Short communication

An internal sequence targets *Trypanosoma brucei* triosephosphate isomerase to glycosomes

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ABSTRACT

In kinetoplastid protists, glycolysis is compartmentalized in glycosomes, organelles belonging to the peroxisome family. The *Trypanosoma brucei* glycosomal enzyme triosephosphate isomerase (TPI) does not contain either of the two established peroxisome-targeting signals, but we identified a 22 amino acids long fragment, present at an internal position of the polypeptide, that has the capacity to route a reporter protein to glycosomes in transfected trypanosomes, as demonstrated by cell-fractionation experiments and corroborating immunofluorescence studies. This polypeptide-internal routing information seems to be unique for the sequence of the trypanosome enzyme: a reporter protein fused to a *Saccharomyces cerevisiae* peptide containing the sequence corresponding to the 22-residue fragment of the *T. brucei* enzyme, was not targeted to glycosomes. In yeasts, as in most other organisms, TPI is indeed exclusively present in the cytosol. These results suggest that it may be possible to develop new trypanocidal drugs by targeting specifically the glycosome import mechanism of TPI.

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Trypanosoma brucei, like all other kinetoplastid organisms, possesses peroxisome-like organelles which, by reference to their special feature of harbouring a major part of the glycolytic pathway, are called glycosomes. Glycolysis is essential for trypanosomes living in the bloodstream of the mammalian host, because it is their sole source of energy [1]. Compartmentalization of glycolytic enzymes inside glycosomes is vital for the parasite as was shown in several studies in which mislocalization of some glycolytic enzymes resulted into parasite death [2–4]. Therefore, both glycolysis and the process of their compartmentalization, i.e. glycosome biogenesis, have been proposed as potential targets for new trypanocidal drugs [5]. Indeed, the diseases caused by these parasites are fatal if untreated, but available drugs are expensive, toxic and have become increasingly inefficient due to the development of resistance.

Genes of glycosomal and peroxisomal proteins are encoded in the nucleus necessitating organellar protein import in a post-translational fashion. This import requires a routing signal. Two main topogenic signals (peroxisome-targeting signal or PTS) that direct the matrix proteins to peroxisomes or glycosomes have been described and are well conserved between species. Most of these proteins use a PTS1, a tripeptide motif present at their C-terminus and which is recognized by the C-terminal tetratricopeptide repeat (TPR) domain of a cytosolic peroxisome-import receptor called peroxin 5 (PEX5). In general, at the C-terminal (−1) position a large hydrophobic residue is found, whereas at the positions −2 and −3, respectively, a positively charged residue and a small uncharged residue are present. The general consensus sequence of PTS1 is (S/C/A)-(K/R/H)-(L/M). The PTS2 is a degenerate nonapeptide [(R/K)-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/A)] that seems to be relatively conserved among species and resides near the N-terminus of the matrix proteins to be imported [6]. This signal is recognized by the receptor peroxin 7 (PEX7). In trypanosomatids, two glycolytic enzymes, hexokinase and aldolase, possess such a signal.

For some peroxisomal matrix proteins the consensus PTS1 or PTS2 motif appears absent. Instead, they are imported upon recognition of a polypeptide-internal signal (I-PTS). Such signals have been described for acyl-CoA oxidase from several yeast species [7–9]. In trypanosomes, a minor phosphoglycerate kinase isoen-

zyme (PGK-A) was shown to be targeted to glycosomes via a polypeptide-internal signal [10]. Triosephosphate isomerase (TPI), although present in glycosomes, is devoid of PTS1 and PTS2 motifs, raising the question whether it also might possess an internal routing signal. TPI is, in nearly all organisms analyzed, a dimeric protein with identical subunits of approximately 27 kDa. The crystal structure of *T. brucei* TPI has been determined [11]. The role of this enzyme in glycolysis is the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, ensuring the complete catabolism of the entire hexose unit rather than just half of it. Here we report our work on the identification of the polypeptide-internal region responsible for TPI targeting to the glycosome.

To determine the amino-acid sequence responsible for the import of *T. brucei* TPI, we transiently transfected procyclic trypanosomes with constructs coding for different segments of the TPI polypeptide fused to the reporter protein chloramphenicol acetyltransferase (CAT). The overall levels of CAT signal in the different clones were approximately equivalent, as determined by a quantitative CAT-ELISA assay. The intracellular localization of the different fusion proteins was determined after separation of the cytosolic and organellar fractions as described in [12]. To that end, trypanosomes were treated with 0.1 mg of digitonin per mg of protein, a concentration that only permeabilizes the plasma membrane but not the glycosomal membrane (and other intracellular membranes such as that of the mitochondrion). After centrifugation, in cells expressing the unmodified CAT reporter, the protein was detected almost exclusively in the cytosol (Fig. 1A). As a positive control, we used the reporter protein extended with the C-terminal tripeptide (-AKL, a prototypical PTS1) of the glycosomal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *T. brucei*. Approximately 60% of the CAT-AKL protein was detected in the organelles-containing pellet fraction indicating import into glycosomes. Similarly, when the entire ORF of TPI was fused N-terminally to CAT, the fusion protein was targeted to organelles. A construct coding for the first 129 amino acids of TPI fused to CAT was not imported but the import could be restored when the N-terminal region was extended to the first 173 amino acids of TPI. When the last 119 amino acids of TPI were fused to CAT [T(132–250)C] the protein was also efficiently routed to the organelles. By making constructs for several C-terminally truncated proteins we could determine that a sequence of 45 amino acids from residue 132 to 176 is important for targeting of TPI to glycosomes. To further define the targeting region, a fragment of 22 amino acids, delimited by residues 140 and 161, was fused to the N-terminus of CAT. This fusion protein was also predominantly found in the glycosome-enriched fraction. Importantly, a fragment containing the corresponding amino acids from *Saccharomyces cerevisiae* TPI, a cytosolic protein, fused to CAT was not at all imported into *T. brucei* glycosomes. Conversely, expression of *T. brucei* TPI in the heterologous host *Hansenula polymorpha* revealed a cytosolic localization of the protein (J. Kiel, M. Veenhuis and SdW, data not shown). We therefore conclude that the heterologous TPI was not imported into *H. polymorpha* peroxisomes, whereas for other glycolytic enzymes of *T. brucei* such as GAPDH and aldolase, that possess a PTS1 and a PTS2, respectively, import was observed (data not shown). These findings suggest that the import of TPI is dependent on specific features present in the trypanosome sequence that are not recognized by a heterologous host because either the targeting information is too divergent or the partner for interaction is absent or too different.

