Glycosome Assembly in Trypanosomes: Variations in the Acceptable Degeneracy of a COOH-terminal Microbody Targeting Signal

Judith Blattner, Bart Swinkels,* Heinz Dörsam, Terry Prospero, Suresh Subramani,* and Christine Clayton

Zentrum für Molekulare Biologie, Im Neuenheimer Feld 282, Postfach 106249, D-6900 Heidelberg 1, Germany; and *Department of Biology, University of California at San Diego, La Jolla, California 92093

Abstract. Trypanosomes compartmentalize most of their glycolytic enzymes in a peroxisome-like microbody, the glycosome. The specificity of glycosomal targeting was examined by expression of chloramphenicol acetyltransferase fusion proteins in trypanosomes and monkey cells. Compartmentalization was assessed by cell fractionation, differential detergent permeabilization, and immunofluorescence. The targeting signal of trypanosome phosphoglycerate kinase resides in the COOH-terminal hexapeptide, NRWSSL; a basic amino acid is not required. The minimal targeting signal is, as for mammalian cells, a COOH-terminal tripeptide related to -SKL. However, the acceptable degeneracy of the signal for glycosomal targeting in trypanosomes is considerably greater than that for peroxisomal targeting in mammals, with particularly relaxed requirements in the penultimate position.

THE trypanosomatid protozoa compartmentalize most of their glycolytic enzymes in a microbody, the glycosome (27). The glycosome is clearly evolutionarily related to the peroxisomes and glyoxysomes found in yeast, insects, mammals, and plants. All have similar morphology and behavior in density gradients, and have a subset of enzymes in common (3). However, only the glycosome contains glycolytic enzymes. While growing in their mammalian host, the pathogenic African trypanosomes are completely dependent upon glycolysis and substrate-level phosphorylation for their energy supply, suggesting glycolysis and glycosome assembly as a promising target for chemotherapy (10).

The uptake of proteins into glycosomes and peroxisomes mostly occurs within 5 min of protein synthesis, and is in general (although there may be exceptions) not coupled to any obligatory posttranslational processing (3, 21). Various variants of the COOH-terminal peptide (serine-lysine-leucine) (SKL)[1] are capable of directing foreign proteins into the peroxisome-like organelles of mammalian, trypanosome, insect, yeast, and plant cells (11, 14, 15) and the microbodies of all these species are labeled by an SKL-specific antibody (18). However, many peroxisomal and glycosomal proteins lack a COOH-terminal SKL-like signal.

African trypanosomes have three genes encoding phosphoglycerate kinase (PGK) (12, 28). One (PGK-B) is a cytoplasmic PGK, and another (PGK-A) is a minor glycosomal enzyme that has an internal 80-amino acid insertion relative to the cytoplasmic PGK (2, 22, 36). The third PGK (PGK-C) is the major glycosomal enzyme. It has a 20-amino acid COOH-terminal extension that is capable of targeting chloramphenicol acetyltransferase to glycosomes (11). The final three amino acids of this COOH-terminal extension are -SSL, which has been shown to be inactive in targeting luciferase to peroxisomes of monkey kidney cells (14). This suggested to us that there might be differences between glycosomes and peroxisomes in their signal recognition properties. To characterize these differences further, we have undertaken a detailed characterization of the precise amino acid requirements for glycosomal targeting using the PGK and SKL signals.

Materials and Methods

Plasmid Constructions and Expression of Plasmids in Trypanosomes and Monkey Cells

Trypanosome expression plasmids were derivatives of the vectors pJP25 or pJP44 (29). The construction of a plasmid expressing the CAT-PGK hybrid protein (pJP 62) has been described previously (11). The hybrid gene was constructed in such a way that the sequence encoding the COOH-terminal 22 amino acids of glycosomal PGK was joined to the CAT coding region at a unique SacII site; a PstI site downstream of the polyadenylation signal in the vector (pJP 44) was destroyed. The targeting signal sequence contains a unique internal PstI site and there is a unique BamHI site immediately downstream of the termination codon (Fig. 1). CAT-PGK hybrids with altered COOH-terminal signals were therefore constructed by cloning double-stranded synthetic oligonucleotides directly between the above-mentioned restriction sites. To obtain a variety of mutants, oligonucleotides degenerate at a single position (the first base of a codon) were used. However, the use

Bart Swinkels' present address is Royal Gist-brocades N.V., Research and Development, Postbus 1, 2600 MA Delft, The Netherlands.

1. *Abbreviations used in this paper*: PGK, phosphoglycerate kinase; SKL, serine-lysine-leucine.

