dependent on *His3* reporter activation in plates lacking histidine and is indicative of interaction. In reverse analysis, growth is indicative of no interaction since the activation of *Ura3* reporter leads to the conversion of 5-fluoroortic acid (5-FOA) in 5-fluorouracil, a compound toxic to yeast. As shown in Fig. 2B, all yeast cells positive for the histidine assay did not grow on 5FOA plates, demonstrating that, as it happens with other microorganisms, TcVps15 and TcVps34 interact with each other.

Development and analysis of transgenic lines

Having biochemically characterized TcVps15 as a catalytically active kinase, we next investigated its functionality in the parasite. For this purpose, C-terminal HA-tagged *TcVps15*, a catalytic site mutant of this gene where lysine 219 was replaced by aspartic acid, and *TcVps34* genes were subcloned into the pRIBOTEX expression vector. *Trypanosoma cruzi* epimastigotes were then transfected with these constructs. Southern blot analysis indicated that *TcVps15* is a single copy gene and confirmed the presence of an extra copy integrated into the genome of TcVps34-HA and TcVps15-HA transfected cells (Data not shown). Afterward, the expression and intracellular localization of these two proteins was investigated first by western blot using an anti-HA antibody. Two protein bands of the expected molecular weight (100 and 148 kDa for TcVps34 and TcVps15, respectively) were detected in the membrane fraction of parasite extracts (Fig. 3A). To investigate whether these proteins represented peripheral or integral membrane proteins, membrane extracts were subjected to independent washes with 1 M NaCl, 0.1% sodium cholate, 0.5% sodium cholate, and 8 M urea for 30 min. As shown in Fig. 3, TcVps15 and TcVps34 were partially released when pellets were washed with 1 M NaCl or 0.1% sodium cholate, and totally released with the other treatments, indicating that TcVps34 and TcVps15 are peripheral proteins weakly associated to membranes (Fig. 3B, C).

Starvation of TcVps15 overexpressing cells leads to an increase in acidification

To investigate if TcVps15-TcVps34 complex has a role in acidification, we used the lysosomotropic agent, orange (AO). We performed FACS analysis of AO-stained-cells using PerCP-A mode (>650 nm) to value the bright red fluorescence and FITC-A mode (500–550 nm) to value the green fluorescence. In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red as described previously. The intensity of the red fluorescence is proportional to the degree of acidity of the cellular acidic compartments. TcVps15-HA cells show more acidification (evaluated as AO staining) in comparison with the mutated TcVps15-K219D-HA and the wild-type parasites after the starvation treatment (Fig. S4A). In addition, it is worth to note that TcVps15-HA parasites have a higher level of basal acidification. To deepen this observation, we performed microscopic fluorescence analysis with AO stained cells under control or starvation conditions. We could observe that under normal cultivation conditions, the staining with AO was very low, needing a long exposure time to capture the images. In contrast, there is a significant puncta staining under starvation conditions, being this signal more intense in TcVps15-HA overexpressing parasites, comparing with wild type and TcVps15-K219D-HA parasites (Fig. S4B). Taken together, these findings suggest an implication of TcVps34 and TcVps15 proteins in acidification. This is an interesting result, since in *T. brucei* it has been reported that autophagy is accompanied by acidocalcisome acidification, so future studies would be worthy to address if TcVps34 and TcVps15 are also implied in this process.

TcVps15 and TcVps34 are involved in autophagy

To further investigate if the acidification increase observed in transgenic parasites is related to autophagy, we evaluate TcVps15 and TcVps34 intracellular localization by indirect immunofluorescence microscopy under different nutritional conditions (Fig. 4A, B). In nutrient-rich LIT growth medium, both proteins were located in the membrane structures distributed around the parasite. However, when overexpressing epimastigotes were exposed to prolonged starvation in PBS, both TcVps15 and TcVps34 localization changed considerably, revealing the presence of several large bright spots, which partially co-localize with the autophagosomal marker TcATG8.1, indicating that these proteins were involved in the autophagy mechanism (Fig. 4A, B, respectively). On the other hand, when epimastigotes were transfected with TcVps15-K219D-HA, the quantity of punctuate structures in starvation was reduced

