Subcellular proteomics of *Trypanosoma cruzi* reservosomes

Celso Sant'Anna[1,3,#], **Ernesto S. Nakayasu**[2,#], **Miria G. Pereira**[1], **Daniela Lourenço**[1,3], **Wanderley de Souza**[1,3], **Igor C. Almeida**[2,*], and **Narcisa L. Cunha-e-Silva**[1,*]

[1] Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

[2] Department of Biological Sciences, The Border Biomedical Research Center, University of Texas at El Paso, El Paso, TX, USA

[3] Diretoria de Programas, Instituto Nacional de Metrologia, Normalização e Qualidade Industrial-INMETRO, Rio de Janeiro-Brazil

Abstract

Reservosomes are the endpoint of the endocytic pathway in *Trypanosoma cruzi* epimastigotes. These organelles have the particular ability to concentrate proteins and lipids obtained from medium together with the main proteolytic enzymes originated from the secretory pathway, being at the same time a storage organelle and the main site of protein degradation. Subcellular proteomics have been extensively used for profiling organelles in different cell types. Here, we combine cell fractionation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify reservosome-resident proteins. Starting from a purified reservosome fraction, we established a protocol to isolate reservosome membranes. Transmission electron microscopy was applied to confirm the purity of the fractions. To achieve a better coverage of identified proteins we analyzed the fractions separately and combined the results. LC-MS/MS analysis identified in total 709 *T. cruzi*-specific proteins; of these, 456 had predicted function and 253 were classified as hypothetical proteins. We could confirm the presence of most of the proteins validated by previous work and identify new proteins from different classes such as enzymes, proton pumps, transport proteins and others. The definition of the reservosome protein profile is a good tool to assess their molecular signature, identify molecular markers, and understand their relationship with different organelles.

Keywords

mass spectrometry; proteomic analysis; reservosomes; *Trypanosoma cruzi*

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, has a complex life cycle, which includes three different developmental stages [1]. Like other eukaryotic cells, *T. cruzi* needs to ingest macromolecules by endocytosis, which constitutes one of the main challenges during its life cycle. In *T. cruzi*, significant nutrient uptake takes place only in epimastigotes and is low or absent in both trypomastigotes and amastigotes [2]. Epimastigotes can ingest exogenous macromolecules by the flagellar pocket and mainly by the

*Corresponding authors: Narcisa L. Cunha-e-Silva: Av. Carlos Chagas Filho 373, bloco G subsolo, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brasil, 21941-902. Tel: 55-21-2562-6593 Fax: 55-21-2260-2364; narcisa@biof.ufrj.br and Igor C. Almeida: 500 W. University Ave, Biosciences building, El Paso, TX 79968, USA; Tel: (915)747-6086, Fax: (915)747-5808; icalmeida@utep.edu.
#These authors contributed equally to the work.

The authors declare that this work does not represent any conflicts of interest of commercial or financial nature.

cytostome [3,4]. From the entry sites, endocytic vesicles bud off and fuse with branched tubular-vesicular early endosomes. Subsequently, the macromolecules are delivered to *T. cruzi* storage organelles, the reservosomes [4].

Reservosomes are the main site for the storage of ingested proteins and lipids, as well as of secretory proteins synthesized by the protozoan [2,5]. Based on characteristics found in mammalian lysosome-related organelles (LROs) [6], we recently proposed that *T. cruzi* reservosomes are members of the LRO group [7]. They have been considered as pre-lysosomal organelles due to the absence of *bona fide* lysosomal molecular markers and to pH evaluation at 6.0 [8]. Reservosomes are round organelles (average diameter of 400 – 600 nm) surrounded by a membrane and mainly localized at the posterior region of epimastigotes. The core of the organelles is composed of an electrondense protein matrix and electronlucent lipid inclusions [2]. Recently, we have demonstrated the presence of inner membranes and described unusual rod-shaped bodies, which are presumably lipids [9]. We have also determined the presence and distribution of transmembrane proteins in the organelle membranes by freeze-fracture. Furthermore, we have described organelles that share typical reservosome properties in trypomastigotes and amastigotes, also characterized as LROs [7]. This finding has inferred that the reservosomes may be a potential chemotherapy target against Chagas' disease.

Few proteins have been identified and characterized in reservosomes. Among them, there are two lysosomal proteases, cruzipain [8,10,11] and serine carboxipeptidase [7,12]. Due to the concentration of these proteases, reservosomes have been hypothesized to be the main site of protein degradation. Chagasin, a tight-binding natural inhibitor of cruzipain, was also localized in the reservosomes, suggesting modulation of proteolytic activity inside the organelle [13]. In addition, two P-type H^+-ATPase isoforms (TcHA1 and TcHA2), usually found in the plasma membrane of plant and yeast cells [14], were shown to be responsible for generating the acidic character of reservosomes [15]. Unexpectedly, TcRab11, a homologue of mammalian rab11, generally found in recycling endosomes [16] was also suggested to be localized in the reservosomes [17].

Even though some reservosome proteins have been identified, a molecular marker for this organelle has not yet been characterized. Seeking a better understanding of the function of this organelle and the determination of possible molecular markers, we performed a comprehensive subcellular proteomic analysis of the purified epimastigote reservosome fraction by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Parasites

T. cruzi epimastigotes from the Dm28c clone were cultivated for 4 days at 28°C in liver infusion tryptose (LIT) medium [18] supplemented with 10% fetal calf serum.

Reservosome fractionation

The reservosome fraction was obtained according to [11]. Briefly, epimastigotes in TMS buffer (20 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 250 mM sucrose) were disrupted by sonication on ice, in an ultrasonic apparatus (Sigma, GEX 600 Model) using a standard probe. After centrifugation at 2,450g for 10 min, the supernatant was mixed with an equal volume of 2.3 M sucrose in TMS buffer, deposited into a Beckman SW28 centrifuge tube, overlaid with 10 mL of 1.2 M, 10 mL of 1.0 M and 5 mL of 0.8 M sucrose (in TMS buffer) and centrifuged at 97,000 g for 150 min. The interface 0.8 M/1.0 M was collected, diluted in TMS buffer and centrifuged at 120,000g for 30 min. The pellet, named B1 reservosome fraction, was

resuspended in TMS, characterized by transmission electron microscopy (TEM) and submitted to LC-MS/MS.

Isolation of reservosome membranes

Isolated reservosome fraction (B1) was disrupted by 5 cycles of freezing in liquid nitrogen and thawing in a water bath at 37°C. Subsequently, the sample was extracted with 200 mM sodium carbonate, pH 11.5, at 4°C for 30 minutes, under mild agitation. The membrane fraction (B1M) was obtained by centrifugation at 120,000g for 2 h at 4°C, characterized by TEM and submitted to LC-MS/MS.

Transmission electron microscopy

Isolated fractions were processed according to [11] and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 1200EX electron microscope.

Protein digestion and peptide fractionation

Total proteins from B1 and B1M fractions were digested with trypsin (TU strategy) or with trypsin and endoproteinase Glu-C (TG strategy). For the TU strategy, the proteins were digested as described [19]. For the TG strategy, samples were digested with the same procedure as for the TU strategy but, after the trypsin digestion, two micrograms sequencing-grade endoproteinase Glu-C were added, and the reaction was allowed to proceed for 24 h at 37°C. The reaction was terminated by adding 0.05% trifluoroacetic acid (TFA), and the digested proteins were desalted using reverse-phase ZipTip columns (POROS R2 50, Applied Biosystems, Foster City, CA) as described [20]. The resulting peptides were fractionated in a strong cation-exchange (SCX) SCX ZipTip (POROS HS 50, Applied Biosystems) by eluting with increasing concentrations of NaCl (25, 50, 100, 200, and 500 mM) [21]. The peptide fractions were dried to remove the acetonitrile (ACN) and desalted in reverse-phase ZipTips.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The LC-MS/MS analysis was performed in a electrospray ionization-linear ion-trap mass spectrometer (ESI-LIT-MS) equipped with a nanospray source (LTQXL, Thermo Fisher Scientific, San Jose, CA). Each SCX fraction was resuspended in 20 μl 0.05% TFA, and 8-μl aliquot from each sample was loaded onto a C18 trap column (0.25 μL C18, OPTI-PAK. Oregon City, OR). Tryptic peptide separation was performed on a capillary reverse-phase column (Acclaim, 3-μm C18, 75 μm × 25 cm, LC Packings/Waters, Amsterdam, The Netherlands) connected to a nanoHPLC system (nanoLC 1D plus, Eksigent, Dublin, CA). The peptides were eluted using a linear gradient from 0 to 40% ACN in 0.1% FA for 200 min and directly analyzed in the ESI-LIT-MS. MS spectra were collected in centroid mode in the 400 to 1700 *m/z* range, and the five most abundant ions of each spectrum were subjected twice to collision-induced dissociation (CID) using 35% normalized collision energy, before dynamic exclusion for 120 sec.

