FEBS Letters 586 (2012) 1765–1771

journal homepage: www.FEBSLetters.org

Trypanosomes contain two highly different isoforms of peroxin PEX13 involved in glycosome biogenesis

Ana Brennand [a], Daniel J. Rigden [b], Paul A.M. Michels [a,*]

[a] Research Unit for Tropical Diseases, de Duve Institute and Laboratory of Biochemistry, Université catholique de Louvain, Avenue Hippocrate 74, Postal Box B1.74.01, B-1200 Brussels, Belgium
[b] Institute of Integrative Biology, Biosciences Building, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK

ARTICLE INFO

Article history:
Received 17 March 2012
Revised 3 May 2012
Accepted 10 May 2012
Available online 26 May 2012

Edited by Wilhelm Just

Keywords:
Trypanosoma
Glycosome
Peroxisome
Biogenesis
Docking complex
Peroxin

ABSTRACT

We previously identified the peroxin PEX13 in *Trypanosoma brucei*. Although lacking some features considered typical of PEX13s, it appeared functional in the biogenesis of glycosomes, the peroxisome-like organelles of trypanosomatids. Here we report the identification of a very different trypanosomatid PEX13, not containing the commonly encountered PEX13 SH3 domain but having other typical features. It is readily detected with the jackhmmer database search program, but not with PSI-BLAST. This is the first time different PEX13 isoforms are reported in a single organism. We show that this PEX13.2, like the PEX13.1 previously described, is associated with glycosomes and that its depletion by RNA interference affects the biogenesis of the organelles and viability of trypanosomes. The features considered typical of PEX13s are discussed.

Structured summary of protein interactions:
Pex19 physically interacts with **Pex13.2** by two hybrid (View interaction)
Pex13.1 physically interacts with **Pex13.2** by two hybrid (View interaction)

1. Introduction

Trypanosoma brucei is a parasitic protist responsible for sleeping sickness, a potentially fatal disease of humans in sub-Saharan Africa, and a similar disease called nagana of livestock animals. It lives in the bloodstream and other extracellular fluids of its mammalian host and is transmitted by the bite of tsetse flies. These parasites belong to the class Kinetoplastea, order Trypanosomatida [1] together with the other human pathogens *Trypanosoma cruzi*, responsible for Chagas' disease and *Leishmania* spp., the causative agents of a variety of disorders collectively called leishmaniases.

Trypanosomatids possess peroxisome-like organelles called glycosomes which, however, are unique in the sense that they contain the majority of the enzymes of the glycolytic pathway [2,3]. In other organisms glycolysis is a cytosolic process. It has been shown that this compartmentalization is essential for the viability of the bloodstream form (BF) of the parasite, for which glycolysis is the only source of ATP, and also for the procyclic form (PF), the life-cycle stage of the parasite present in the midgut of the tsetse fly, when in vitro cultured in the presence of glucose. Even a small degree of mislocalisation of glycolytic proteins from the glycosome into the cytosol can cause cell death (reviewed in [4,5]).

Peroxisomal and glycosomal matrix proteins are encoded in the nucleus and synthesized on free ribosomes in the cytosol. The post-translational import of these proteins into the organellar matrix is dependent on an amino-acid sequence motif, a so-called peroxisomal-targeting signal (PTS). More commonly this may be a PTS1, a specific C-terminal sequence such as SKL or three amino acids with similar physicochemical properties. The alternative PTS2 is a more complex nonapeptide sequence found in the N-terminal region of a number of other peroxisomal/glycosomal proteins. Targeting can also be mediated by a polypeptide internal sequence or I-PTS. However, the I-PTS motifs of different proteins do not share a consensus sequence and the means of import of proteins possessing such a PTS has not been unambiguously established: it may, at least in some cases, involve piggy-back transport on a PTS1 or PTS2 containing protein [6,7]. The PTS1 and PTS2 of newly synthesized proteins to be imported are recognized by the cytosolic receptors PEX5 and PEX7, respectively. The receptors, with their cargo-proteins bound, interact with an assembly of proteins associated with the peroxisomal/glycosomal membrane known as the docking complex. This complex comprises PEX13, PEX14 and in yeasts also PEX17. The docking leads to a number of successive conformational changes, protein–protein interactions (reviewed in [5]) and the

