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Function of N-terminal import signals in trypanosome microbodies

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Abstract The glycosomes of trypanosomes are related to eukaryotic peroxisomes. For many glycosomal and peroxisomal proteins, a C-terminal SKL-like tripeptide known as PTS-1 serves as the targeting signal. For peroxisomes, a second N-terminal signal (PTS-2) was demonstrated on rat 3-ketoacyl-CoA thiolase. Several glycosomal proteins do not bear a PTS-1. One such protein, fructose bisphosphate aldolase, has a PTS-2 homology at its N-terminus. To find out whether the PTS-2 pathway exists in trypanosomes, we expressed chloramphenicol acetyltransferase fusion proteins bearing N-terminal segments of either rat thiolase or trypanosome aldolase. The mammalian PTS-2 clearly mediated glycosomal import. The aldolase N-terminus mediated import with variable efficiency depending on the length of the appended sequence. These results provide evidence for the existence of the PTS-2 pathway in trypanosomes.

Key words: Glycosome; Protein import; N-Terminal signal sequence; *Trypanosoma brucei*

1. Introduction

Microbodies are found in all but the most primitive eukaryotes. They have a single bounding membrane typically surrounding a small subset of the cell's enzymatic pathways; which pathways are present, and to what degree varies considerably both between and within species, according to the growth conditions and developmental stage. In trypanosomatid protozoa, the first seven enzymes of glycolysis are compartmentalised in such a microbody, the glycosome. It is believed (though unproven) that this unique compartmentation contributes to the very high glycolytic rate of bloodstream form trypanosomes, which rely solely on glycolysis for their energy supply [1]. Glycosomes are morphologically [2], enzymatically [3] and evolutionarily [4] related to peroxisomes and glyoxysomes present in yeast, insects, mammals and plants.

Import of peroxisomal matrix proteins occurs post-translationally [5]. Many contain at their C-termini the PTS-1 (peroxisomal targeting signal 1): either −SKL or a related sequence [6]. Results from experiments using permeabilised cells [7], together with analysis of yeast mutants, suggest that PTS-1 is recognised on fully folded proteins by a soluble cytoplasmic receptor, the product of the *pas 8* (*Pichia pastoris*) [8] or *pas 10* (*Saccharomyces cerevisiae*) [9] genes. Yeast PTS-1 receptor mutants, however, are still able to incorporate 3-ketoacyl-CoA thiolase into their peroxisomes, implying not only that PTS-1-type proteins are not an essential part of the peroxisomal import apparatus (at least for non-PTS-1 proteins), but also that another type of import signal exists. This signal, PTS-2, is located at the N-terminus of thiolase; a homologue has also been identified on watermelon malate dehydrogenase [10]. The *pas 7* mutants of *S. cerevisiae*, which fail to import thiolase but still import PTS-1 proteins [11], are deficient in a protein that is a good candidate for a PTS-2 receptor [12]. Mutants of both PTS-1 (complementation group I) and PTS-2 (group IV) type are also found among human Zellweger syndrome cells [13]. Peroxisomal membrane proteins, which lack both types of signal, are presumably inserted by a separate pathway [14].

The presence of PTS-1-type signals has previously been demonstrated on many glycosomal proteins, although the spectrum of tolerated amino acid substitutions appears to be rather enlarged in comparison to other 'higher' eukaryotes [15,16]. As trypanosomatids are evolutionarily the earliest-branching eukaryotes to contain microbodies [17], it is of considerable interest to determine to what extent the import apparatus is conserved. Here we provide evidence that the PTS-2 pathway is present in trypanosomes, indicating that it has been conserved throughout evolution.