Database search and peptide identification/validation

MS/MS spectra from peptides with 600–3500 Da and at least 100 counts and 15 fragments were converted into DTA files using Bioworks v.3.3.1 (Thermo Scientific). The DTA files were submitted to a database search using TurboSequest [22] (available in Bioworks) against a database composed of *T. cruzi,* bovine, human keratin and porcine trypsin sequences (downloaded March 17th, 2008 from GenBank), in the forward and reverse orientations, forming a dataset of 191,762 sequences. Database search parameters included: i) trypsin or endoproteinase Glu-C cleavage in both peptide termini with one allowed missed cleavage site; ii) carbamidomethylation of cysteine residues as a fixed modification; iii) oxidation of

methionine residues as a variable modification; and iv) 2.0 Da and 1.0 Da for peptide and fragment mass tolerances, respectively. The following filters were applied in Bioworks: DCn ≥ 0.085; protein probability ≤1E-3; consensus score; and Xcorr ≥1.5, 2.2, and 2.7 for singly-, doubly-, and triply-charged peptides, respectively. The false-positive rate (FPR) was calculated as previously described [21].

Bioinformatic analysis of identified protein sequences

All valid *T. cruzi*-specific protein sequences were compared to sequences deposited in the GenBank using the Blast tool (http://www.ncbi.nlm.nih.gov/BLAST/). Gene ontology (GO) annotation was assigned by similarity searches against the Swiss-Prot and TrEMBL databases (invertebrate taxonomy, which includes all eukaryotic entries, except those from vertebrates, fungi, and plants) using GOblet tool [23], which is available online at http://goblet.molgen.mpg.de. Only GOs from proteins with evalues ≤1e-10 for database search were accepted. This analysis was performed on October 3rd, 2008. The sequences were also analyzed to determine potential transmembrane domains using the TMHMM server v2.0 software (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

RESULTS

Ultrastructure of isolated reservosomes and their isolated membranes

In order to identify reservosome-resident proteins, we initially purified reservosomes to obtain the intact organelles (B1). Subsequently, we established a new protocol to isolate reservosome membranes. Isolated organelles were disrupted by freezing and thawing, the associated proteins were removed with sodium carbonate buffer, and the membrane (B1M) fraction was recovered by centrifugation. To assess the purity and preservation of isolated fractions, B1 and B1M fractions were analyzed by TEM (Fig. 1b,c). The B1 fraction displayed intact reservosomes with a characteristic morphology observed for *in situ* reservosomes (Fig. 1a). Some disrupted organelles were also visualized, as previously reported by Cunha-e-Silva and co-workers [11], probably due to the fractionation procedures. While examining several randomly collected ultrathin sections, the presence of other structures such as mitochondria, kinetoplast, endoplasmic reticulum cisternae, glycosomes, acidocalcisomes, nuclei or flagella were not detected, thus indicating that the preparation was virtually free of these contaminating organelles (data not shown). The B1M fraction showed highly purified total reservosome membranes (Fig. 1c) because no intact organelle was detected. The B1 and B1M fractions were then separately subjected to LC-MS/MS analysis.

Identification of reservosome proteins by LC-MS/MS

B1 and B1M fractions were digested with two distinct strategies: i) trypsin, with urea as a denaturing agent (TU), and ii) double digestion with trypsin and endoproteinase Glu-C (TG). After digestion, resulting peptides were fractionated by SCX chromatography, analyzed LC-MS/MS, and submitted to search through a database containing forward and reverse sequences of *T. cruzi*, bovine, keratin and porcine trypsin sequences. The FPR was determined as described in the Materials and Methods. Our data were validated with a FRP = 2.41%. Figure 2 shows a schematic summary of the approach used in our analyses.

In total, LC-MS/MS data allowed the unambiguous identification of 869 proteins, with 709 being *T. cruzi*-specific proteins and 160 as proteins from the culture medium and contaminants from the sample preparation (see complete list in Supplementary Table 1). In the case of *T. cruzi*, 456 proteins were assigned as having a predicted function, and 253 were assigned as having an unknown function or being hypothetical proteins. Of these proteins, cruzipain [8, 10, 11], serine carboxipeptidase [7, 12], ABC transporters [24], and a protein tyrosine phosphatase [25] have already been previously described to be present in reservosomes. Our

proteomic analysis of reservosomes allowed the identification of a novel isoform of P-type H$^+$-ATPase, TcHA3. We also confirmed the presence of TcHA1 and TcHA2, the other proton pump described in reservosomes [15]. We could identify several additional hydrolases, such as cysteine peptidases, α-mannosidases, acid phosphatase, acid phosphatase 2, calpain cysteine peptidases, lipase, and serine carboxypeptidase S28. In addition, a calcium translocation pump and calcium-binding proteins were found. A chloride channel protein, also identified in mammalian endosomes [26], was detected. Members of the ABC family, which act in lipid metabolism [27], also appeared, as well as a P-glycoprotein. Surprisingly, a multidrug resistance (MDR) protein was identified. An interesting finding was the presence of p67, a glycoprotein molecular marker of *T. brucei* lysosome [28]. In addition, we detected a glycoprotein, which thus far has an undefined function, which is also present in the Golgi complex and lysosomes.

In our analysis, the reservosomes exhibited endosomal integral membrane proteins and proteins involved in vesicular traffic, such as COP-coated vesicle membrane proteins erv25 and gp25L, mu-adaptin 1, huntingtin-interacting protein (HIP), vesicle-associated membrane proteins (VAMPs), and the GTP-binding proteins Rab1, Rab2a, Rab7, and Rab18.

Also, our reservosome proteomic data showed proteins related to lipid metabolism, including sterol 24-c-methyltransferase, fatty acyl CoA synthetase, phospholipid-translocating ATPase, C-8 sterol isomerase, fatty acyl CoA syntetase 1, and phosphatidylcholine:ceramide cholinephosphotransferase, which suggests that these organelles play a role in lipid synthesis in addition to storing lipids ingested by the endocytic pathway [11]. We also found enzymes that act as regulators of signal transduction pathways in most eukaryotic cell types, e. g., protein tyrosine phosphatase, regulatory subunit of protein kinase A, casein kinase, MCAK-like kinesin, activated protein kinase C receptor, protein kinase, and serine/threonine protein kinase.

Transmembrane proteins from the plasma membrane, such as dispersed gene family protein 1 (DGF-1), ferric reductase, hexose transporter, folate/pteridine transporter and GPI-anchored p63, were identified in the reservosome proteome, confirming a membrane-trafficking relationship between the plasma membrane and reservosomes. We also identified cytoskeleton components essential for vesicular traffic, among other functions, such as alpha and beta tubulin, cytoskeleton-associated protein CAP5.5, actin, and cofilin/actin depolymerizing factor.

Other proteins that were detected can be characterized as non-reservosomal, including mitochondrial, glycosomal or flagellar proteins. This suggests a functional relationship between the organelles, a recycling process of these proteins in the reservosomes, or a possible contamination, commonly observed in subcellular fractionation protocols. However, only very few of these presumed contaminants are transmembrane proteins, thus suggesting a low level of contamination with other organelle membranes. Predictably, we could also identify bovine serum proteins, which are supposed to be derived from the culture medium and likely reached the reservosomes by an endocytic process.

Gene ontology analysis

In order to understand their function and to identify processes related to reservosomes, we performed gene ontology (GO) annotation of all proteins identified in the proteome analysis (Fig. 3 and Supplemental material 1). Catalytic activity was prevalent in the reservosome molecular function. Reservosomes are also rich in proteins involved in binding and hydrolase activity. Proteins found in the reservosome proteome are involved in transporter, carrier and ion-transporter activities, each representing about 12% of the proteins identified. Our analysis found significant relationships to nucleotide-binding, transferase activity, ion-binding, and oxidoreductase activity. A large number of reservosomal proteins were related to metabolism

as well as to physiological and cellular processes. Membrane localization of the proteins identified by LC-MS/MS analysis was remarkable. Reservosome proteins were also predicted to be localized in the proton-transporting complex.