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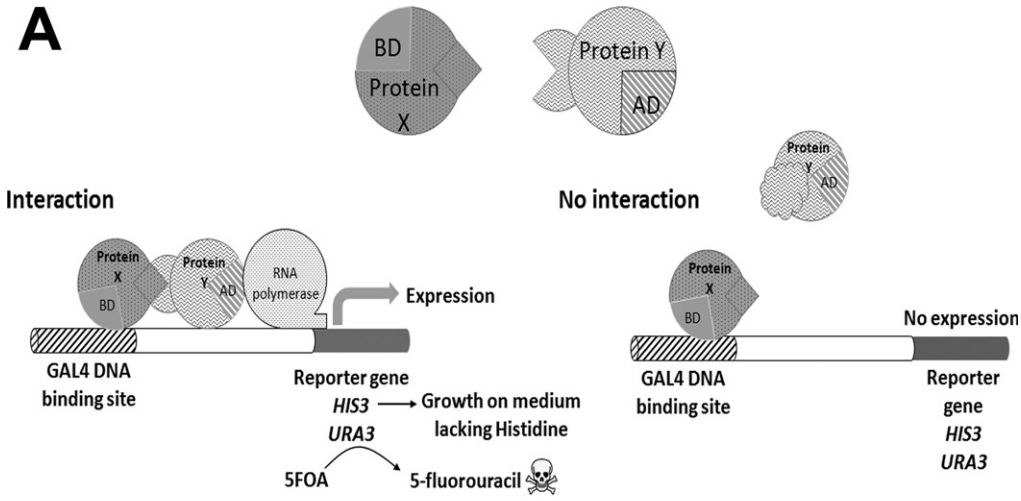