2. Materials and methods

2.1. Plasmid constructions

To construct the expression vector pAldE a PCR was performed with pAld17 [18] as template. The primers CZ031 5′TTCACAAGCTTCACAATGTCCAAGCGTGTTGAAGTT3′ and CZ049 5′ATATCCAGTGATTTTTTTCTCGAGCGCTTCATATGGCGTCTTCAG3′ (restriction sites underlined) were used to amplify a fragment encoding the 24 N-terminal amino acids of aldolase joined to the first six amino acids of CAT starting with glutamic acid (the amino acid immediately following the initiator methionine). pJP44 [19] was used as template in a second PCR to amplify the CAT gene with the primers CZ030 5′CTGAAGACGCCATATGAAGCGCTCGAGAAAAAAATCACTGGATAT3′ and CC12 5′GTTTCGTTCCTCCGAGGCGC3′. The product is a CAT coding region that can hybridise to the first via a junction segment containing an *Xho*I site. The full-length hybrid gene was produced in a third PCR using the first two products as templates and the primers CZ031 and CC12. The hybrid gene contains a unique *Xho*I site (encoding a leucine residue) at the junction point between the aldolase and CAT sequences. It was digested with *Hin*dIII and *Bam*HI and cloned into pJP62 [20] cut with the same enzymes. The vectors pHD436, pHD438 and pHD456 were generated by PCR amplification of aldolase 5′ fragments with genomic DNA of the AnTat1.1 strain as template, using the 5′ primer CZ031 and 3′ primers CZ354 5′GGCTACTCGAGACCCTTACCGGGGGC3′, CZ365 5′GGCTACTCGAGTTGGGTAAGCAGAAC3′ and CZ353 5′GGCTACTCGAGCTCCGCTTCATATGG3′ respectively, followed by cloning of the amplified fragments into pAldE via their *Hin*dIII and *Xho*I sites. Thiolase-CAT derivatives were excised as *Hin*dIII–*Bgl*II fragments from previously described pRSV-FLT-CAT vectors [10] and cloned into trypanosome expression vectors cut with *Hin*dIII and *Bam*HI. The FLT3-CAT was subcloned into pAldE (pHD448). The FLT4-CAT N-terminus was first transferred to pRSV-FLT3-CAT as a *Hin*dIII–*Xho*I fragment, then transferred to pAldE

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(pHD443). The FLT5-CAT hybrid was cloned into pHD356 (pHD377). pHD356 was obtained (H.-R. Hotz, ZMBH, unpublished) by inserting the Klenow treated *SmaI–Asp*718 fragment containing the rRNA promoter from pHD118 [21] into the filled *Xho*I site of pHD330 [22]. To generate the plasmids encoding the aldolase N-terminus fused to CAT via the same four amino acid linker as is found in the thiolase-CAT constructs, the 5′ aldolase fragment was amplified by PCR, with genomic DNA of the AnTat1.1 strain as template, using the 5′ primer CZ031 and the 3′ primers CZ373 5′GGCTACTCGAGAGTTGGGTAAGCAG3′ (pHD442) or CZ378 5′GGCTACTCGAGAGGCGGTTGTACGCAGGG3′ (pHD457). The amplified fragments were digested with *Hin*dIII and *Xho*I for cloning into pRSV-FLT3-CAT, and the resulting hybrid genes transferred to pAldE as before.

2.2. Trypanosome transfection, cell lines, subcellular fractionation and immunofluorescence

All methods for analysis of compartmentation of fusion proteins were exactly as previously described [16]. For the transient transfections 10–20 μg DNA were electroporated in duplicate into procyclic forms of the AnTat1.1 strain, the cells digitonin-fractionated the next day and the CAT activity of the fractions measured (3–10 independent experiments per DNA). In one case (pHD377) a permanently transformed trypanosome cell line was used; cells were transformed with 100 μg DNA linearised at a *Not*I site for insertion into the tubulin locus, and selected with hygromycin as described [22]. For this cell line four independent CAT measurements were performed.

For further analysis of crude glycosomal fractions the digitonin pellets were centrifuged through a 35% sucrose cushion and the resulting pellets subsequently fractionated on a 3.2 ml 30–60% sucrose gradient with a cushion of 70% sucrose (45,000 rpm for 90 min at 4°C in a swing-out rotor). 330 μl fractions were taken from the top of the gradient and CAT (100 μl) and GPDH (200 μl) of each fraction were assayed as described before [20].

