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Glycosomal membrane proteins and lipids from *Leishmania mexicana*

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ABSTRACT

Constituents of the glycosomal membrane from *Leishmania mexicana* should play a critical role in the coordination of metabolic processes occurring in the cytosol and those compartmentalized within glycosomes. We have made an inventory of glycosomal membrane-associated proteins using approaches specific for enriching both integral and peripheral membrane proteins. Surprisingly, 70% of the proteins were recovered in the hydrophobic fraction of membranes solubilized with Triton X-114, while 20% were present in the soluble fraction obtained upon treatment with Na_2CO_3. 14 major polypeptides, ranging in molecular weight from 65 to 16 kDa, were found to be associated with the membrane, nine of them behaving as integral membrane proteins. Assessment of their topology in the membrane indicated that the polypeptides of 56, 50, 46 and 32 kDa have no domains exposed to the cytosol. The 50 kDa protein is the most abundant one of the glycosomal membrane, where it is peripherically located at the matrix face. The major phospholipids of glycosomal membranes are phosphatidylethanolamine, phosphatidyl-choline and phosphatidyl-serine, with smaller proportions of sphingomyelin and phosphatidyl-inositol. The sterols found were of 5-dehydroepisterol, ergosta-5,7,24(24^1)-trien-3β-ol, and also their precursors, consistent with the notion that these organelles are involved in *de novo* biosynthesis of sterols in trypanosomatids.

1. Introduction

The Trypanosomatidae family comprises protists belonging to the order Kinetoplastea, which represents one of the earliest evolutionary branches in the eukaryotic lineage (Hannaert et al., 2003). All trypanosomatids known are parasites, some of them causing serious diseases in man (*Leishmania* spp., *Trypanosoma cruzi* and the African trypanosomes *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*), domestic animals (many African trypanosome and *Leishmania* species) and plants (*Phytomonas* spp.) (Besteiro et al., 2002).

Trypanosomatids have as specific feature that most glycolytic enzymes are sequestered in organelles, the glycosomes, that are related to peroxisomes. The glycosomal compartments are surrounded by a single lipid bilayer membrane. The trafficking of metabolites across the glycosomal membrane requires specific macromolecules, both transporters and channel-forming proteins (Michels et al., 2000; Gualdrón-López et al., 2013). Translocation of cofactors such as adenine nucleotides and NAD(P)(H) over the membrane is considered a slow process; the ATP consumption and synthesis are balanced inside the glycosomes and an ATP/ADP ratio different from that in the cytosol is maintained (Bakker et al., 1999). With the NADH generated during glycolysis, the redox balance inside glycosomes is maintained by auxiliary redox enzymes such as malate dehydrogenase (MDH), fumarate reductase (FR) and a glycerol-3-phosphate dehydrogenase (GPDH) (Concepción et al., 2001a; Barros-Alvárez et al., 2014). The GPDH is part of a system that enables the reoxidation of glycosomal NADH by molecular oxygen through a dihydroxyacetone-phosphate/glycerol-3-phosphate shuttle and a mitochondrial FAD-dependent GPDH that, in *Trypanosoma* species, is part of a glycerol oxidase complex also containing an alternative oxidase (Tielens and Van Hellemond, 1998). However, this oxidase is absent from *Leishmania* species. The transporter that exchanges glycerol 3-phosphate for dihydroxyacetone-phosphate remains to be identified, but its existence is inferred from the low permeability of lipid membranes and the theoretical requirement for strict coupling of the fluxes by which the two triosephosphates are exchanged (Bakker et al., 1999).