Survival	LWH- (3AT)	5FOA
Interaction	+	-
No interaction	-	+

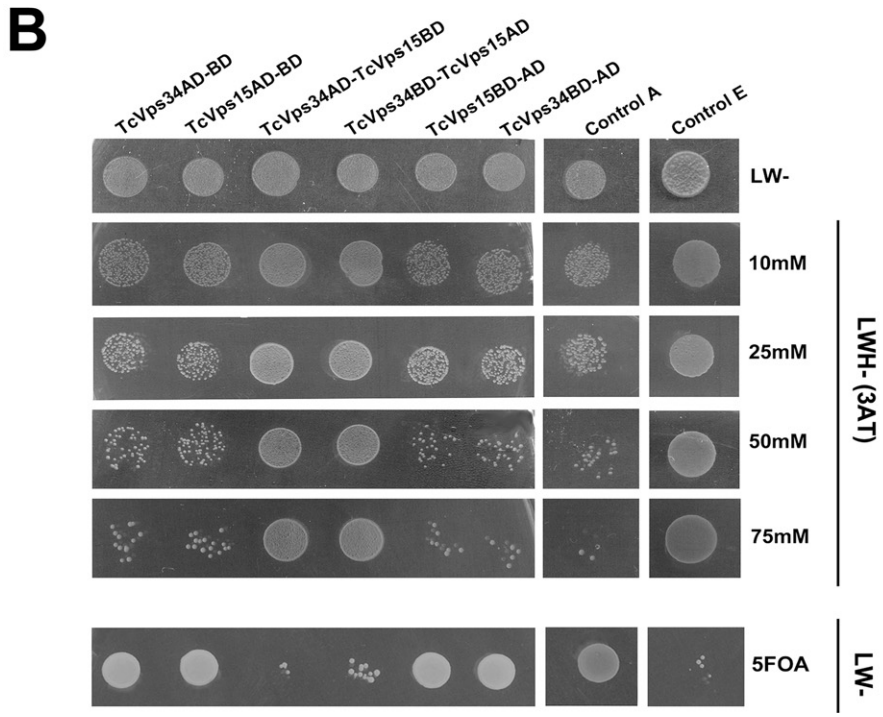

Figure 2 Interaction between TcVps15 and TcVps34 by yeast two-hybrid system. (**A**) Yeast two-hybrid system. This scheme illustrates the bases of the yeast two-hybrid system used to screen interaction between two proteins. Each protein gene is encoded in a separate plasmid with different selectable markers. One is expressed as a fusion with the DNA-binding domain of the transcription factor GAL4 (BD), and the other is attached to the activation domain (AD) of the same transcription factor. The plasmids are double-transformed into yeast and the interactions are analyzed by different selection markers. If there is interaction between the two proteins (X and Y in the scheme), the activation domain activates the transcription of the reporter genes HIS3 and URA3. The first one allows the yeast to produce histidine, which makes it possible to assess the interaction in a histidine free growth medium (Forward analysis). The URA3 reporter allows the conversion of 5-fluoroortic acid (5FOA) into the toxic compound 5-fluorouracil, hence the yeast does not survive in the presence of 5FOA (Reverse analysis). (**B**) Interaction between TcVps15 and TcVps34 by yeast two-hybrid system. Each protein pair was tested as indicated in DB and AD configuration. Double transformants were evaluated for forward and reverse yeast two-hybrid analysis. Forward, activation of *His3* reporter in LWH$^-$ with increasing 3AT concentration plates. Reverse, activation of *Ura3* reporter in LW$^-$ 0.5% FOA plates. Control A (empty DB and AD) and Control E (DB full-length GAL4 protein) indicates ProQuest's standard negative and positive controls, respectively.

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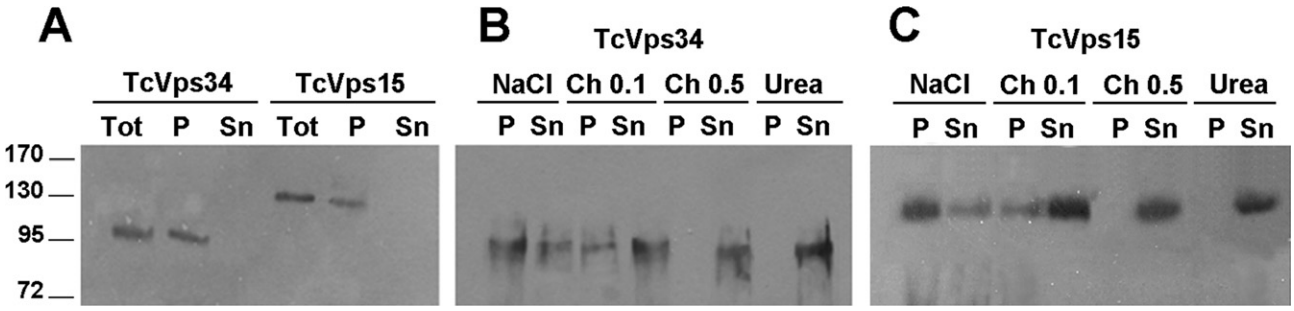

Figure 3 Membrane association of recombinant TcVps15 and TcVps34. The association of TcVps15 and TcVps34 with membranes was analyzed by western blot assay. 50 μg of total extracts (tot), soluble fractions (Sn) or pellet (P) were resolved by SDS-PAGE (8% gels), electrotransferred on to Hybond C membranes and revealed with rat anti-HA antibody (**A**) Afterward, membranes were extracted to release each protein by independent washes with 1 M NaCl, 0.1 or 0.5% (w/v) sodium cholate, or 8 M urea (**B** and **C**).