3. Results

3.1. Identification of an aldolase PTS-2 signal

Trypanosome fructose bisphosphate aldolase is a glycosomal enzyme yet lacks a PTS-1 signal. Indeed, the presence of a C-terminal signal may be precluded by strict catalytic requirements at the extreme C-terminus [23]. Comparison of trypanosome aldolase with mammalian aldolases (which are of course cytoplasmic) revealed the presence of a trypanosome-specific N-terminal extension [18,24]. An alignment of this sequence with known or predicted PTS-2 sequences is shown in Fig. 1. Mutagenic analysis has demonstrated that non-conservative changes in the residues at positions 4 (Arg), 5 (small, non-polar) and 12 (Leu) can severely reduce peroxisomal targeting [25]. These residues, together with others, are conserved in the trypanosome aldolase N-terminus, which completely conforms to the PTS-2 consensus. We therefore set out to see both whether trypanosome glycosomes would accept a mammalian PTS-2, and whether the aldolase N-terminus had the predicted function. This was done by expressing chloramphenicol acetyltransferase (CAT) fusion proteins bearing either rat thiolase or trypanosome aldolase sequences at the N-terminus.

3.2. Function of the PTS-2 in glycosomal targeting

Trypanosomes were transfected with plasmids designed to express a variety of different fusion proteins and the compartmentation of CAT measured the next day by digitonin fractionation. Results are shown in Fig. 2. In cells expressing CAT without signal (pJP44) very little enzyme was detected in the glycosomes, whereas in the positive control (cells expressing CAT containing a PTS-1 (pJP62)) over 80% of the CAT activity was associated with the glycosomes. In contradiction to a recent report [26], in cells expressing the thiolase-CAT fusion proteins approximately 70–80% of the enzyme activity was associated with the glycosomes (pHD443, pHD448). In fact, the results for glycosomal import of thiolase fusion proteins paralleled those observed for peroxisomal import of the same proteins in mammalian cells [10]. Thus, both the first 15 or 11 amino acids were sufficient for import, but the 11 amino acid prepiece seemed to be a slightly less efficient targeting signal than the 15 amino acids; and the first five amino acids were no longer able to mediate glycosomal targeting (pHD377). These results show that trypanosome glycosomes have an import system that recognises mammalian PTS-2 signals.

Experiments using aldolase fusion proteins gave less clear-cut results. Maximal import was seen with a construct bearing the first 18 amino acids of aldolase, joined to the CAT initiator methionine via a four amino acid bridge (SSGT) of the same sequence as that used in the thiolase constructs (pHD457). Import of this protein, with about 30%, was at a level similar to that observed using a minimal C-terminal tripeptide signal from trypanosomal phosphoglycerate kinase, −SSL [16]. Inclusion of either more (pAldE, pHD436, pHD456) or fewer aldolase residues (pHD438, pHD442) clearly diminished import. Nevertheless, the latter constructs, including the likely minimal signal (up to the conserved leucine residue), gave import that was significantly above background levels. The presence or absence of the four amino acid linker and the CAT initiator methionine did not much alter the import efficiency of the otherwise identical fusion proteins.

The fact that the fusion proteins are actually associated with glycosomes was corroborated by the transient transfection of trypanosomes with either pHD448 (thiolase-CAT) or pHD457 (aldolase-CAT) and subsequent fractionation of the digitonin pellet on a sucrose gradient. In both cases the pelleted CAT co-fractionated with the glycosomal marker glycerol phosphate

	1												12							
MSESVGRTSAM	-	H	R	L	Q	V	V	L	G	H	L	-	A	G	R	P...	Rat thiolase A
M	-	H	R	L	Q	V	V	L	G	H	L	-	A	G	R	S...	Rat thiolase B
M	-	Q	R	L	Q	V	V	L	G	H	L	-	R	G	P	A...	Human thiolase
M	S	Q	R	L	Q	S	I	K	D	H	L	-	-	-	V	L...	*S. cerevisiae* thiolase
M	-	D	R	L	N	Q	L	S	G	Q	L	-	-	-	-	-...	*C. tropicalis* thiolase
M	-	D	R	L	N	N	L	A	T	Q	L	-	-	-	-	-...	*Y. lipolytica* thiolase
MQPIPDV	N	Q	R	I	A	R	I	S	A	H	L	H	P	P	K	S...	watermelon malate dehydrogenase
M	S	K	R	V	E	V	L	L	T	Q	L	P	A	Y	N	R...	*T. brucei* aldolase
		±	R	sn		sn			±	L						...	Consensus
			*	*						*							

Fig. 1. Sequence alignments of proven or putative PTS-2 signals. Universally conserved amino acids are in outline type and partially conserved in bold type. ± stands for polar; sn for small neutral.

