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Identification and characterization of three peroxins—PEX6, PEX10 and PEX12—involved in glycosome biogenesis in *Trypanosoma brucei*☆

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Abstract

Protozoan Kinetoplastida such as the pathogenic trypanosomes compartmentalize several important metabolic systems, including the glycolytic pathway, in peroxisome-like organelles designated glycosomes. Genes for three proteins involved in glycosome biogenesis of *Trypanosoma brucei* were identified. A preliminary analysis of these proteins, the peroxins PEX6, PEX10 and PEX12, was performed. Cellular depletion of these peroxins by RNA interference affected growth of both mammalian bloodstream-form and insect-form (procyclic) trypanosomes. The bloodstream forms, which rely entirely on glycolysis for their ATP supply, were more rapidly killed. Both by immunofluorescence studies of intact procyclic *T. brucei* cells and subcellular fractionation experiments involving differential permeabilization of plasma and organellar membranes it was shown that RNAi-dependent knockdown of the expression of each of these peroxins resulted in the partial mis-localization of different types of glycosomal matrix enzymes to the cytoplasm: proteins with consensus motifs such as the C-terminal type 1 peroxisomal targeting signal PTS1 or the N-terminal signal PTS2 and a protein for which the sorting information is present in a polypeptide-internal fragment not containing an identifiable consensus sequence.

Keywords: *Trypanosoma*; Glycosome biogenesis; Peroxin; RNA interference

1. Introduction

Trypanosomes are parasitic organisms belonging to the protozoan order Kinetoplastida. All members of this taxonomic group possess a number of distinctive structural and metabolic properties such as the presence of peculiar organelles. The unique organelles comprise the kinetoplast, a subcompartment of the single mitochondrion containing DNA uniquely organized and expressed [1–3], and glycosomes, peroxisomes which have as their most distinctive feature the presence of the majority of the enzymes of the glycolytic pathway [4,5]. In other organisms, glycolysis occurs in the cytosol. In addition to glycolytic enzymes, the glycosomes may contain enzymes or (parts of) other pathways, such the gluconeogenic and pentosephosphate pathways, pyrimidine biosynthesis and purine salvage, and in common with peroxisomes in other eukaryotes, enzymes of peroxide metabolism, fatty-acid oxidation and ether-lipid biosynthesis [4]. The classification of glycosomes within the organelle family of peroxisomes, also including yeast microbodies, plant glyoxysomes and fungal Woronin bodies, is further based on the fact that all these organelles have a single boundary membrane, contain no DNA, and follow similar routes of biogenesis [4–7].

The matrix proteins of the organelles are encoded by nuclear genes, synthesized on free ribosomes in the cytosol and post-translationally imported usually without any detectable form of processing. Previous research, mainly performed on different

Abbreviations: ALD, aldolase; ENO, enolase; gGAPDH, glycosomal glyceraldehyde-3-phosphate dehydrogenase; PEX, peroxin; PTS, peroxisome-targeting signal; PYK, pyruvate kinase; TIM, triosephosphate isomerase

☆ The new nucleotide sequence data reported in this paper are available in the DDJB/EMBL/GenBank databases under the accession numbers DQ226512 (PEX6), DQ226513 (PEX10) and DQ226514 (PEX12). (2) Slightly different systems are being used for abbreviations of proteins and genes in research on peroxisomes/glyoxysomes/glycosomes in mammals, plants, yeasts and protozoa. Throughout this paper, we used the official nomenclature system for trypanosomatid protozoa (upper case for proteins, italicized upper case for genes) [58].

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yeasts and mammalian cells, has shown that the import process can conveniently be considered as comprising a number of distinct steps [6–11]. (1) Recognition of the proteins by a receptor; (2) docking of the receptor with its bound cargo on the membrane; (3) membrane translocation of the receptor–protein complex; (4) cycling of the discharged receptor back to the cytosol. The process is mediated by proteins called peroxins, abbreviated PEX. In the first step, two different cytosolic receptors, PEX5 and PEX7 recognize proteins to be imported through their peroxisome-targeting signals, PTS1 and PTS2, respectively. PTS1 is the most commonly used signal; it consists of a C-terminal tripeptide -SKL or a sequence of amino acids with similar physico-chemical properties. Yet, the ~10 residues preceding the tripeptide may to some extent affect the efficiency of import. PTS2 is a less frequently used signal; it comprises a nonapeptide motif—(R/K)(L/H/V)X_5(QH)(LA)—close to the N-terminus of the protein. Some peroxisomal proteins do not have a PTS1 or PTS2, but may use either a non-conserved sequence, often internal to the polypeptide (I-PTS), to interact with PEX5 or PEX7, or enter by forming a heteromeric complex with PTS1- or PTS2-bearing proteins ('piggy-backing'). Indeed, it has been shown that peroxisomal proteins may enter the organelle in folded and even oligomeric form [12]. The cargo–receptor complex, possibly even in conjunction with auxiliary proteins, interacts with the membrane through a docking complex minimally comprising PEX13 and PEX14. The complex is then transported through the membrane, probably mediated by the so-called translocation complex of integral membrane RING-finger proteins, PEX2, PEX10 and PEX12, followed by cargo release at the matrix side of the membrane. Although the notion that the receptor moves through the membrane is generally accepted, some controversy exists as to whether it effectively enters the matrix or only releases the cargo at the membrane's inner surface [8]. But consensus exists that the receptors PEX5 and PEX7 return to the cytosol, mediated minimally by an integral membrane protein (PEX15 in yeast or the non-homologous PEX26 in mammalian cells) and two soluble peroxins belonging to the AAA+ protein family, PEX1 and PEX6. Several recently reported observations suggest that mono- or di-ubiquitylation of the import receptors functions as a signal for PEX1 and PEX6 to transfer them back to the cytosol [reviewed in [11]]. It should be noted that an alternative function invoked for the RING-finger proteins PEX2, PEX10 and PEX12 is an involvement in this return of the receptors to the cytosol, rather than in the import of the receptor–cargo complex [10]. In this respect, it is important to note that RING-finger domains are characteristic elements of E3-ubiquitin ligases.

