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Ubiquitination of the glycosomal matrix protein receptor PEX5 in *Trypanosoma brucei* by PEX4 displays novel features

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ABSTRACT

Trypanosomatids contain peroxisome-like organelles called glycosomes. Peroxisomal biogenesis involves a cytosolic receptor, PEX5, which, after its insertion into the organellar membrane, delivers proteins to the matrix. In yeasts and mammalian cells, transient PEX5 monoubiquitination at the membrane serves as the signal for its retrieval from the organelle for re-use. When its recycling is impaired, PEX5 is polyubiquitinated for proteasomal degradation. Stably monoubiquitinated TbPEX5 was detected in cytosolic fractions of *Trypanosoma brucei*, indicative for its role as physiological intermediate in receptor recycling. This modification's resistance to dithiothreitol suggests ubiquitin conjugation of a lysine residue. *T. brucei* PEX4, the functional homologue of the ubiquitin-conjugating (UBC) enzyme responsible for PEX5 monoubiquitination in yeast, was identified. It is associated with the cytosolic face of the glycosomal membrane, probably anchored by an identified putative TbPEX22. The involvement of TbPEX4 in TbPEX5 ubiquitination was demonstrated using procyclic Δ*PEX4* trypanosomes. Surprisingly, glycosomal matrix protein import was only mildly affected in this mutant. Since other UBC homologues were upregulated, it might be possible that these have partially rescued PEX4's function in PEX5 ubiquitination. In addition, the altered expression of UBCs, notably of candidates involved in cell-cycle control, could be responsible for observed morphological and motility defects of the Δ*PEX4* mutant.

1. Introduction

Human African trypanosomiasis is an infectious disease caused by the parasitic protist *Trypanosoma brucei* and transmitted by bites of tsetse flies. *T. brucei* belongs to the Kinetoplastea clade and the Trypanosomatidae family. This group also includes *Trypanosoma cruzi*, the causative agent of Chagas disease in Latin America, and *Leishmania* species which are responsible for several forms of leishmaniasis in tropical and subtropical areas of the world [1–3].

The *T. brucei* life cycle involves several successive developmental changes which occur when the parasite alternates between living extracellularly in the mammalian host, where its main form is the proliferative so-called long-slender bloodstream form and in the insect, where procyclic forms are the replicating stage in the fly's midgut. This differentiation, which involves physiological, morphological and metabolic remodelling, allows the parasite to adapt to the different environments [4,5].

One of the unique features that distinguish members of the kinetoplastids from other eukaryotes is the metabolic compartmentalisation of the majority of glycolytic enzymes and some other pathways of core metabolism within organelles called glycosomes [6–8]. Remarkably, correct compartmentalisation of glycolysis in glycosomes of the bloodstream form of *T. brucei* is absolutely essential for survival of the parasites [9,10].

Glycosomes are microbodies that belong to the organelle family of peroxisomes, together with the peroxisomes found in representatives of all eukaryotic lineages. These organelles are bounded by a single phospholipid bilayer membrane and do not contain any detectable DNA. Peroxisomal and glycosomal matrix and membrane proteins are synthesised in the cytosol and post-translationally imported into the organelles. The formation of these organelles involves different proteins (so far a non-redundant set of 33 proteins have been identified in different organisms) commonly named 'peroxins' (abbreviated PEX), which accomplish the different steps of the process: peroxisome

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; DUB, deubiquitinating; FBPase, fructose-1,6-bisphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green-fluorescent protein; HXK, hexokinase; NEM, N-ethylmaleimide; PEX, peroxin; PFK, phosphofructokinase; PTS, peroxisomal-targeting signal; ORF, open-reading frame; Tet, tetracycline; TPI, triosephosphate isomerase; TPR, tetratricopeptide repeat; UBC, ubiquitin-conjugating

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membrane formation, import of peroxisomal matrix proteins and peroxisome proliferation [11–13].