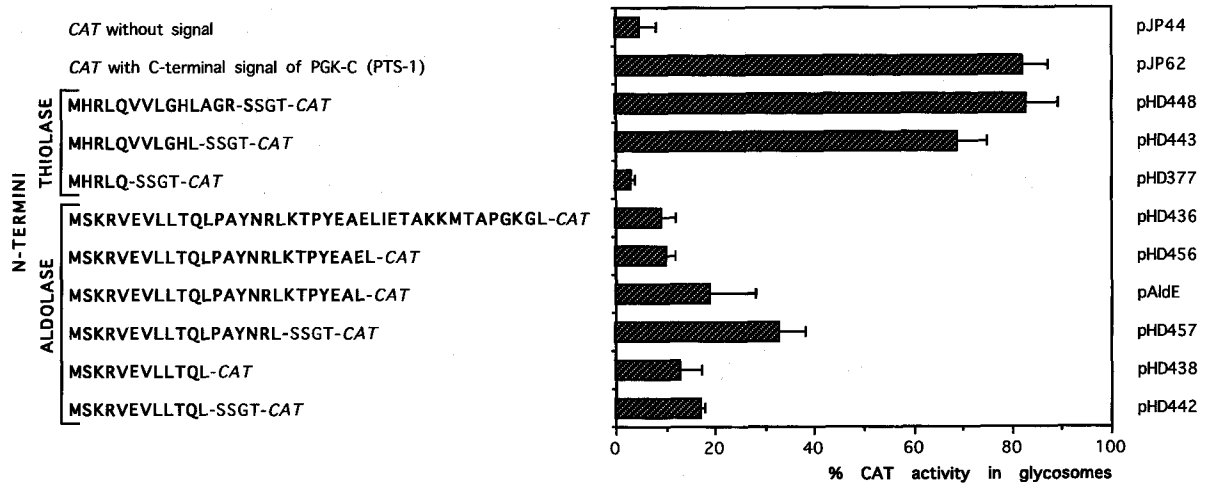

Fig. 2. Import of hybrid CAT proteins into glycosomes. The percentage of CAT activity found in glycosomes after transient transfection and digitonin fractionation is expressed as the mean ± S.D. for at least three independent experiments. For the stable cell line pHD377 the mean ± S.D. is shown for four independent measurements. The sequences joined to CAT are indicated on the left and plasmid numbers on the right. In the hybrid proteins consisting of an N-terminal fragment of thiolase or aldolase fused to CAT via a four amino acid linker, CAT starts with a methionine; in those without the linker CAT starts with glutamic acid.

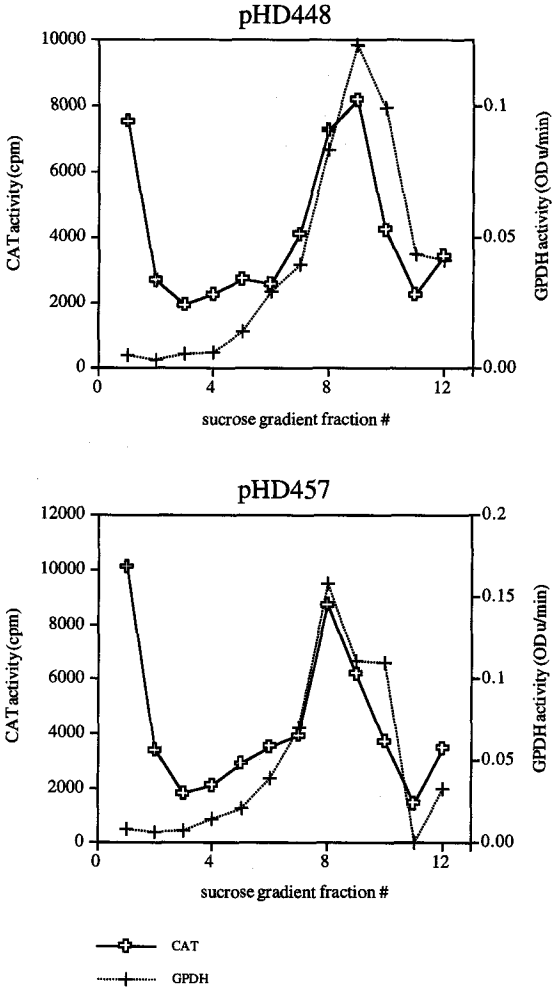

Fig. 3. Co-migration of a thiolase-CAT (pHD448) and an aldolase-CAT (pHD457) fusion protein with the glycosomal marker enzyme GPDH. After transient transfection, the digitonin pellet was fractionated by sucrose gradient centrifugation. CAT and GPDH activity of the fractions are shown.