The first two steps of the process by which proteins are imported into the glycosomal matrix of the kinetoplastids *Trypanosoma brucei* and *Leishmania donovani* have already received some attention in previous research. Cloning and characterization of PEX5 and PEX14 of these parasites have been reported, and the interaction between these peroxins and with PTS1 proteins has been studied in detail [13–20]. Also, the importance of these peroxins for proper compartmentation of glycosomal enzymes, and for trypanosomatid metabolism and viability has been analyzed [17,21,22]. In contrast, little is known about the peroxins involved in later steps of import of glycosomal matrix proteins. Only studies on trypanosomatid PEX2 homologues have been reported [23–26].

Here, we present our work on the identification and preliminary characterization of three *T. brucei* proteins homologous to peroxins involved in the third and fourth steps of import of proteins into the peroxisomal matrix: PEX10 and PEX12, presumably responsible for the translocation process, and PEX6, involved in receptor cycling. Moreover, we provide evidence that indeed these trypanosomal proteins are essential for proper glycosome biogenesis.

2. Materials and methods

2.1. Trypanosomes and growth conditions

Bloodstream and procyclic form *T. brucei* 427, cell line 449 [27], constitutively expressing the *Escherichia coli* tetracycline repressor gene integrated in its genome, were used in this study. Bloodstream forms were cultured in HMI-9 medium containing 10% heat-inactivated foetal calf serum (Invitrogen) and 0.2 μg mL^{-1} phleomycin (Cayla), the selectable marker for the tetracycline repressor construct, at 37 °C under water-saturated air with 5% CO_2. Procyclic trypanosomes were grown in normal SDM-79 medium [28] supplemented with 10% foetal calf serum and 0.5 μg mL^{-1} phleomycin at 28 °C under water-saturated air with 5% CO_2. Cultures were always harvested in the exponential growth phase, i.e., at densities lower than 2×10^6 cells mL^{-1} for bloodstream forms and 1×10^7 cells mL^{-1} for procyclic cells, by centrifugation at 1900×*g* for 10 min.

2.2. Identification of sequences for the trypanosomatid homologues of peroxins PEX6, PEX10 and PEX12

The database of the *T. brucei* (strain TREU927/4) genome project, when still at a stage of partial completion, was searched for sequences homologous to those of various yeast and mammalian peroxins. Short fragments of putative candidates were identified in searches performed with PEX6, PEX10 and PEX12 sequences. The corresponding fragments were amplified from genomic DNA of *T. brucei* strain 427, using *Taq* DNA polymerase (TaKaRa), ligated in the pGEM-T Easy vector (Promega), cloned and sequenced with a Beckmann CEQ2000 apparatus (Beckman Instruments, Inc.) using the CEQ DTCS Dye Terminator Cycle sequencing kit (Amersham Biosciences). A purified recombinant plasmid was then used as a probe to screen a genomic *T. brucei* 427 library prepared in *E. coli* strain MB406 with phage λGEM11 [29]. Positive clones were isolated, and DNA fragments subcloned and sequenced. Protein sequences were aligned using the program Clustal W [30].

2.3. Molecular biological methods

For most experiments in molecular biology standard methodologies were used [31], or protocols were followed as provided by suppliers of enzymes used for various forms of DNA and RNA manipulation (Fermentas, Roche Applied Science, New England Biolabs, Promega, Invitrogen, TaKaRa). *E. coli* strain XL-1-Blue (Stratagene) was used for all plasmid cloning.

2.4. Construction of expression systems, purification of recombinant proteins, and antiserum production

Truncated versions of *T. brucei* 427 *PEX6* (993 bp from positions 1956 to 2949 and 1014 bp from 1935 to 2949, both constructs comprising the AAA motifs D1 and D2) were amplified by PCR using Invitrogen Platinum *Pfx* DNA polymerase, a sense oligonucleotide containing an *Nde*I restriction site just upstream of the chosen (or introduced) ATG start codon and an antisense