Abbreviations: PEX, peroxin; PTS, peroxisomal-targeting signal; RNAi, RNA interference; Tet, tetracycline; BF, bloodstream form; PF, procyclic form

* Corresponding author. Fax: +32 2 7626853.
E-mail address: paul.michels@uclouvain.be (P.A.M. Michels).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.05.019

formation of a transient pore [8] resulting in the delivery of the cargo proteins into the matrix of peroxisomes/glycosomes. After releasing their cargos, the receptors PEX5 and PEX7 are retrieved from the organelle into the cytosol by a ubiquitination dependent process to become available for mediating new rounds of import. The energy required for the import seems to be mainly provided in the later steps of the cyclic process, by ATP that is hydrolyzed by the AAA^+-ATPases PEX1 and PEX6 for the retrieval of the ubiquitinated receptors from the organelles.

PEX13 is part of the docking complex and participates in the import of peroxisomal matrix proteins. It is an integral peroxisomal membrane protein that is only poorly conserved between different organisms. Its N- and C-termini are exposed to the cytosol. Common features in the N-terminal region are a proline-rich portion, often harbouring a KPWE motif, followed by a Gly-rich segment containing multiple YG motifs. The C-terminal region typically contains two membrane-spanning helices followed by a SH3 domain that interacts with PEX5 in yeast.

Peroxisome biogenesis has been studied in a variety of organisms, most notably in yeasts. A combined, not-redundant set of 33 peroxins (PEX) involved in the process have been identified in *Saccharomyces cerevisiae* and other organisms. Research on the biogenesis of *T. brucei* glycosomes has resulted in the identification and characterization of about 10 peroxins involved in matrix import, including PEX13 (reviewed in [4,5]). TbPEX13 shares very low overall sequence identity when compared to its counterparts from other organisms and is unusual in several respects: (1) it lacks the proline-rich motif usually found near the N-terminus; (2) unlike other PEX13s it has at its C-terminus a PTS1-like signal (TKL) that is conserved in all trypanosomatids [9]. The function for this signal is still unknown since the association of this PEX, being a peroxisomal membrane protein, with the glycosomes is supposed not to be dependent on a PTS1 or PTS2, but on another type of signal (a mPTS or membrane peroxisomal-targeting signal) that is recognized by another cytosolic receptor, PEX19. TbPEX13 has been confirmed to be a glycosomal membrane protein, to interact with TbPEX5 and TbPEX14 and to be involved in the import of glycosomal matrix proteins. By RNA interference (RNAi) it was shown to be essential for the survival of both bloodstream and procyclic cells [9].

In this paper we report the identification of another TbPEX13, called TbPEX13.2. It has a higher overall sequence identity to PEX13 from other organisms than does TbPEX13.1, also possesses a YG motif-containing, Gly-rich region and two predicted transmembrane regions, but lacks a SH3 domain and a PTS1. Its glycosomal localization and involvement in glycosome biogenesis were experimentally demonstrated. This is the first report from any species of two distinct, very different PEX13 isoforms being involved in the biogenesis of organelles of the peroxisome family.

2. Materials and methods

2.1. Bioinformatics

Iterative database searches were done with the jackhmmer program of the HMMER3 suite [10] or PSI-BLAST [11] in the UniProt database [12], varying inclusion thresholds – 1×10^{-3}, 1×10^{-6} or 1×10^{-9} – but leaving other parameters as default values. Motif searches were done with ps_scan [13], transmembrane helices predicted with TM-HMM [14], and alignments visualized and manipulated with Jalview 3 [15].