dehydrogenase (GPDH) (Fig. 3). The CAT activity at the top of the gradient is also seen when the pellet fraction of trypanosomes transfected with CAT without a glycosomal signal is assayed [20]. It is presumably due to a combination of leakage and material adhering to the outside of the organelles.

3.3. Immunolocalisation of the PTS-2-CAT fusion proteins

To rule out the possibility that the glycosomal association of the fusion proteins was based on an external attachment to the organelle, the fractionation results were confirmed by indirect immunofluorescence. Cells transiently expressing CAT (pJP44) yielded bright cytoplasmic fluorescence that was visible whether the cells were permeabilised with TritonX-100 (not shown) or digitonin (Fig. 4a), whereas CAT-PTS-1 (pJP62) showed a punctate pattern characteristic of glycosomes that was only detectable after Triton permeabilisation (Fig. 4b) [16]. The thiolase-CAT protein was also normally located within glycosomes (Fig. 4c, pHD443; data for pHD448 not shown). Staining of this PTS-2-CAT was possible after permeabilisation of the fixed cells with Triton but not after permeabilisation with digitonin, confirming that the protein was within the organelle, not stuck to the outside. Curiously, however, thiolase-CAT was also occasionally seen in the cytoplasm (Fig. 4d). In such cells, punctate fluorescence was seen over a background of fainter cytoplasmic fluorescence; the cytoplasmic protein was only accessible after digitonin treatment (Fig. 4e). Clearly this cytoplasmic CAT cannot have represented a significant proportion of the total enzyme activity or this would have been reflected in the digitonin fractionation results. The same phenomenon has been described for mammalian cells expressing the minimal PTS-2 fused to CAT, where in addition to punctate fluorescence some cytosolic fluorescence was detected in many cells [10].

The tendency for dual localisation was much stronger for the aldolase-CAT constructs (Fig. 4f) (pHD442); here, background cytoplasmic fluorescence was common, reflecting the results of the cell fractionation. Nevertheless, it was clear that import of CAT could be mediated by the aldolase N-terminal signal.