(Fig. 4C). The number of TcAtg8.1-positive puncta per cell was manually counted for wild type, TcVps15-K219D-HA and TcVps15-HA parasites (Fig. 4D). Quantification, revealed an increased number of puncta in TcVps15-HA parasites with respect to wild type. In turn, the mutant TcVps15-K219D-HA parasites showed an intermediate number of autophagosomes between control parasites and those that overexpress the wild-type version of TcVps15. Similar results were obtained by counting HA-positive puncta per cell with TcVps15-K219D-HA and TcVps15-HA parasites (results not shown). These results are consistent with those observed with the kinetic assays, where although the mutant protein still possesses kinase activity, it is reduced compared to the wild-type version of the protein. Next, the protein expression levels were studied by western blot analysis and compared with cells overexpressing the nonmutated gene. As Fig. 4E shows, both specific protein levels in TcVps15-HA and TcVps15-K219D-HA expressing epimastigotes reveal that the translation of TcVps15 is not affected by the substitution of lysine 219 by aspartic acid. It is worth noticing that protein kinase activity in extracts of TcVps15-K219D-HA-expressing cells was significantly reduced when compared with extracts from cells overexpressing the wild-type version of TcVps15 (1.75 ± 0.89 nmoles/min, 8.62 ± 0.17 nmoles/min, 10.34 ± 0.90 nmoles/min and 7.24 ± 0.65 nmoles/min for wild type, TcVps34-HA, TcVps15-HA and TcVps15-K219D-HA cells, respectively) (Fig. 4F). Moreover, and to strengthen the relationship between TcVps34 and TcVps15, we analyzed Vps34 activity in wild-type cells, TcVps15-HA and TcVps15-K219D-HA extracts. Interestingly, lipid kinase activity was enhanced in TcVps15-HA parasites comparing to wild type, but it was less significant in TcVps15-K219D-HA parasites. This is a remarkable result, since it is yet not known how Vps15 regulates the activity of Vps34 in other organisms. Finally, to corroborate the role of TcVps15 in autophagy, we performed fluorescence microscopy using the autofluorescence drug monodansylcadaverine (MDC), which has been reported as a specific in vivo marker for autophagic vacuoles. As shown in Fig. 5A, starved wild type, TcVps15-K219D-HA and TcVps15-HA parasites showed a positive label of autophagic vacuoles, which was not observed in parasites cultivated in LIT medium. To further evaluate differences between the three lines of parasites, intracellular MDC was quantified by fluorescent photometry (see Materials and Methods) (Fig. 5B). These results again showed increased levels of autophagy in parasites that overexpressed TcVps15 compared to wild type, and an intermediate level of autophagy in parasites that have the mutated version of this protein. Taken together, these results demonstrate that TcVps15 has a role in autophagy in *T. cruzi*, and that its overexpression enhances this process.

DISCUSSION

As *T. cruzi* passes through its digenetic life cycle, it encounters extreme environmental fluctuations which are accompanied by extensive metabolic and morphological changes of the parasite. Previously, we have identified the first PI3K in *T. cruzi*, TcVps34, which plays a prominent role in vital processes such as osmoregulation, vesicular acidification, and vesicular trafficking (Schoijet et al. 2008). This enzyme has also been involved in other organisms in the processes of autophagy (Jaber et al. 2012; Kim et al. 2013). In the last few years, ATG homologs have been discovered in *Trypanosoma cruzi* (Alvarez et al. 2008a), *T. brucei* (Li et al. 2012), and *L. major* (Besteiro et al. 2006; Williams et al. 2009). Autophagy in these parasites has a more important role than the basal turnover of proteins to eliminate damaged cell constituents, due to their metabolic capacity during the successive transitions in their complicated life cycle. In fact, it has been reported that autophagy is triggered during *T. cruzi* epimastigote to metacyclic trypomastigote differentiation (Alvarez et al. 2008a).

The class III PI3K Vps34 and its interacting partners ATG6/Vps30 and Vps15, which participate in the vesicle nucleation step, possess homologs in the trypanosomatid genomes but their role in autophagy has not been studied until now. In this study, we propose that the PI3K TcVps34 and its serine/threonine protein kinase TcVps15 are involved in autophagy in *T. cruzi*.