Peroxisomal matrix protein import is a multistep process that involves successive interactions between several peroxins in the cytosol and at the peroxisomal membrane. The matrix proteins are targeted to the organelles through their peroxisomal-targeting signals: PTS1, consisting of the motif SKL or physicochemically conserved variants of this tripeptide, with also contributions by adjacent residues, more precisely a dodecamer sequence, located at the extreme C-terminus of the great majority of matrix proteins [14]; PTS2 is a nonapeptide (R/K)(L/V/I/Q/)xx(L/V/I/H/Q)(L/S/G/A/K)X(H/Q)(L/A/F) near the N-terminus of a smaller subset of matrix proteins [13,15,16]. The matrix proteins are recognised in the cytosol through their targeting signals PTS1 or PTS2 by different receptors, PEX5 and PEX7, respectively. PEX5 is a predominantly cytosolic protein that possesses two domains: a C-terminal domain composed of 6–7 tetratricopeptide repeat (TPR) motifs which interact with the PTS1 of the cargo, and a structurally disordered N-terminal domain that functions in the receptor's docking, at the organelle's membrane, through its conserved pentapeptide (WxxxF/Y) repeat motifs, and in the receptor's recycling (see below). PEX7 is also a cytosolic protein that becomes transiently internalised inside the peroxisomes [17,18] belonging to the WD protein family. For the PTS2-protein import, it requires species-specific auxiliary proteins also known as co-receptors: PEX18 and PEX21 in *Saccharomyces cerevisiae*, PEX20 in *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha* and *Neurospora crassa* or a longer splice variant of the PTS1 receptor PEX5 in plants and mammals. The co-receptors form a ternary complex with the cargo-loaded PEX7 in the cytosol and direct the complex to the peroxisomal membrane [19]. A small fraction of proteins that lack canonical PTS1 and 2 motifs may be imported through either their association with PTS-containing proteins ("piggy backing") or via the binding to the N-terminal region of PEX5 (non-PTS import) [20].

The next step in matrix protein import is the association of the receptor–cargo complex, formed in the cytosol, with the peroxisomal membrane. A docking subcomplex minimally composed of PEX14, PEX13 and in *S. cerevisiae* also PEX17, is responsible for this step [21]. The docking subcomplex has been shown to physically associate with a subcomplex of three RING-finger proteins, PEX2-PEX10-PEX12, in order to form an import-competent complex, called the importomer [13,15]. The docking and RING-finger subcomplexes are associated in a PEX8 and PEX3-dependent manner in *S. cerevisiae* and *P. pastoris*, respectively [22].

Peroxisomes are able to import folded proteins and also large oligomeric proteins into the peroxisomal matrix [23,24]. Recently, Meinecke et al. [25] demonstrated that the membrane-associated import receptor PEX5 from *S. cerevisiae* together with the docking-complex protein PEX14 forms a dynamic channel that is opened to a diameter up to 9 nM upon docking of the cytosolic receptor–cargo complex. This discovery explains how peroxisomes are able to import completely folded and even oligomeric proteins of different sizes and illustrates the extremely important, multiple roles of PEX5 in the peroxisomal matrix protein import process.

The next step in matrix protein import is the cargo release in the peroxisomal matrix, the mechanism of which is poorly understood. Several lines of evidence indicate a possible function of PEX8 in the dissociation of cargo proteins from the PEX5–cargo complex; however, this issue remains to be solved [16]. After the cargo release, the two receptors, PEX5 and PEX7, must be retrieved from the peroxisomes. Whereas PEX7 is fully internalised into the matrix [18], PEX5 does not enter the lumen with its cargo, but seems to release it while remaining inserted in the membrane. PEX5 is thus a cycling protein that translocates to the peroxisomal membrane from where it is recycled to the cytosol [26]. The molecular machinery that is involved in this process was defined as the exportomer [16]. It comprises two cytosolic AAA^+-ATPases, PEX1 and PEX6, which are anchored to the peroxisomal membrane through PEX15 in yeasts or PEX26 in mammalian cells, and which have been identified as the mechanoenzymes responsible for the removal of PEX5 from the peroxisomal membrane [27]. Also part of the exportomer is a member of the E2 ubiquitin-conjugating enzyme family, a function performed in yeasts and plants by PEX4 together with its membrane anchor protein PEX22 [28–30]. In mammalian cells, this function is accomplished by the cytosolic ubiquitin-conjugating enzymes UbcH5a/b/c [31]. Additionally involved in the receptor recycling are the RING-finger peroxins which have E3-ubiquitin ligase activity [32].