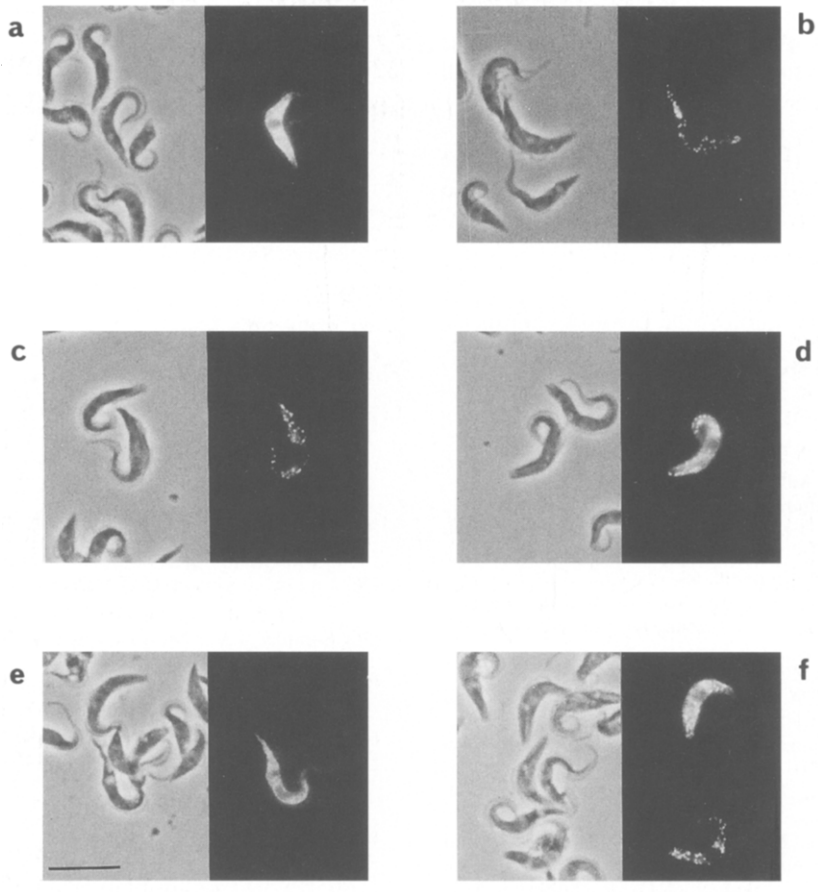

Fig. 4. Immunofluorescent staining of transiently transfected trypanosomes expressing thiolase-CAT or aldolase-CAT hybrid proteins. Phase illumination (left panel) and fluorescein immunofluorescence pictures (right panel) are shown for trypanosomes stained with a monoclonal anti-CAT antibody after permeabilisation with Triton or digitonin. Bar = 10 μm. (a) CAT (pJP44), digitonin-permeabilised. (b) CAT-PGK (pJP62), Triton-permeabilised. (c) Thiolase-CAT (pHD443), Triton-permeabilised. (d) Thiolase-CAT (pHD443), Triton-permeabilised. (e) Thiolase-CAT (pHD443), digitonin-permeabilised. (f) Aldolase-CAT (pHD442), Triton-permeabilised.

We made several different attempts to express an N-terminally truncated version of aldolase, by either transient transfection or generation of permanent cell lines. However, the predicted product was never detectable. Therefore we were not able to determine whether the aldolase PTS-2 is absolutely required for the glycosomal targeting of the protein, or whether additional signals are also present.

4. Discussion

Our experiments demonstrate clearly that PTS-2-type peroxisomal targeting signals exist and can function in *Trypanosoma brucei*. However, the functioning of the aldolase signals we tested was not as efficient as that of PTS-2 signals in mammalian cells. Cytoplasmic localisation was observed both by cell fractionation and immunofluorescence.

Rat peroxisomal thiolase B is synthesised as a larger precursor with an N-terminal extension of 26 amino acids which is proteolytically processed after import into the organelle [27,28]. Processing of thiolase hybrid proteins, however, is undetectable in CV-1 cells [10], and either inefficient or undetectable in CHO cells [29]; also, watermelon malate dehydrogenase is not processed when expressed in *Hansenula polymorpha* [30]. In addition, all glycosomal proteins, including aldolase, are synthesised as their mature size [31]. The thiolase-CAT fusion proteins that we expressed in trypanosomes do not include the cleavage point so no cleavage is to be expected.

Cytoplasmic fluorescence is never seen in trypanosomes expressing CAT with a fully functional PTS-1, whereas it is not infrequent for thiolase-CAT and very common for aldolase-CAT. There are three possible explanations for this. One possibility is that the PTS-2 sequences do not function well as a consequence of joining to CAT in the artificial fusion proteins. Especially for the aldolase-CAT constructs, it seems quite likely that some conformational effects may be reducing the accessibility (or recognisability) of the signal sequence, as alterations of the length of the N-terminal sequences used had marked effects on import efficiency. Secondly, at least for aldolase, it is possible that two sequences are necessary for import. These two, non-exclusive explanations seem to us most probable, as we have also seen dual location for weakened PTS-1 signals, such as −SSL.

A third possibility is that we are actually observing 'overflow' from the glycosomes into the cytoplasm, because we have saturated the available PTS-2 receptors. As very few PTS-2 proteins have been found, it is quite conceivable that the pool of available receptors is also lower than that for PTS-1. Saturation of PTS-2 receptors would presumably lead to exclusion not

only of the hybrid protein but also of newly synthesised protein, from the glycosome. Only expression of various levels of the proteins in an inducible system (still under development) will answer this question.

In conclusion, PTS-2-type signals are recognised in trypanosomes, implying that the receptor is also present. Aldolase features an N-terminal PTS-2 signal but other factors may also promote its import.

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