TcVps15 is a single copy gene which shares a considerable degree of similarity with its mammalian,

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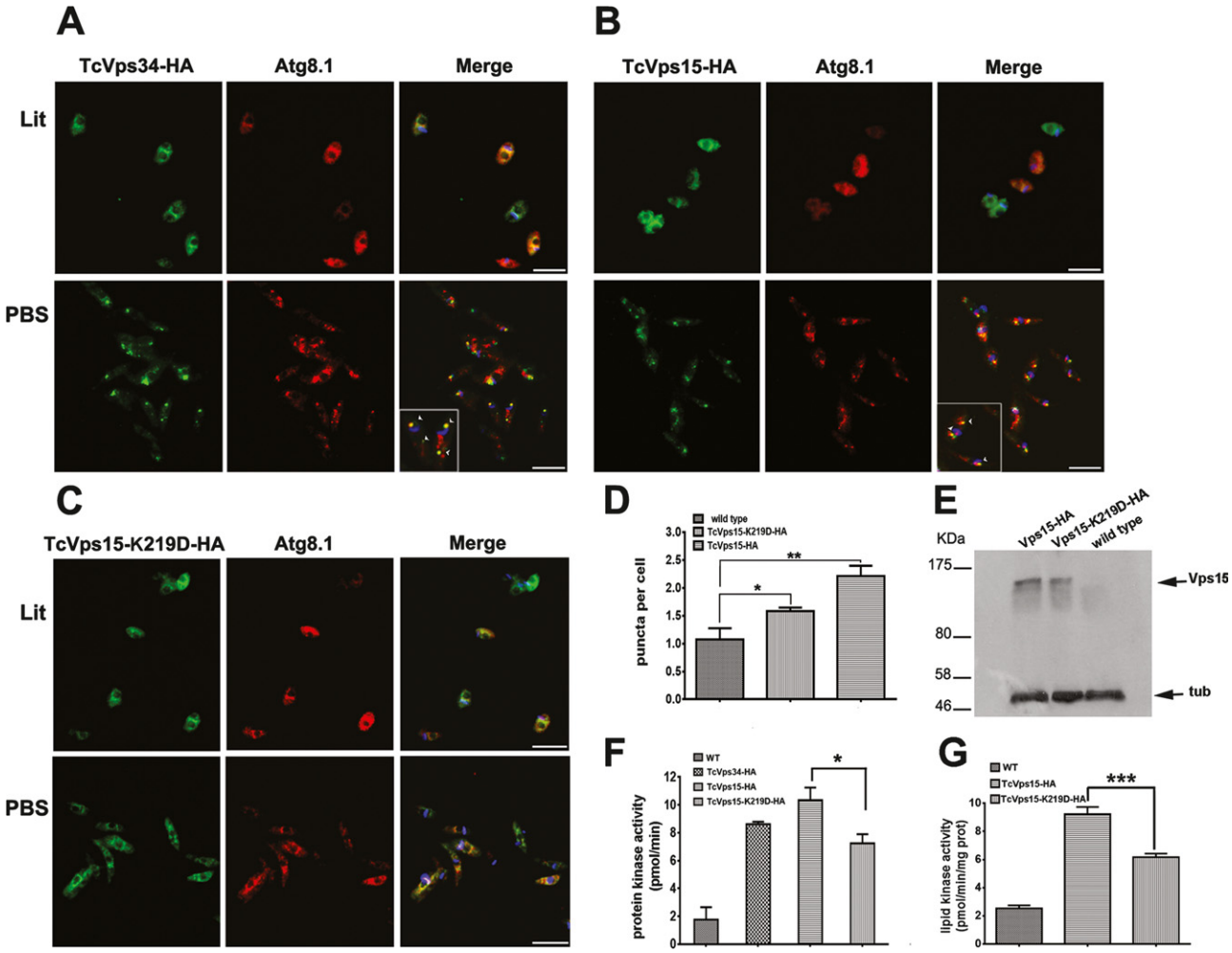