Glycosomes, like all peroxisomes, do not contain DNA (Opperdoes, 1987). Their matrix proteins and some membrane proteins are synthesized in the cytoplasm and imported into pre-existing glycosomes, while other membrane proteins are probably inserted into the endoplasmic reticulum before routed by vesicles to growing organelles, in a similar way as occurs for peroxisomes (reviewed by Galland and Michels, 2010; Gualdrón-López et al., 2013). The mechanism of matrix protein import is similar in all organelles of the peroxisome family

Abbreviations: ALAT, alanine amino-transferase; FR, fumarate reductase; GAT, glycosomal ABC transporter; GPDH, glycerol-3-phosphate dehydrogenase; HK, hexokinase; MDH, malate dehydrogenase; PBS, phosphate-buffered saline; PEPCK, phosphoenolpyruvate carboxykinase; PEX, peroxin; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PK, proteinase K; PTS1, type 1 peroxisomal-targeting signal; PPDK, pyruvate phosphate dikinase; TEA, triethanolamine-HCl.

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and, involves a cascade of actions executed by several proteins called peroxins (PEX) which are homologous even in distantly related organisms. This import machinery of proteins recognizes three types of signal sequences present in the nascent proteins. A type 1 peroxisomal-targeting signal (PTS1) is a tripeptide located at the C-terminal end with the consensus sequence Serine–Lysine–Leucine (SKL) or variations thereof (Gould et al., 1989). The PTS2 sequence is present near the N-terminus and has the consensus motif (R/K)(L/V/I)X5(H/Q)(L/A/F) (Lazarow, 2006). For the third type of targeting signal no consensus motif has been identified; it is found for peroxisomal and glycosomal proteins without a PTS1 or PTS2. The targeting of these proteins may be achieved by a polypeptide internal sequence or I-PTS; at least in some cases this may involve piggy-back transport on a PTS1 or PTS2 containing protein (Galland et al., 2010).

A non-redundant set of 33 PEX proteins are known to mediate, in different organisms such as yeasts, fungi, plants and mammals, the distinct processes responsible for peroxisome biogenesis (Galland and Michels, 2010). Several peroxins are soluble, in the cytosol or organellar matrix, but many are associated with the peroxisomal membrane where some are involved in the import of matrix proteins and others in the insertion of additional membrane proteins. Orthologues of several peroxisomal PEX proteins have been identified in trypanosomatids as being responsible for glycosome biogenesis (Galland and Michels, 2010; Gualdrón-López et al., 2013).

The import of peroxisomal and *T. brucei* glycosomal matrix proteins has been shown to involve several consecutive steps, in which membrane peroxins interact with other peroxins and the proteins to be imported through specific protein-interaction domains, such as RING-finger domains (in the case of a complex of the three integral membrane proteins PEX2, PEX10 and PEX12) or a SH3 domain as present in the integral membrane protein PEX13. This latter peroxin forms together with PEX14, another protein associated to the peroxisomal/glycosomal membrane, a docking complex for PTS1- and PTS2-proteins bound to respectively the cytosolic receptors PEX5 and PEX7. All these proteins have also been identified in trypanosomatids (reviewed by Galland and Michels, 2010; Gualdrón-López et al., 2013).

Targeting of peroxisomal membrane proteins is known to involve motifs (so called mPTS) different from those of matrix proteins (PTS1 and PTS2). Three peroxins have been shown to be involved in the insertion of proteins into the peroxisomal membrane: PEX3, PEX19 and PEX16 (Michels et al., 2005). *T. brucei* homologues of PEX19 and PEX16 have also been identified. However, in contrast to the mechanism of matrix protein import, very little is known yet about the precise mechanism by which peroxisomal/glycosomal membrane proteins are inserted.

Additionally, three proteins of the PEX11 family have been identified in *T. brucei*; PEX11 itself, required for glycosome proliferation (Lorenz et al., 1998) and the related GIM5A and GIM5B (Maier et al., 2001; Voncken et al., 2003) of which the precise function is still under debate. Homologues of some of these membrane-associated proteins involved in glycosome biogenesis have also been identified in *Leishmania* spp. (Flaspohler et al., 1997, 1999).