To confirm our results, immunolocalization studies were performed using bloodstream-form cell lines stably expressing some of the same fusion constructs as were used in the import/cell-fractionation studies of procyclic cells shown in Fig. 1A. Two positive controls were used, the reporter protein fused to the GAPDH PTS1 (CAT-AKL) and the full-length TPI fused to CAT (TC). The subcellular localization of the recombinant proteins in *T. bru-*

cei was determined by immunofluorescence microscopy using an antibody directed against CAT (red labeling) and an Alexa Fluor 488 coupled antibody (green labeling) against aldolase, serving as a glycosomal marker. A typical punctate pattern and co-localization could be observed for both CAT-AKL and TC with aldolase indicating glycosomal import of the respective fusion proteins (Fig. 1B, panels a and b). However, for the construct where the first 129 amino acids of TPI were fused to CAT [T(1–129)C] the typical punctate pattern was absent, and instead staining was found throughout the entire body of the parasite. The lack of co-localization between aldolase and CAT [T(1–129)C] corroborates the cytosolic localization of this fusion protein (Fig. 1B, panel c). In contrast, when the 22 amino acids long peptide, containing the targeting signal as identified in the cell-fractionation experiments, was fused to CAT, some punctate structures that co-localized with aldolase were observed (Fig. 1B, panel d). A minor part of this fusion protein did however not co-localize with aldolase and was found in the cytosol. These results are in agreement with those of the experiment of Fig. 1A, where not more than 60% of the various TPI–CAT fusion proteins, including T(140–161)C, were detected in the glycosome-enriched pellet fraction. This incomplete compartmentalization may be due to a decreased efficiency of the glycosome-routing signal caused by a non-native conformation of the TPI peptide when fused to the reporter protein.

To further analyze the identified 22 amino acids long *T. brucei* TPI import signal, we aligned the sequence with the corresponding region of TPIs from other species. As shown in Fig. 2A, the sequence is well conserved between different kinetoplastids (*T. brucei*, *Trypanosoma cruzi*, *Leishmania major* and *Leishmania mexicana*) in which TPI is imported into glycosomes. However, alignment with non-kinetoplastid species like *S. cerevisiae*, *Arabidopsis thaliana* and mammals, where TPI is typically a cytosolic protein, reveals a low degree of conservation, comparable with that of the overall sequence (on average about 50%), except for five residues highlighted in the figure by a black background. Moreover, the kinetoplastid sequences appear to contain some additional residues not present in other species. To investigate further which residues are involved in glycosome-targeting, specific amino acids were mutated. In a first mutated peptide, the non-charged residues L144 and T145 (residues indicated by arrows in Fig. 2A) were substituted by the charged amino acids E and R, respectively, found at the corresponding positions in the yeast sequence. These substitutions were chosen because the corresponding fragment of the yeast protein was unable to direct the reporter protein CAT to the glycosomes (as shown in Fig. 1A). However, these mutations did not affect glycosomal import of the reporter protein fused to TPI (data not shown). Similar results were obtained for another mutated form from which both the amino acids K156 and A157, which contribute to a two-residues specific insertion in kinetoplastid TPIs, were deleted, and by substitution of a lysine residue (indicated by a dashed arrow in Fig. 2A), conserved in kinetoplastids, into a glutamate as present in the yeast sequence (K153E). The import efficiency of this latter mutated protein was also not significantly decreased (data not shown). Hence, mutagenesis of specific residues in the 22 residues-long fragment did not allow us to identify more specifically which amino acids play a crucial role in the glycosome-routing process. Moreover, no sequence similarity could be observed between the 22 amino-acid fragment of TPI and the 68 residues-long fragment that contains the glycosome-targeting information for PGK-A [10].

In the three-dimensional structure of *T. brucei* TPI [11], the 22 amino-acid fragment contributes to the formation of α-helix no. 5 and a loop followed by a 3_{10}-helix (Fig. 2B). The α-helix no. 5 is a structurally conserved element of TPI proteins. The loop and the 3_{10}-helix connect this α helix with β strand no. 6 that is buried in the protein structure. As shown in Fig. 2B several residues