Figure 4 Punctate distribution of TcVps34 and TcVps15 in starved *Trypanosoma cruzi* epimastigotes. (**A–C**) Localization of TcVps34 (A), TcVps15 (B), and TcVps15-K219D (C) fused to an HA tag by immunofluorescence microscopy in epimastigotes of *T. cruzi*. Studies were performed using rat anti-HA monoclonal antibody (green) and polyclonal anti-TcAtg8.1 serum (red). Nuclear and kinetoplast DNA were visualized by DAPI staining. The resulting merged images are shown. The upper half represents the subcellular distribution of the overexpressed proteins in nutrient-rich LIT media, whereas the lower half corresponds to the pattern observed after starvation in PBS. Scale bar = 10 μm (**D**) Quantification of the number of TcAtg8.1 puncta per cell (autophagosomes) in wild type, TcVps15-HA, and TcVps15-K219D-HA epimastigotes. Average number of puncta per cell was obtained by counting 200 cells from each indicated conditions in three independent experiments. Error bars represent the standard error of the mean (SEM) (*t*-test, $p < 0.05$, *asterisks*). (**E**) Western blot analysis of wild type, TcVps15-HA, and TcVps15-K219D-HA-expressing epimastigotes. Total proteins extracts (50 μg) were resolved by SDS-PAGE (8% gels), electrotransferred onto Hybond C membranes and revealed with anti-HA antibody. The blot was at the same time probed with antitubuline antibody as a loading control (arrow). (**F**) Kinase activity in transgenic parasites. Kinase activity assays were performed as described under Material and Methods using H2As as substrate and 20 μg of total protein of wild type (WT), TcVps34-HA, TcVps15-HA, and TcVps15-K219D-HA parasite extracts. There was a statistically significant (*t*-test, *p* 0.0336, *asterisk*) decrease in TcVps15-K219D-HA kinase activity comparing to TcVps15-HA. Error bars represent the standard error of the mean (SEM) of three experiments. (**G**) Enhanced PI3K activity in TcVps15-HA-transfected parasites. PI3K activity assays were performed as described under Material and Methods using 50 μg of wild type (WT), TcVps15-HA, and TcVps15-K219D-HA parasite extracts. There was a statistically significant (*t*-test, *p* 0.0005, *asterisk*) decrease in TcVps15-K219D-HA kinase activity comparing to TcVps15-HA.

S. cerevisiae, *T. brucei*, and *L. major* orthologs. Moreover, its protein sequence conserves the protein domain characteristic structure with an N-terminal putative myristoilation site, the catalytic domain shared by the serine/threonine family of protein kinases and the C-terminal WD repeats region.

The recombinant enzyme was able to phosphorylate exogenous peptide and protein substrates in a Mn^{+2}-dependent manner. The preference for Mn^{+2} resembles the Mn^{+2} dependence of Vps15p and p150 for autocatalytic activity (Panaretou et al. 1997). These findings support the hypothesis that similar regulatory mechanisms exist for the protein kinase activity of the yeast, human, and *T. cruzi* proteins.

Kinetic data indicate that TcVps15 is a catalytically active kinase with a Km value, 2.08 ± 0.25 nM. In addition, the