The depletion of each of the membrane-associated peroxins PEX6, PEX10, PEX12, PEX13 and PEX14 affected growth of both mammalian bloodstream-form and insect-form (procyclic) *T. brucei* and resulted in the partial mislocalization of different types of glycosomal matrix enzymes to the cytosol (Moyersoen et al., 2003; Krazy and Michels, 2006; Galland et al., 2007; Brennand et al., 2012; Verplaetse et al., 2012). The importance of the proteins present in this membrane has previously also been evidenced in procyclic trypanosomes by knocking down the expression of PEX14, a peroxin involved in the import of matrix proteins (Moyersoen et al., 2003). These procyclic cells depleted for PEX14 were unable to grow in the presence of glucose as the source of carbon (Furuya et al., 2002). Thus, interference with glycosome protein import makes glucose toxic to trypanosomes (Furuya et al., 2002).

Other glycosomal membrane proteins identified in *T. brucei*, with orthologues in other trypanosomatids, are three half-size ABC transporters, GAT1–3, of which GAT1 has been shown to be involved in fatty-acyl-CoA import into the organelles (Yernaux et al., 2006; Igoillo-Esteve et al., 2011), similarly as homologues in peroxisomes of other eukaryotes (Theodoulou et al., 2006). Furthermore, Gualdrón-López et al. (2012b) demonstrated that solubilized, purified glycosomal membrane preparations of *T. brucei* contain proteins with channel-forming activities, similar to what has been shown for peroxisomes (Antonenkov and Hiltunen, 2012).

Some glycosomal membrane proteins from *T. cruzi* have been described by Quiñones et al., (2004), while also proteome analyses of total glycosomal preparations from *Leishmania tarentolae* and *T. brucei* revealed a number of membrane proteins (Colasante et al., 2013; Güther et al., 2014).

The lipid composition is relevant for the structure and properties of membranes, like their fluidity. For *Leishmania* spp., the lipid composition of the plasma membrane has been determined (Lux et al., 2000), whereas quantitative analyses of lipids present in the glycosomal membrane have been reported for *T. cruzi* (Quiñones et al., 2004) and *T. brucei* (Colasante et al., 2013). Neutral lipids have distinctive features in trypanosomatids compared to the mammalian cells, since the parasites synthesize ergosterol instead of cholesterol (Urbina, 1997), and this feature is being exploited in rational strategies for development of drugs against *T. cruzi* and *Leishmania* spp. Knowledge of the glycosomal membrane lipid composition and understanding the properties of this membrane is relevant since the organelle contains parts of pathways of lipid synthesis (Urbina et al., 2002).

In this paper, we report an inventory of the protein and lipid composition of the glycosomal membrane from *Leishmania mexicana*. Membrane protein preparations were obtained by two different approaches, with Triton X-114 and sodium carbonate, in order to be able to distinguish between integral and peripheral proteins; the topology of these proteins in the glycosomal membrane was evaluated by protease protection assays. Since the integrity of the glycosomal membrane is important to the survival of the trypanosomatids, knowledge about the composition of the proteins present in the membrane of this organelle could be useful for designing a chemotherapeutic strategy against these parasites.

2. Materials and methods

2.1. Growth conditions of parasites

L. mexicana promastigotes (AZB strain) were cultivated in Schneider's medium (Sigma-Aldrich) as described by Bates (1994), prepared following the manufacturer's instructions. The medium was supplemented with 20% fetal bovine serum. The cells were grown at 28 °C with agitation at 30 rpm and collected when the culture reached an optical density at 600 nm of 0.6–0.7, i.e. after approximately five days.

2.2. Glycosome purification and fractionation of membrane proteins from highly purified glycosomes

Glycosomes from *L. mexicana* were purified using the same procedure we reported previously (Quiñones et al., 2004). Highly purified glycosomes were then treated in different ways to obtain specific membrane protein preparations, as previously described by Quiñones et al. (2004). One experimental protocol involved treatment with Triton X-114. Another protocol allowed to obtain the glycosomal membrane with its integral proteins by treatment with sodium carbonate, a protocol originally described for endoplasmic reticulum membrane of rats by Fujiki et al. (1982).

2.3. Glycosome labeling with biotin

Purified glycosomes displaying highly latency (>90%) for activity of their matrix enzymes pyruvate phosphate dikinase (PPDK) and