2.2. Trypanosome growth

Bloodstream-form (BF) *T. brucei* cells of strain Lister 427, cell line 90–13 [16] possessing a chromosomically integrated T7 polymerase gene and the tetracycline (Tet) repressor gene from *Escherichia coli* were cultured in HMI-9 medium containing 10% foetal calf serum (Gibco) and 2.5 μg/mL G418 (Gibco). The cells were incubated at 37 °C with 5% CO_2.

Procyclic-form (PF) cells of *T. brucei* Lister 427, cell line 449 [17] with the *E. coli* Tet repressor gene integrated in its genome were cultured in SDM-79 medium [18] containing 15% foetal calf serum and 0.5 μg/mL phleomycin (Cayla). PF trypanosomes of cell line 29–13 [16] were cultured in SDM-79 containing 15% foetal calf serum, 50 μg/mL hygromycin (Sigma–Aldrich) and 15 μg/mL G418. Cells were incubated at 28 °C with 5% CO_2.

2.3. Preparation, cloning and expression of DNA constructs

A construct was made with the trypanosome-specific pGC1 vector that allows the expression of an N-terminally GFP-tagged protein upon induction with Tet [19]. To that end the *PEX13.2* gene was amplified by PCR using the primers 5′-TTAAGCTTTT*ATG*TCGGCAGCATGG-3′ (forward) and 5′-AAGGATCC*CTA*ACGAGTAATCCTTTTT-3′ (reverse); the *Hind*III and *Bam*HI restriction sites are underlined and the start and stop codons are italicized in the primer sequences. The amplification conditions were: first a DNA denaturing step at 95 °C for two min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for one min with a final extension step of 72 °C for five min. The amplified DNA was verified by sequencing (Macrogen, South-Korea). Clones of transfected PF cells having the construct incorporated in the genome were selected for blasticidin (Invitrogen) resistance. For fluorescence microscopy experiments GFP-tagged PEX13.2 was expressed by addition of 5 μg/mL Tet to the medium for 24 h.

Another trypanosome-specific construct was created using the vector p2T7–177 to generate RNAi against PEX13.2 in BF cells. The same amplified DNA was used to place the gene between two opposite Tet-inducible T7 promoters to generate two complementary RNA strands that will direct the degradation of mRNA matching the sequence and consequently the depletion of the protein from the cells. Positive clones were selected by resistance to phleomycin. RNAi was induced by the addition of Tet (1 μg/mL) to the medium.

2.4. Confocal fluorescence microscopy

For acquisition of images a Cell Observer Spinning Disk microscope was used. Poly-L-lysine was used to coat the slides. Trypanosomes were fixed with 4% formaldehyde and permeabilized with 1% Triton X-100. Anti-PEX11 (gift from F. Vonken), a glycosomal membrane protein, was used at a 1:5 dilution (in PBS containing 2% BSA) to assess for colocalization with GFP-tagged PEX13.2. Secondary antibody Alexa 568 anti-rabbit IgG (Molecular Probes) were used at a dilution of 1:800. Mowiol was used as the mounting medium.

2.5. Subcellular location of proteins by selective membrane permeabilization with digitonin

To study the effects of depleting PEX13.2 on the subcellular distribution of proteins, BF trypanosomes possessing an integrated RNAi p2T7-177-*PEX13.2* construct were grown in the presence or absence of Tet (1 μg/mL in DMSO). After 24–48 h samples were collected and the total protein concentration was determined. Samples of trypanosomes, each corresponding to 100–200 μg of total protein, were treated with the detergent digitonin. All samples were exposed for the same period, 4 min, to digitonin, each at a different concentration of the detergent. This allowed the permeabilization of the different membranes of the cells (plasma, glycosomal and mitochondrial membrane, successively) each to a different extent, according that membrane's sterol content. The incubation was then stopped by centrifugation (2 min, 13,000×*g*)