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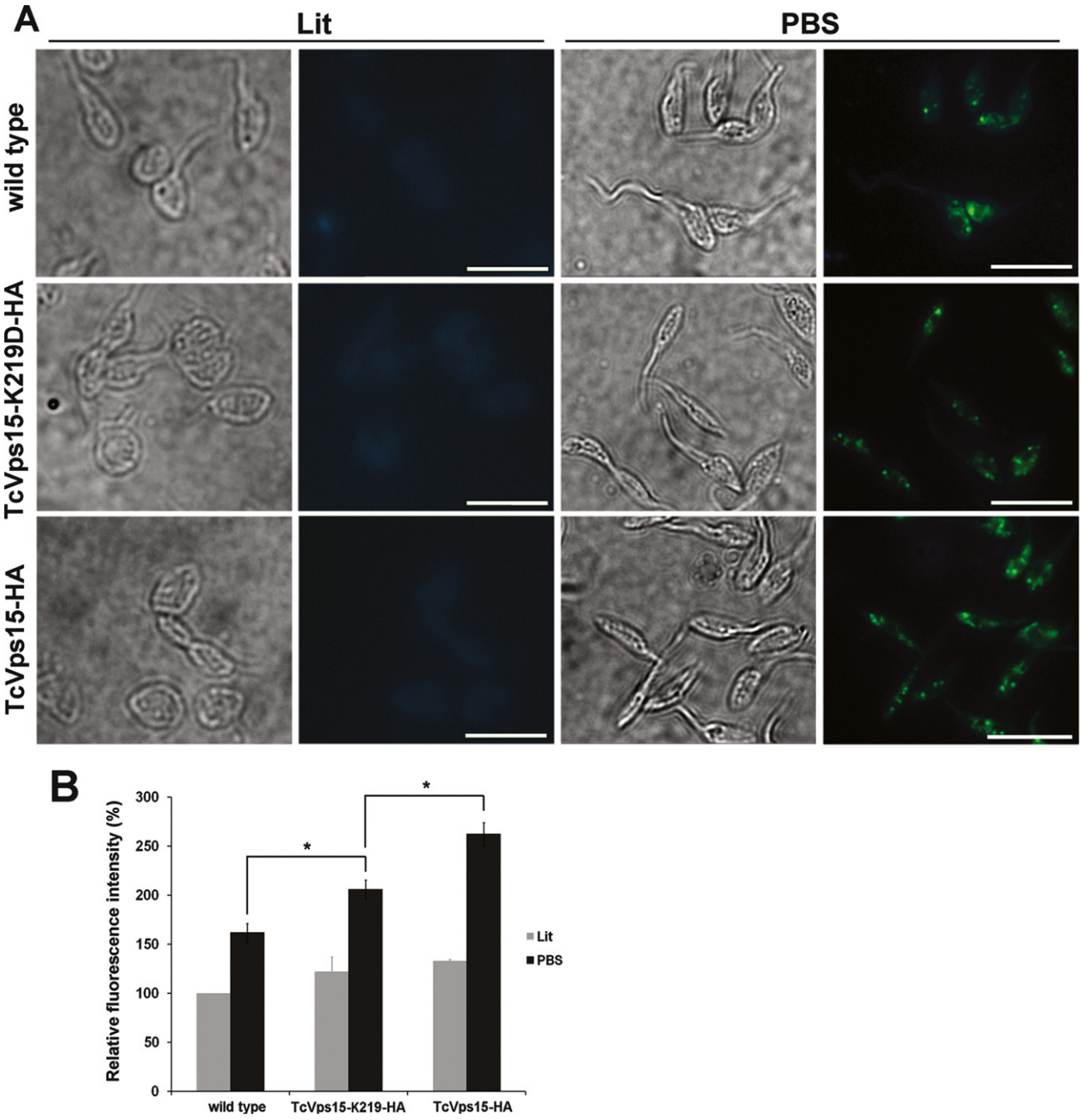

Figure 5 Accumulation of MDC in transgenic parasites. (**A**) Epimastigote cells were incubated in LIT medium (control cells) or in PBS (starved cells) for 16 h, washed with PBS and then incubated with 0.05 mM MDC for 10 min at 28 °C. Afterward cells where washed four times with PBS pH 7.4 and were immediately analyzed by fluorescence microscopy. Scale bar = 10 μm. (**B**) Cells treated as indicated above and then lysed in 10 mM Tris–HCl, pH 8 containing 0.1% Triton X-100. Intracellular MDC was measured by fluorescence photometry as indicated in Materials and Methods. The data represent the mean ± SEM of three independent experiments ($p < 0.05$, *asterisk*).

substitution of the lysine 219 by aspartic acid in its catalytic site increase de Km value of the enzyme, although do not modify the Vmax, indicating that this mutation alters the geometric structure of the protein, resembling to a competitive inhibitor. In addition and unlike *S. cerevisiae* Vps15p, we have shown here that TcVpsl5 is incapable of catalyzing a specific autophosphorylation in vitro.