DISCUSSION

Subcellular proteomics have been extensively used to identify the molecular composition of several cytoplasmic organelles and has been considered an effective approach to understand cellular processes and integrated cell function. It takes advantage of the subcellular fractionation strategies, which are based on sequential and/or density-gradient centrifugation, allowing the separation of different population of organelles based on their size, density and charge, in combination with mass spectrometry analysis [29]. This strategy greatly reduces sample complexity in comparison with whole cell proteomic analysis.

Sub-cellular fractionation methodologies have been widely employed in protozoan parasites [30] in order to carry out parasite-specific organelles characterization and understand their role in parasite cell biology. These organelles have particular interest since their absence in higher eukaryotes may comprise a potential chemotherapeutic target against human and animal parasitism. By centrifugation in a sucrose gradient, our group reported an accurate and reproducible protocol to obtain a highly purified *T. cruzi* epimastigote reservosome fraction [11]. Afterwards, the same protocol was successfully used to measure proton transport in isolated reservosomes [15]. The purity of the fraction was assessed using three strategies: (i) enzymatic assays by means of enzymes recognized as markers of cell organelles in mammal cells and protozoa such as acid phosphatase (lysosomes), hexokinase (glycosomes), vacuolar H^+-pyrophosphatase (acidocalcisomes) and succinate-cytochrome *c* reductase (mitochondria); (ii) Western blot, using antibodies against cruzipain (reservosomes) and lipopeptidephosphoglycan (plasma membrane) and (iii) ultrastructure analysis by transmission electron microcopy, a powerful mean for judging the efficiency of the method. In conclusion, we could essentially assure the high degree of purity and reproducibility of the reservosome fraction obtained.

Taking advantage of efficiency of the cell fractionation of reservosome [11], we isolated for the first time a highly purified reservosome fraction from Dm28c epimastigotes (Fig. 1b). In addition, to enrich the membrane protein identification and avoid the contamination of the proteomic analysis with a great number of medium-derived peptides, we developed a methodology to purify total reservosome membranes (Fig. 1c). Our morphological analysis did not allow discrimination between reservosome-surrounding membranes and membranes derived from inside the organelle. Reservosomes had been described as an organelle where few or no inner membranes were seen [2]. However, we have recently demonstrated, using different electron microscopy approaches, the presence of vesicles and planar membranes in the lumen of reservosomes [9]. Nevertheless, the composition and function of these structures need to be clarified and are objects of our studies. Intending to achieve better proteome profiling of reservosomes, we used both B1 and B1M fractions in our proteomic analysis.

Proteomic analysis of B1 and B1M fractions led to the identification of 709 *T.* cruzi-specific proteins. Several identified proteins are expected components for endocytic organelles, which interact and fuse with vesicles coming from the secretory pathway and/or the plasma membrane as part of the endocytic process.

We also found cytoskeleton proteins, as did other proteomic analyses of *T. cruzi* subcellular fractions [31]. Since the pool of unpolymerized tubulin is very small in epimastigote forms [32], this finding may represent an association of reservosomes with microtubules, which are responsible for traffic and intracellular organelle positioning.

Rab 1, Rab 2a, Rab 7, and Rab 18, small GTPases of the Rab family, which are crucial regulators of vesicle traffic in endocytic and secretory pathways [33], were another important class of proteins found in our proteomic analysis. Rab 1 and Rab 2 have been implicated in the transport from the ER to the Golgi complex [34]. Orthologues of mammalian Rab 1 and Rab 2 were characterized in *T. brucei* [35], and it was shown that TbRab1 and TbRab2 play a role in the early events of the secretory pathway, suggesting a conserved function. Rab 7 has been regarded as a mediator of early to late endosome transport [36], and evidence has accumulated suggesting that Rab 7 may work in the transport from late endosomes to lysosomes [37]. In *Leishmania* [38] and *T. brucei* [39], the orthologue of mammalian Rab 7 was localized in the endosomal/ lysosomal system. Surprisingly, *T. cruzi* Rab 7 was found in high concentration in the Golgi complex by ultrastructural localization [40]. Our analysis detected TcRab 7 in the reservosome proteome, which suggests a possible role of this small GTPase in the membrane traffic between Golgi complex/reservosomes. Unexpectedly, TcRab 11, previously suggested to be localized in the reservosomes [17], was not detected in our proteomic analysis. The suggestion of TcRab11 localization in reservosomes was based exclusively on immunofluorescence images. No further confirmation by immunoelectronmicroscopy or western blot using isolated reservosome fraction was published since then. We cannot exclude that the absence of this protein in reservosome proteomic profile be a consequence of sample preparation, but this possibility does not seem to be probable, as sample processing did not affect other Rab proteins.

While mammalian Rab 18 has been involved in endocytic transport and in association with lipid droplets [41], *T. brucei* Rab 18 seems to function in Golgi transport [42]. The presence of Rab18 in the reservosome proteomic profile may point to its role in the mobilization of the abundant lipid storage [9,11], similar to Rab18 function in adipocytes [41]. Undoubtedly, the presence of several Rab proteins associated with reservosomes argues for the dynamic nature of this organelle and is compatible with the nature of a LRO [7] with a secretory character, sending molecules to other cytoplasmic compartments or the extracellular medium, while simultaneously receiving molecules from their cell "progenitors" [43], the Golgi complex, and endocytic pathway [44]. Furthermore, proteins that belong to the fusion machinery, such as syntaxyn, NSF, SNAP and others whose genes were found in the *T. cruzi* genome, were not identified during our analysis. This virtual absence may be justified by the low level of protein expression, differences in the sequences from different strains (the *T. cruzi* genome project was carried out with the CL Brener strain, while we used the Dm28c clone), or protein modifications that cannot or are difficult to be identified by LC-MS/MS. We also cannot exclude the possibility of losing this sort of protein during the subcellular fractionation procedure.

We also found isoforms of ABC transporters that, in mammalian cells, work in lipid metabolism, and P-glycoprotein (Table 1). Recently, our group reported the intracellular trafficking of heme [45]. Using a specific inhibitor, cyclosporin A, it was hypothesized that a P-glycoprotein transporter is likely to be the responsible for heme transport through the plasma membrane. In addition, heme is stored in reservosomes, probably by the action of a second transporter. The identification of P-glycoprotein in the proteomic profile of reservosomes reinforces this hypothesis. Moreover, reservosomes store high levels of neutral lipids, such as cholesteryl esters [11], as a result of the endocytic process. The high concentration of lipids in the reservosome lumen seems to be involved in the formation of rectangular lipid bodies [9]. Like in mammals, ABCA1 could serve to control the rate of exogenous cholesterol internalized into early and late endosomes, back to plasma membrane or, yet, out of the cell [27].

LC-MS/MS analysis confirmed the reservosomal localization of cruzipain and its inhibitor chagasin and serine carboxypeptidase, shown by previous works to be present [7,10,13] and functional [11,12] in reservosomes. Novel hydrolases were found, such as lysosomal α-mannosidase, cysteine proteases, calpain cysteine peptidase, cysteine protease C, and

membrane-bound acid phosphatase. Interestingly, in a previous paper, acid phosphatase was not detected in reservosomes by ultrastructural cytochemistry. Based on the absence of this enzyme and other lysosomal structural proteins, reservosomes were classified as non-lysosomal endocytic organelles [8]. Recently, a typical lysosome enzyme, aryl sulphatase, was shown to be functional in epimastigote small vesicles but not in reservosomes [46], reinforcing the reservosome non-lysosomal character. Nevertheless, our proteomic results confirmed that reservosomes are the main site of lysosomal hydrolases and are hence working as an important endogenous protein regulator.

On the other hand, reservosomes concentrate lysosomal hydrolases, and they have been considered the main site of protein degradation and regulation. Therefore, it is reasonable that the reservosome constitutes an important site of protein recycling, justifying the presence of such proteins in the proteomic analysis. An autophagic process was recently reported in *T. cruzi* [47,48], showing that autophagy is essential for parasite survival during starvation and differentiation. This work suggested that part of the cytoplasm and organelles are degraded in reservosomes.

We could detect some proteins that are not related to endocytic compartments. These proteins are constituents of the cytoplasm, mitochondrion, glycosome, acidocalcisome, nucleus, endoplasmic reticulum or flagellum. The presence of contaminants derived from other organelles is the main challenge faced during the subcellular fractionation, and it is a possible explanation for the presence of several proteins from different origin in the reservosome proteomics. In order to demonstrate the low level of cross-contamination with membranes from different organelles we predicted transmembrane domains in non-reservosomal proteins. In this way, we managed to show that our fractionation protocol led to very low degree of contamination with non-reservosomal membranes.