Unlike the docking event, which is ATP independent, the receptor recycling requires the free energy from the hydrolysis of ATP, both by the AAA^+-ATPases and for the activation of ubiquitin. Recently, it has been proved that the ATP-dependence of the ubiquitination-mediated export of *S. cerevisiae* PEX18, via the mechanistic linkage, drives the translocation of the PEX7 receptor-bound cargo into the matrix [33], supporting the export driven protein import model proposed by Schliebs et al. [34].

In yeast PEX5 is mono- or polyubiquitinated at the peroxisomal membrane. The monoubiquitination of the receptor serves as the signal for its recycling and depends on the concerted action of the peroxisomal membrane-bound PEX4 and PEX12, which ubiquitinate PEX5 at a conserved cysteine residue (Cys6) located in its N-terminal part. The polyubiquitination has been demonstrated to occur as a quality control mechanism that serves to signal the removal of not properly recycled receptor molecules. This type of ubiquitination depends on the redundant activities of the cytosolic ubiquitin-conjugating enzymes UBC4/5/1 and membrane-bound PEX2 and occurs at different lysine residues located also in the N-terminal part of the protein (Lys18 and Lys24 in *S. cerevisiae* and Lys 21 in *H. polymorpha*) [28,29,32,35–37]. Importantly, in *S. cerevisiae* the co-receptor PEX18 has been proved to be also monoubiquitinated at a conserved cysteine residue (Cys6) as a signal for its recycling and polyubiquitinated at lysine residues (Lys13 and Lys20) for its proteasome-dependent degradation [33]. Very recently, it has been shown that the *P. pastoris* co-receptor PEX20 is also mono- or diubiquitinated (Cys8) and polyubiquitinated (Lys19) in a PEX4 and PEX4–PEX7 dependent manner, respectively, providing new roles for PEX4 in the ubiquitination cascade of the PTS2 import pathway [38].

PEX5 in mammalian cells, as with its counterpart in yeast, is also monoubiquitinated at the peroxisomal membrane at a well conserved cysteine residue (Cys11) [39]. However, the ubiquitin-conjugating enzyme involved in this modification is the cytosolic broad range UbcH5a/b/c [31]. It was recently proved that mammalian PEX10 is necessary for PEX5 ubiquitination as well [40]. Interestingly, in mammalian cells two different monoubiquitinated PEX5 species were detected in distinct cellular locations. While the cysteine-monoubiquitinated [dithiothreitol (DTT) sensitive] form of PEX5 was exclusively detected in the organellar fraction of mammalian cells, another monoubiquitinated form (DTT resistant), for which no function has yet been ascribed, was located in the cytosol [40,41].

In trypanosomes, several of the peroxins involved in the glycosomal matrix protein import have been identified and characterised. These include TbPEX2, TbPEX5, TbPEX6, TbPEX7, TbPEX10, TbPEX12, TbPEX13 (two different isoforms, TbPEX13.1 and TbPEX13.2) and TbPEX14. All these peroxins are essential for glycosome biogenesis and therefore for the survival of the parasites (reviewed in [9,10,42]). Nevertheless, few data are available yet about the mechanism of the import process and how some of these peroxins operate in TbPEX5 recycling. In this paper we provide the first experimental evidence of TbPEX5 ubiquitination and present the identification and functional characterisation of the *T. brucei* orthologue of the peroxisomal ubiquitin-conjugating enzyme TbPEX4.

2. Materials and methods

2.1. Parasite cultures, transfections and cell growth measurements

Monomorphic bloodstream and procyclic-form *T. brucei* strain Lister 427 (hereafter called *T. brucei* 427), cell lines 449 [43] that were used in