Using yeast two-hybrid experiments, we showed the direct interaction of TcVps15 with TcVps34 in *T. cruzi*, which leads us to propose that in this parasite both proteins function as a complex, similar to what was shown for *S. cerevisiae* (Budovskaya et al. 2002). To support this hypothesis, we demonstrated that in nutrient-rich LIT medium, both proteins were located in membrane

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structures distributed in the cytosol. In the future it will be interesting to study the identity of these membrane structures, since Vps34 exists in different complexes with different subcellular localizations, and concerning Vps15 there are very few studies regarding its localization (Kim et al. 2013; Morris et al. 2015). Remarkably, when transfected epimastigotes were exposed to prolonged starvation in PBS, TcVps15, and TcVps34 localization changed considerably, revealing a partial co-localization with autophagosomal vesicles. This feature suggests that these two proteins are involved in the autophagic pathway alongside the Atg8 genes previously identified in trypanosomatids. Moreover, it is worth to remark an increase in the number of TcAtg8.1-autophagosome labeling in TcVps15-HA parasites with respect to wild-type parasites, and an intermediate level in TcVps15-K219D-HA parasites. In addition, it should be pointed that although the expression at protein level of the mutated TcVps15 was not affected, as was evidenced by western blot, the global kinase activity of the TcVps15-K219D-HA transgenic parasites was reduced compared to the parasites overexpressing the wild-type version of TcVps15 (Fig. 4E, F). Moreover, Vps34 activity was reduced in parasites expressing the mutated version of TcVps15. Together, these results suggest that the point mutation in the catalytic site of TcVps15 partially affects its activity and subcellular localization without affecting its levels of expression in the parasite. In addition, these data revealed a role of TcVps15 as a key regulator of TcVps34 activity, providing information on the regulation of this protein complex not known until now in *T. cruzi* and in most other organisms. Moreover, we found through monodansylcadaverine (MDC) staining that TcVps15 overexpression causes an accumulation of autophagic vacuoles, which together with TcAtg8.1 co-localization microscopy assays support a role for TcVps15 in autophagy in *T. cruzi*.

Complementary, by using the vital cell staining acridine orange, under starvation conditions, we observed an increase in the labeling of acidic compartments in TcVps15-HA parasites compared to wild-type ones. These results could indicate either, an upregulation in the autophagosome formation due to a general increase in lysosomal acidity, which is accompanied by an increase in autophagy, or a delay in some step of this degradative pathway. Moreover, the parasites overexpressing the mutated version of TcVps15 showed a reduced red-acridine orange staining compared to TcVps15-HA parasites, which is consistent with the TcAtg8.1-labeling described above. It is important to highlight that acidocalcisome acidification was reported in *T. brucei* during autophagy (Li and He 2014), so a possible acidocalcisome regulation may also be taking place in these transgenic parasites, which would be attractive to investigate hereafter.

Taken together, in this study we characterized the serine/threonine protein kinase TcVps15 of *T. cruzi*, which functions as part of a complex in concert with TcVps34, and more important, we demonstrate for the first time that TcVps15 regulates TcVps34 activity and that both proteins are involved in autophagy. Autophagy-related cell death has been observed by ultrastructural analysis in trypanosomes treated with certain drugs (Menna-Barreto et al. 2009; Uzcategui et al. 2007) and neuropeptides (Delgado et al. 2009). Our studies provided molecular evidence for autophagy in starvation induced parasites, together with the Atg8 gene previously identified.

Normally autophagy is reversible and acts as a self-protection mechanism. However, in tripanosomatids previous data has been provided that supports the essential role of autophagy in the transition between successive differentiation steps of their life cycle. Moreover, the unique features of *T. cruzi* autophagy mechanism lead us to propose an intriguing possibility of targeting autophagy pathway proteins as promising targets for the chemotherapy of Chagas disease.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Domain architecture of the Vps15 ortholog proteins from Human, *S. cerevisiae* and *Trypanosoma cruzi*.
Figure S2. Comparison of TcVps15 protein sequence with other Vps15 kinases.
Figure S3. Purification of recombinant TcVps15.
Figure S4. Detection of acidic organelles in acridine orange-stained cells.

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