Subcellular proteomics have contributed to determine protein localization and function [49]. However, finding proteins in multiple locations helps to determine the relationship between different organelles or structures, such as organelles from the endocytic pathway. Recently, it was estimated that up to 39% of organelle proteins can be detected in multiple intracellular compartments [50]. The authors suggested some hypotheses to explain these findings: i) proteins are indeed found in different compartments; ii) contaminants are created during the fractionation process; and iii) proteins are mistakenly identified. It is important to point out that reservosomes result from the fusion of vesicles originating in the plasma membrane during the endocytic pathway with vesicles from the endoplasmic reticulum-Golgi complex via the secretory pathway [44]. Therefore, the protein profile of the reservosomes may reflect the dynamic traffic between these structures.

Among the transmembrane proteins identified in the reservosomal proteome, we can suggest P-type H^+-ATPase (TcHA3) and p67 as possible molecular markers. In *T. cruzi*, only two isoforms of P-type H^+-ATPases, TcHA1 and TcHA2, were characterized and had their localization determined [15]. TcHA1 was found in the plasma membrane and also in the endocytic pathway, while TcHA2 plays a role exclusively in the acidification of reservosomes. Besides TcHA1 and TcHA2, our proteomic data using Dm28c clone detected the presence of TcHA3 in the reservosome membranes with a high coverage, what may imply a high abundance. Due to the absence of these proteins in mammalian cells and their presence in fungi and trypanosomatids, these pumps may be considered as potential chemotherapeutic targets.

p67 is a type I transmembrane glycoprotein that has been described as a *T. brucei* lysosome molecular marker [28]. It presents a general structure similar to LAMPs (lysosome-associated membrane proteins) in mammalian cells, although without sequence homology. In African trypanosomes, p67 has been related to the formation of lysosomal glycocalyx, protecting

against hydrolases. The p67 gene was found in *T. cruzi*, and its reservosomal localization was demonstrated in our proteomic analysis. Because orthologues of p67 are not found in mammals, it might be a candidate for chemotherapy as well.

In protozoan parasites, subcellular proteomic analyses were successfully applied to the characterization of *Entamoeba histolytica* [51] and *Dictyostelium discoideum* [52] phagosome. In trypanosomatids, we can highlight the proteome of flagella [53], glycosomes [54], and the comparative proteomic analysis of glycosomes and mitochondria [55] of *T. brucei*. Recently, Ferella and co-workers [31] prepared a subcellular fraction from *T. cruzi* CL Brener epimastigotes that was enriched in acidocalcisomes and glycosomes but also contained endoplasmic reticulum and Golgi components. Using 1D and 2D electrophoresis followed by MS analysis, they identified proteins corresponding to 396 genes, 258 of which were annotated as previously described and 138 were hypothetical proteins. Among the identified proteins, only three are presumably reservosomal: glutathione S-transferase [56], cysteine peptidase [10], and serine carboxypeptidase [7,12]. These studies yielded valuable information about protein localization and reported the identification of novel proteins in these organelles. In the current paper, we performed the proteomic analysis of *T. cruzi* epimastigote reservosomes, which is the first protein profiling of an endocytic organelle in a trypanosomatid family member. This analysis offers a new perspective in the identification of reservosomal molecular markers and contributes to the understanding of the biogenesis and dynamic interactions of these organelles with other *T. cruzi* structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. De Souza W. From the cell biology to the development of new chemotherapeutic approaches against trypanosomatids: dreams and reality. Kinetoplastid Biol Dis 2002;1:3. [PubMed: 12234386]

2. Soares MJ, De Souza W. Cytoplasmic organelles of trypanosomatids: a cytochemical and stereological study. J Submicrosc Cytol Pathol 1988;20:349–361. [PubMed: 3135113]

3. Soares MJ, de Souza W. Endocytosis of gold-labeled proteins and LDL by *Trypanosoma cruzi*. Parasitol Res 1991;77:461–468. [PubMed: 1656428]

4. Porto-Carreiro I, Attias M, Miranda K, De Souza W, Cunha-e-Silva N. *Trypanosoma cruzi* epimastigote endocytic pathway: cargo enters the cytostome and passes through an early endosomal network before storage in reservosomes. Eur J Cell Biol 2000;79:858–869. [PubMed: 11139150]

5. Cunha-e-Silva N, Sant'Anna C, Pereira MG, Porto-Carreiro I, et al. Reservosomes: multipurpose organelles? Parasitol Res 2006;99:325–327. [PubMed: 16794853]

6. Raposo G, Marks MS, Cutler DF. Lysosome-related organelles: driving post-Golgi compartments into specialisation. Curr Opin Cell Biol 2007;19:394–401. [PubMed: 17628466]

7. Sant'Anna C, Parussini F, Lourenço D, de Souza W, et al. All *Trypanosoma cruzi* developmental forms present lysosome-related organelles. Histochem Cell Biol. 200810.1007/s00418-008-0486-8

8. Soares MJ, Souto-Padron T, De Souza W. Identification of a large pre-lysosomal compartment in the pathogenic protozoon *Trypanosoma cruzi*. J Cell Sci 1992;102:157–167. [PubMed: 1500438]

9. Sant'Anna C, Pereira MG, Lemgruber L, de Souza W, Cunha e Silva NL. New insights into the morphology of *Trypanosoma cruzi* reservosome. Microsc Res Tech 2008;71:599–605. [PubMed: 18452191]

10. Souto-Padron T, Campetella OE, Cazzulo JJ, de Souza W. Cysteine proteinase in *Trypanosoma cruzi*: immunocytochemical localization and involvement in parasite-host cell interaction. J Cell Sci 1990;96:485–490. [PubMed: 2229199]

11. Cunha-e-Silva NL, Atella GC, Porto-Carreiro IA, Morgado-Diaz JA, et al. Isolation and characterization of a reservosome fraction from *Trypanosoma cruzi*. FEMS Microbiol Lett 2002;214:7–12. [PubMed: 12204365]

12. Parussini F, Garcia M, Mucci J, Aguero F, et al. Characterization of a lysosomal serine carboxypeptidase from *Trypanosoma cruzi*. Mol Biochem Parasitol 2003;131:11–23. [PubMed: 12967708]

13. Santos CC, Sant'Anna C, Terres A, Cunha-e-Silva NL, et al. Chagasin, the endogenous cysteine-protease inhibitor of *Trypanosoma cruzi*, modulates parasite differentiation and invasion of mammalian cells. J Cell Sci 2005;118:901–915. [PubMed: 15713748]

14. Serrano R, Villalba JM, Palmgren MG, Portillo F, et al. Studies of the plasma membrane H(+)-ATPase of yeast and plants. Biochem Soc Trans 1992;20:562–566. [PubMed: 1426592]

15. Vieira M, Rohloff P, Luo S, Cunha-e-Silva NL, et al. Role for a P-type H^+-ATPase in the acidification of the endocytic pathway of *Trypanosoma cruzi*. Biochem J 2005;392:467–474. [PubMed: 16149915]

16. Ullrich O, Reinsch S, Urbe S, Zerial M, Parton RG. Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol 1996;135:913–924. [PubMed: 8922376]

17. Mendonca SM, Nepomuceno da Silva JL, Cunha e-Silva N, de Souza W, Gazos Lopes U. Characterization of a Rab11 homologue in *Trypanosoma cruzi*. Gene 2000;243:179–185. [PubMed: 10675626]

18. Camargo EP. Growth and Differentiation in *Trypanosoma cruzi*. I. Origin of Metacyclic Trypanosomes in Liquid Media. Rev Inst Med Trop Sao Paulo 1964;12:93–100. [PubMed: 14177814]

19. Stone, KL.; Williams, KR. Enzymatic digestion of proteins in solution and in SDS polyacrylamide gel. In: Walker, JM., editor. The protein protocol handbook. Humana Press; New Jersey: 1996. p. 415-425.

20. Jurado JD, Rael ED, Lieb CS, Nakayasu E, et al. Complement inactivating proteins and intraspecies venom variation in *Crotalus oreganus helleri*. Toxicon 2007;49:339–350. [PubMed: 17134729]

21. Rodrigues ML, Nakayasu ES, Oliveira DL, Nimrichter L, et al. Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. Eukaryot Cell 2008;7:58–67. [PubMed: 18039940]

22. Eng JK, McCormack AL, Yates JR III. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom 1994;5:976–989.

23. Groth D, Lehrach H, Hennig S. GOblet: a platform for Gene Ontology annotation of anonymous sequence data. Nucleic Acids Res 2004;32:W313–317. [PubMed: 15215401]

24. Torres C, Perez-Victoria FJ, Parodi-Talice A, Castanys S, Gamarro F. Characterization of an ABCA-like transporter involved in vesicular trafficking in the protozoan parasite *Trypanosoma cruzi*. Mol Microbiol 2004;54:632–646. [PubMed: 15491356]

25. Cuevas IC, Rohloff P, Sanchez DO, Docampo R. Characterization of farnesylated protein tyrosine phosphatase TcPRL-1 from *Trypanosoma cruzi*. Eukaryot Cell 2005;4:1550–1561. [PubMed: 16151248]

26. Jentsch TJ. CLC chloride channels and transporters: from genes to protein structure, pathology and physiology. Crit Rev Biochem Mol Biol 2008;43:3–36. [PubMed: 18307107]

27. Attie AD. ABCA1: at the nexus of cholesterol, HDL and atherosclerosis. Trends Biochem Sci 2007;32:172–179. [PubMed: 17324574]

28. Alexander DL, Schwartz KJ, Balber AE, Bangs JD. Developmentally regulated trafficking of the lysosomal membrane protein p67 in *Trypanosoma brucei*. J Cell Sci 2002;115:3253–3263. [PubMed: 12140257]

29. Dreger M. Subcellular proteomics. Mass Spectrom Rev 2003;22:27–56. [PubMed: 12768603]

30. De Souza W, Cunha-e-Silva N. Cell fractionation of parasitic protozoa – a review. Mem Inst Oswaldo Cruz 2003;98:151–170.

31. Ferella M, Nilsson D, Darban H, Rodrigues C, et al. Proteomics in *Trypanosoma cruzi* -localization of novel proteins to various organelles. Proteomics 2008;8:2735–2749. [PubMed: 18546153]

32. da Silva RA, Bartholomeu DC, Teixeira SM. Control mechanisms of tubulin gene expression in *Trypanosoma cruzi*. Int J Parasitol 2006;36:87–96. [PubMed: 16233898]

33. Zerial M, McBride H. Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2001;2:107–117. [PubMed: 11252952]

34. Tisdale EJ, Bourne JR, Khosravi-Far R, Der CJ, Balch WE. GTP-binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. J Cell Biol 1992;119:749–761. [PubMed: 1429835]

35. Dhir V, Goulding D, Field MC. TbRAB1 and TbRAB2 mediate trafficking through the early secretory pathway of *Trypanosoma brucei*. Mol Biochem Parasitol 2004;137:253–265. [PubMed: 15383296]

36. Feng Y, Press B, Wandinger-Ness A. Rab 7: an important regulator of late endocytic membrane traffic. J Cell Biol 1995;131:1435–1452. [PubMed: 8522602]

37. Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B. Rab7: a key to lysosome biogenesis. Mol Biol Cell 2000;11:467–480. [PubMed: 10679007]

38. Denny PW, Lewis S, Tempero JE, Goulding D, et al. Leishmania RAB7: characterisation of terminal endocytic stages in an intracellular parasite. Mol Biochem Parasitol 2002;123:105–113. [PubMed: 12270626]

39. Morgan GW, Hall BS, Denny PW, Field MC, Carrington M. The endocytic apparatus of the kinetoplastida. Part II: machinery and components of the system. Trends Parasitol 2002;18:540–546. [PubMed: 12482539]

40. Araripe JR, Cunha e Silva NL, Leal ST, de Souza W, Rondinelli E. *Trypanosoma cruzi*: TcRAB7 protein is localized at the Golgi apparatus in epimastigotes. Biochem Biophys Res Commun 2004;321:397–402. [PubMed: 15358190]

41. Martin S, Parton RG. Characterization of Rab18, a lipid droplet-associated small GTPase. Methods Enzymol 2008;438:109–129. [PubMed: 18413244]

42. Jeffries TR, Morgan GW, Field MC. TbRAB18, a developmentally regulated Golgi GTPase from *Trypanosoma brucei*. Mol Biochem Parasitol 2002;121:63–74. [PubMed: 11985863]

43. Hu ZZ, Valencia JC, Huang H, Chi A, et al. Comparative Bioinformatics Analyses and Profiling of Lysosome-Related Organelle Proteomes. Int J Mass Spectrom 2007;259:147–160. [PubMed: 17375895]

44. Sant'Anna C, de Souza W, Cunha-e-Silva N. Biogenesis of the reservosomes of *Trypanosoma cruzi*. Microsc Microanal 2004;10:637–646. [PubMed: 15525436]

45. Lara FA, Sant'anna C, Lemos D, Laranja GA, et al. Heme requirement and intracellular trafficking in *Trypanosoma cruzi* epimastigotes. Biochem Biophys Res Commun 2007;355:16–22. [PubMed: 17292866]

46. Adade CM, de Castro SL, Soares MJ. Ultrastructural localization of *Trypanosoma cruzi* lysosomes by aryl sulphatase cytochemistry. Micron 2007;38:252–256. [PubMed: 16860560]

47. Alvarez VE, Kosec G, Sant Anna C, Turk V, et al. Blocking autophagy to prevent parasite differentiation: a possible new strategy for fighting parasitic infections? Autophagy 2008;4:361–363. [PubMed: 18212533]

48. Alvarez VE, Kosec G, Sant'Anna C, Turk V, et al. Autophagy is involved in nutritional stress response and differentiation in *Trypanosoma cruzi*. J Biol Chem 2008;283:3454–3464. [PubMed: 18039653]

49. Andersen JS, Mann M. Organellar proteomics: turning inventories into insights. EMBO Rep 2006;7:874–879. [PubMed: 16953200]

50. Foster LJ, de Hoog CL, Zhang Y, Zhang Y, et al. A mammalian organelle map by protein correlation profiling. Cell 2006;125:187–199. [PubMed: 16615899]

51. Okada M, Huston CD, Mann BJ, Petri WA Jr, et al. Proteomic analysis of phagocytosis in the enteric protozoan parasite *Entamoeba histolytica*. Eukaryot Cell 2005;4:827–831. [PubMed: 15821141]

52. Gotthardt D, Warnatz HJ, Henschel O, Brückert F, et al. High-resolution dissection of phagosome maturation reveals distinct membrane trafficking phases. Mol Biol Cell 2002;13:3508–3520. [PubMed: 12388753]

53. Broadhead R, Dawe HR, Farr H, Griffiths S, et al. Flagellar motility is required for the viability of the bloodstream trypanosome. Nature 2006;440:224–227. [PubMed: 16525475]

54. Colasante C, Ellis M, Ruppert T, Voncken F. Comparative proteomics of glycosomes from bloodstream form and procyclic culture form *Trypanosoma brucei brucei*. Proteomics 2006;6:3275–3293. [PubMed: 16622829]

55. Vertommen D, Van Roy J, Szikora JP, Rider MH, et al. Differential expression of glycosomal and mitochondrial proteins in the two major life-cycle stages of *Trypanosoma brucei*. Mol Biochem Parasitol 2008;158:189–201. [PubMed: 18242729]

56. Ouaissi MA, Dubremetz JF, Schöneck R, Fernandez-Gomez R, et al. *Trypanosoma cruzi*: a 52-kDa protein sharing sequence homology with glutathione S-transferase is localized in parasite organelles morphologically resembling reservosomes. Exp Parasitol 1995;81:453–461. [PubMed: 8542986]
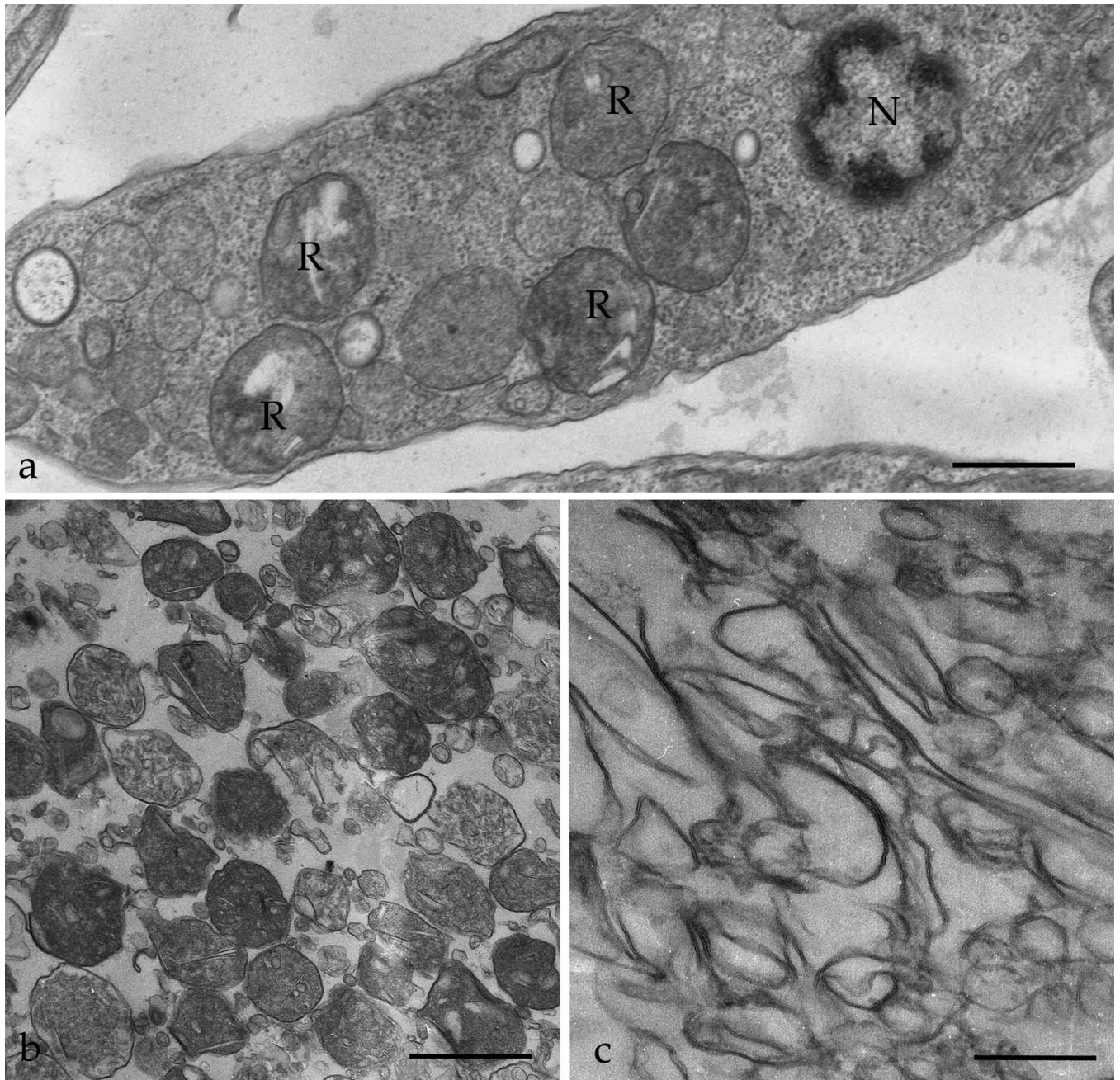
Figure 1.
Transmission electron microscopy of *T. cruzi* reservosomes. (**a**) Ultrathin section of an epimastigote showing reservosomes (R) in situ, with their typical morphology and position, between nucleus (N) and posterior end of the cell. (**b**) Purified reservosome fraction (B1). (**c**) subcellular fraction containing reservosome membranes (B1M). Bars represent 0.3 μm (**a**) and 1 μm (**b,c**).

Epimastigote Dm28c

↓

Isolation of reservosomes (B1 fraction)

↓

Isolation of reservosome membrane (B1M fraction)

↓

Protein digestion | Trypsin-urea (TU)
Trypsin + Glu-C (TG)

↓

SCX
(25, 50, 100, 200, 500 mM NaCl fractions)

↓

C18-RP nanoHPLC

↓

ESI-linear trap-MS
(2D LC-MS/MS)

↓

TurboSequest database search
(TcruziDB, GenBank)

↓

Blast
(GenBank, GeneDB)

↓

Protein Identification

↓

Gene Ontology analysis
Transmembrane-domain prediction

Figure 2.
Schematic representation of the strategy used for the identification of proteins of the reservosomes of *T. cruzi* epimastigotes.

Figure 3.
Functional classification of *T. cruzi* reservosome proteins by gene ontology. The gene ontology prediction was performed using GOblet and the most representative categories (≥3% from the total sequences) were plotted in the graph. For the complete list see Supplemental Table 1.

Table 1

Proteins of *T. cruzi* epimastigote reservosomes identified by proteomic analysis and with annotated function or cellular localization

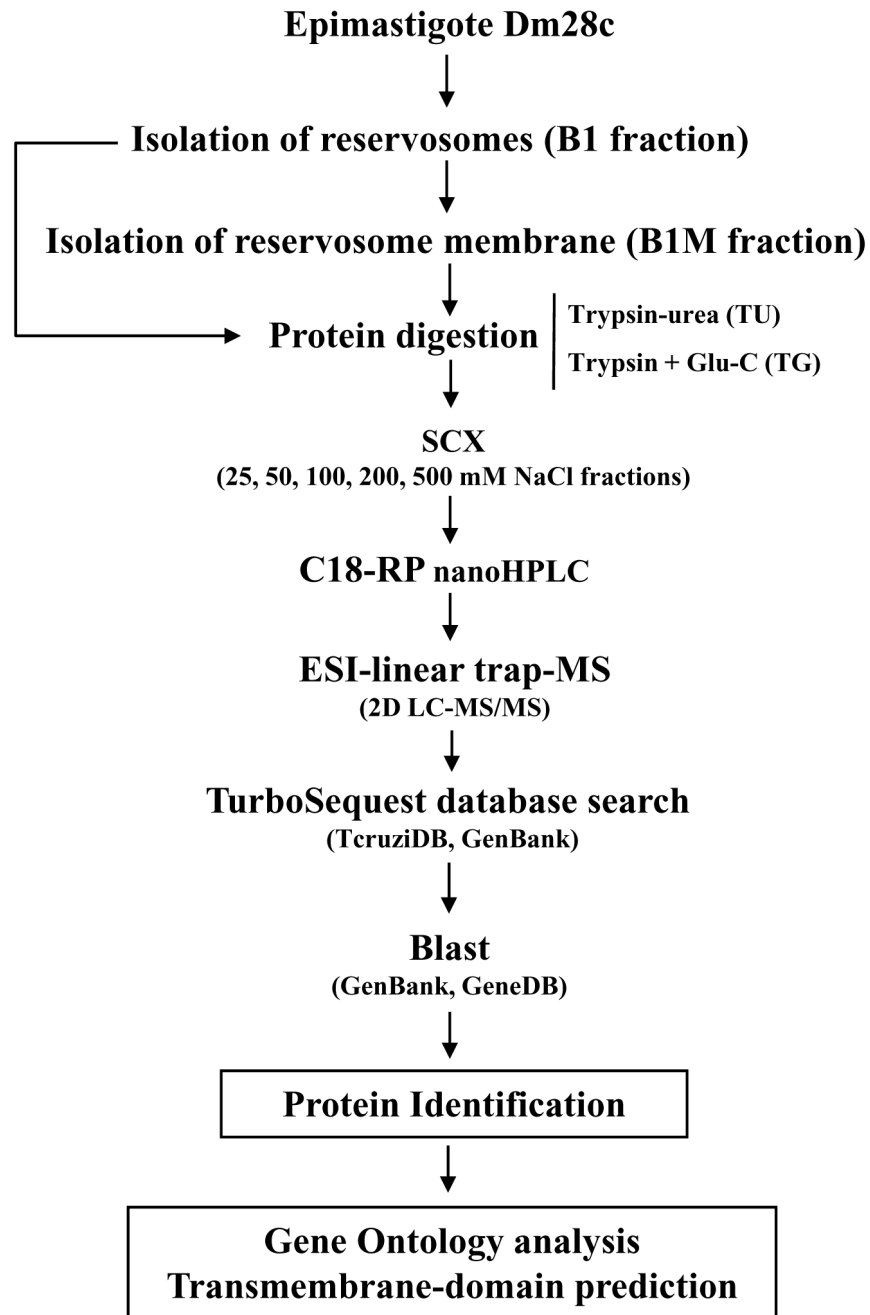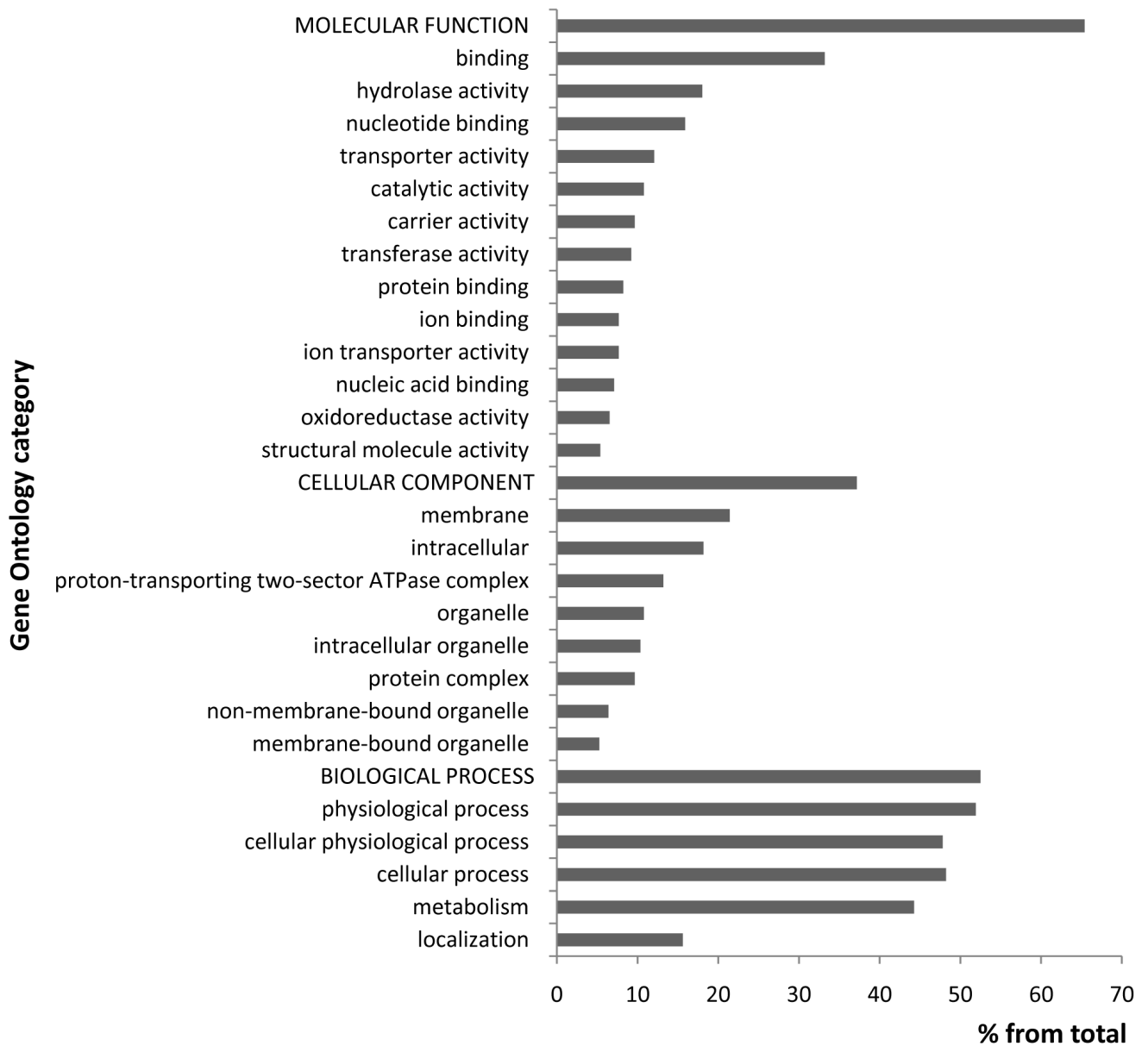| Hit number	Protein group	GenBank accession number	Function/Cellular localization	Transmembrane domain (number of domains)[1]
	(1) Protein metabolism			
1	Serine carboxypeptidase	XP_803003	Lysosomal, peptidase family S10	N
2	Lysosomal alpha-mannosidase	XP_819105	Lysosomal, carbohydrate metabolism	N
3	Histidine ammonia-lyase	XP_820336	Convert histidine into ammonia and urocanic acid	N
4	Cysteine proteinase	XP_811957	Lysosomal, similar to endopeptidase papain	Y (1)
5	Cysteine peptidase	XP_821954	Lysosomal integral to membrane, proteolysis	Y (1)
6	S-Adenosylhomocysteine hydrolase	XP_809153	Adenosylhomocysteine hydrolase activity	N
7	Cysteine peptidase C	XP_812694	Lysosomal, proteolysis	N
8	Calpain cysteine peptidase	XP_816696	Calcium-dependent cysteine-type endopeptidase activity	N
9	Membrane-bound acid phosphatase	XP_808329	Catalytic activity	N
10	Membrane-bound acid phosphatase 2	XP_815570	Lysosomal, acid phosphatase activity	Y (1)
11	Serine carboxypeptidase S28	XP_818592	Peptidase S28	N
12	Cruzipain	AAF75547	Cysteine peptidase, clan CA, family C1	Y (2)
13	Tartrate-resistant acid phosphatase type 5	XP_811082	Metallophosphoesterase	N
14	Ubiquitin hydrolase	XP_821344	Protein deubiquitination	N
15	Glutamamyl carboxypeptidase	XP_804169	Metallopeptidase activity, proteolysis	N
16	Peptidase M20/M25/M40	XP_813635	Metallopeptidase activity	N
17	Chagasin	2FO8	Cysteine Protease Inhibitor	N
	(2) Endosomal/lysosomal membrane proteins			
18	Golgi/lysosome glycoprotein	XP_817916	Golgi/lysosome membrane protein	Y (1)
19	Lysosomal/endosomal membrane protein p67	XP_803464	Lysosomal/endosomal membrane protein	Y (1)
20	Endosomal integral membrane protein	XP_817908	Integral to endosomal membrane	Y (9)
21	Endosomal integral membrane protein	XP_813892	Endosomal protein	Y (11)
22	Huntingtin interacting protein (HIP)	XP_816878	Endocytosis, metal ion binding	Y (4)
	(3) Vesicular traffic			
23	Vesicle-associated membrane protein	XP_816323	Integral to plasma membrane, vesicle targeting	Y (1)
24	Vesicle-associated membrane protein	XP_812489	Exocytosis	Y (1)
25	Rab1	XP_805990	ER, small GTPase mediated signal transduction	N
26	Rab-2a	XP_809735	ER, Small GTPase mediated signal transduction	N
27	COP-coated vesicle membrane protein erv25	XP_821489	Coated vesicle membrane protein	Y (1)
28	COP-coated vesicle membrane protein gp25l	XP_805665	ER to Golgi vesicle-mediated transport	Y (1)
29	Small GTP-binding protein	XP_808772	ER to Golgi vesicle-mediated transport	N
30	Rab7	XP_821619	Small GTPase mediated signal transduction	N
31	Rab18	XP_818013	Small GTPase mediated signal transduction	N
32	Mu-adaptin 1	XP_818899	Adaptor complexes 1 subunit	N
	(4) pumps and channel proteins			
33	P-type H+-atpase (tcha3)	XP_812631	ATPase, transmembrane proton transport	Y (6)
34	P-type H+-atpase (tcha1)	XP_804256	ATPase, transmembrane proton transport	Y (3)
35	P-type H+-atpase (tcha2)	AAL87542.1	ATPase, transmembrane proton transport	Y (8)
36	ABC transporter	XP_818638	ATPase, transmembrane transport of substances	Y (12)
37	P-glycoprotein	AAC09044.1	ABC transporter protein	Y (8)
38	Multidrug resistance protein E	XP_815145	Xenobiotic-transporting ATPase activity	Y (4)
39	Proton motive atpase	AAB70152	Cation transport ATPase, P-type ATPase	N
40	Vacuolar-type proton pyrophosphatase	XP_813460	Hydrogen-translocating pyrophosphatase activity	Y (15)
41	Ferric reductase	XP_808251	Electron transport, oxidoreductase activity	Y (12)
42	Chloride channel protein	XP_816207	Voltage-gated chloride channel activity	Y (10)
43	Hexose transporter	XP_814821	Glucose transmembrane transporter activity	Y (12)
44	V-type atpase, A subunit	XP_807670	Lysosomal proton-transporting V-type ATPase complex	N
45	Folate/pteridine transporter	XP_810759	Biopterin transport	Y (12)
46	Vacuolar ATP synthase	XP_808900	Hydrogen-exporting ATP activity, phosphorylative mechanism	N
47	Amino acid permease	XP_802539	Transmembrane amino acid transporter protein	Y (10)

Hit number	Protein group	GenBank accession number	Function/Cellular localization	Transmembrane domain (number of domains)[1]
48	MFS transporter	XP_804323	Transport small solutes across membranes	Y (7)
49	Amino acid permease/transporter	XP_819801	Amino acid transport	Y (10)
50	Calcium-translocating P-type atpase	XP_814228	Calcium-transporting ATPase activity	Y (3)
(5) Cell surface proteins				
51	Surface protease GP63	XP_821289	Neutral zinc metallopeptidases	Y (1)
52	Dispersed gene family protein 1 (DGF-1)	XP_820404	Cell surface protein	Y (9)
53	Procyclic form surface glycoprotein	XP_803886		Y (3)
54	Kinetoplastid membrane protein KMP-11	XP_808865	kinetoplastid membrane protein	N
(6) Lipid metabolism				
55	Sterol 24-c-methyltransferase	XP_802864	Biosynthesis of ergosterol	N
56	Fatty acid elongase	XP_813971	Long-chain fatty acid biosynthetic process	Y (7)
57	3-oxo-5-alpha-steroid 4-dehydrogenase	XP_802815	Very-long-chain fatty acid metabolic process	Y (4)
58	Fatty acyl coa synthetase	XP_817096	Long-chain-fatty-acid-CoA ligase activity	N
59	Serine-palmitoyl-coa transferase	XP_812739	Sphingolipid metabolic process	N
60	Sterol C-24 reductase	XP_816888	Ergosterol biosynthesis	Y (9)
61	Phospholipid-translocating atpase	XP_817305	Phospholipid-translocating P-type ATPase, flippase	Y (12)
62	Fatty acyl coa synthetase 2	XP_802708	Long-chain fatty acid activation	N
63	C-8 sterol isomerase	XP_821186	Ergosterol biosynthetic process	Y (1)
64	Fatty acyl coa syntetase 1	XP_812351	Fatty acid metabolic process	N
65	Phosphatidylcholine:ceramide cholinephosphotransferase	XP_821506	Sphingomyelin biosynthetic process	Y (5)
66	Phosphatidic acid phosphatase protein	XP_814714	Phospholipid metabolic process	Y (5)
67	Lipase domain protein	XP_814907	Lipid metabolic process	Y (7)
(7) Carbohydrate metabolism				
68	UDP-Gal or UDP-glcnac-dependent glycosyltransferase	XP_814599	Transfer galactose to GlcNAc terminal chains	N
69	Enolase	XP_819700	Glycolysis, phosphopyruvate hydratase activity	N
70	Hexokinase	XP_808992	Carbohydrate metabolic process	N
71	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IB	XP_811964	Protein amino acid N-linked glycosylation	N
72	Beta galactofuranosyl glycosyltransferase	XP_810392		N
73	Lectin	XP_807732	Mannose binding	N
74	Glucokinase 1	XP_821474	Glucokinase activity	N
75	Glucosamine-6-phosphate isomerase	XP_807857	N-acetylglucosamine catabolic process	N
(8) Cell signaling				
76	Protein kinase	XP_807538	Protein amino acid phosphorilation	N
77	Protein tyrosine phosphatase	XP_816679	Protein tyrosine phosphatase activator factor	N
78	Serine/threonine protein kinase	XP_822060	Protein amino acid phosphorilation	N
79	Serine/threonine protein phosphatase	XP_809469	Calcium-dependent protein serine/threonine phosphatase	Y (2)
80	Rac serine-threonine kinase	XP_815140		N
81	Regulatory subunit of protein kinase a	XP_810045	Protein kinase activity	N
82	Casein kinase delta isoform	XP_803179	Casein kinase activity	N
83	Activated protein kinase C receptor	XP_817733	Protein kinase C binding	N
84	Casein kinase	XP_803179	Protein amino acid phosphorylation	N
(9) Cytoskeleton-associated proteins				
85	Beta tubulin	XP_816690	Microtubule cytoskeleton organization and biogenesis	N
86	Alpha tubulin	XP_802499	Microtubule cytoskeleton organization and biogenesis	N
87	Actin	XP_807262	Microfilament organization and biogenesis	N
88	MCAK-like kinesin	XP_813221	Microtubule associated complex	N
89	Cytoskeleton-associated protein CAP5.5	XP_809814	Cytoskeleton-associated protein	N
90	Cofilin/actin depolymerizing factor	XP_806100	Actin filament organization	N
91	I/6 autoantigen	XP_813169	Calcium ion binding, structural constituent of cytoskeleton	N
(10) Cytosolic proteins				
92	Elongation factor alpha G5	AAU47272	Calcium mediated signaling, microtubule-based process	N
93	Tryparedoxin peroxidase	XP_802393	Trypanothione-disulfide reductase activity	N
94	Hypoxanthine-guanine phosphoribosyltransferase	XP_813397	Nucleotide transport and metabolism	N
95	Methylthioadenosine phosphorylase	XP_819927	Amino acid salvage	N

Hit number	Protein group	GenBank accession number	Function/Cellular localization	Transmembrane domain (number of domains)[1]
96	6-phosphogluconate dehydrogenase	XP_808031	Pentose-phosphate shunt	N
97	Disulfide isomerase	XP_821173	Protein folding	N
	(11) Glycosomal proteins			
98	Glutamate dehydrogenase	XP_818855	Glutamate biosynthesis, using glutamate synthase (NADPH)	N
99	Glycosomal phosphoenolpyruvate carboxykinase	XP_810815	Gluconeosenesis, microtubule binding	N
100	Glyceraldehyde 3-phosphate dehydrogenase	XP_812137	Glycolysis	N
101	NADH-dependent fumarate reductase	XP_810232	Glucose metabolic process	N
102	Gim5A protein	XP_821649	Glycosomal membrane	N
103	Glycosomal malate dehydrogenase	XP_812467		N
104	Fructose-bisphosphate aldolase	XP_809369	Fructose-bisphosphate aldolase activity	N
105	Glucose-6-phosphate isomerase	XP_821969	Gluconeogenesis, glycolysis	N
106	6-phospho-1-fructokinase	XP_822053	Glycolysis	N
	(12) Mitochondrial proteins			
107	Heat shock 70 kda protein	XP_806221	Membrane protein folding	N
108	Malate dehydrogenase	XP_809210	Tricarboxilyc acid cycle	N
109	Mitochondrial processing peptidase, beta subunit	XP_816361	Mitochondrial processing peptidase activity	N
110	Pyruvate dehydrogenase E1 beta subunit	XP_811646	Pyruvate dehydrogenase (acetyl-transfering) activity	N
111	Succinyl-coa ligase [GDP-forming] beta-chain	XP_802641	Succinate-CoA ligase (GDP-forming) activity	N
112	P22 protein precursor	XP_813311	Transcription regulator activity	N
113	ADP, ATP carrier protein 1	XP_812264	Transport, mitochondrial inner membrane	Y (3)
114	RNA-binding protein 2	XP_818766	Mitochondrial RNA binding	N
115	Acyl carrier protein	XP_814961	Fatty acid biosynthetic process	N
116	Oligo_U binding protein TBRGG1	XP_809536		N
117	ATP synthase, epsilon chain	XP_818987	Proton-transporting ATP synthase complex	N
118	Chaperonin HSP60/CNP60	XP_804030	Protein import into mitochondrial matrix	N
119	Succinyl-coa ligase [GDP-forming] beta-chain	XP_818583	Tricarboxylic acid cycle	N
120	Aldehyde dehydrogenase	XP_813115	Oxidation of aldehydes	N
121	ADP, ATP carrier protein 1	XP_819458	Mitochodria inner membrane, transport	Y (3)
	(13) others			
122	Glucose-regulated protein 78	XP_809817	Endoplasmic reticulum, protein folding	N
123	Calreticulin	XP_812571	Protein folding, sugar binding	N
124	Calcium-binding protein	XP_808304	Flagellar calcium binding protein	N
125	Malic enzyme	XP_814410	Malate metabolic process	N
126	Cyclophilin A	XP_821578	Protein folding	N
127	S-phase kinase-associated protein	XP_806972	Ubiquitin-dependent protein catabolic process	N
128	Calmodulin	XP_874377	Calcium ion binding	N
129	Centrin	XP_813165	Basal body, calcium ion binding	N

[1] N, no; Y, yes