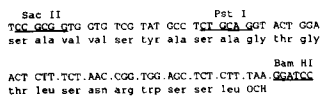

Figure 1. Translated COOH-terminal sequence of the CAT-PGK fusion protein gene, showing the junction with CAT and the restriction sites used in subsequent manipulations. OCH denotes an ochre termination codon.

of mixed oligonucleotides within 6 bases of the SacII site invariably resulted in deletions. The COOH-terminal regions were sequenced using primers hybridizing 30–50 bases upstream or downstream. Oligonucleotides were synthesised by the ZMBH oligonucleotide synthesis facility.

Plasmids expressing variants of the SKL signal were derived from constructs expressing the same proteins in mammalian cells (14, 35). The CAT fusion genes were excised as HindIII–BglII fragments and cloned into the HindIII–BamHI sites of pJP25.

To obtain trypanosomes permanently expressing CAT, we excised the gene, surrounded by the PARP 5′ and 3′ RNA processing signals, from pJP 44 (29). We then ligated the resulting cassette into the XbaI site of pUC Tbneo 3 (37). The plasmid was linearized at the KpnI site before transfection into Antat 1.1 trypanosomes and selection with G418. Attempts to express CAT-PGK with an analogous construct failed. The CAT-PGK gene was therefore cloned between the 5′- and 3′-processing signals of the actin gene, and set downstream of the hygromycin resistance gene under control of the PARP promoter (bNsp-H-α) (23; L. E. Wirtz and C. Clayton, unpublished data). This plasmid was transfected in the form of supercoiled DNA and cells were selected with hygromycin (23).

For expression of CAT-PGK in monkey cells, the dihydrofolate reductase gene in pSV2-DHFR (31) was replaced by the CAT-PGK gene (HindIII–BamHI fragment) using standard procedures to give pSV2-CAT-PGK. The plasmids pSV2-CAT and pSV2-CAT-SKL have been described (14).

DNA Transfection and CAT Assays

DNA was transfected into trypanosomes, and CAT assays were performed as previously described (29). The amount of DNA transfected (10–50 μg per cuvette) was adjusted at least partially to compensate for varying expression levels of the different CAT fusion proteins. DNA transfection of CV-1 cells was done using TransfectAce (Bethesda Research Laboratories, Gaithersburg, MD). In the CAT assay, the level of CAT was assessed from the rate of production of butyryl chloramphenicol over the linear range of the assay. If the postnuclear supernatant sample had a CAT activity of less than four times the background the results were discarded.

CAT immunofluorescence for CV-1 cells was done as described (17) using a commercial anti-CAT antibody (5′-3′ inc.) and FITC-conjugated goat anti-rabbit antiserum from Vector laboratories, Inc. (Burlingame, CA). Trypanosomes were stained after fixation with 3% formaldehyde in PBS (10–15 min). Fixed parasites were preincubated for 15 min with 0.1 M glycine in 0.1 M phosphate buffer, pH 7.2–7.4, before permeabilization for 5 min with either 0.1% Triton X-100 or 3.75 ng/ml digitonin in PBS. Antibodies used were a mouse monoclonal anti-CAT, rabbit polyclonal antibody to purified aldolase (6), rhodamine-conjugated goat anti-rabbit antibody and FITC-conjugated goat anti-mouse antiserum (Becton-Dickinson and Co., Mountain View, CA).

Cell Fractionation

To measure compartmentation, the *T. brucei* were suspended in isotonic buffer (10% sucrose in 25 mM Tris, pH 7.2, 1 mM EDTA, 2 μg/ml leupeptin) and either broken in a mini-glass-bead beater (5 s) or permeabilized with digitonin (150 μg digitonin/ml, 5×10^6 trypanosomes/ml; 60 min on ice with mixing). In the case of the glass-bead procedure, nuclei and unbroken cells were removed by low speed centrifugation, then the glycosomes and other smaller particles were separated from the soluble components by high speed centrifugation (11). Digitonin-permeabilized cells were centrifuged for 10 min at 15,000 rpm in a Heraeus microfuge (Heraeus-Amersil, Inc., Sayerville, NJ), and the supernatant separated from the pellet. The efficiency of the methodology was determined by assessing the compartmentation of CAT, CAT-PGK, and the glycosomal enzyme glycerolphosphate dehydrogenase (GPDH) as previously described (11). To assay for GPDH, glycosomes were permeabilized with Triton. The CAT assay works without prior membrane permeabilization, possibly because the substrates are able to penetrate the glycosomal membrane (11).

In Vivo Labeling and Immunoprecipitation

CV-1 cells were labeled with 100 μCi [^{35}S]methionine/ml on 3.3-cm petri dishes for 2 h, freeze thawed three times in extraction buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 0.1% sodium deoxycholate, 0.1% Triton X-100, 1 mM PMSF, 0.05% BSA, alpha2 macroglobulin, leupeptin, aprotinin, pepstatin) and CAT immunoprecipitated using a polyclonal antibody (5′-3′ Inc.) and Protein A–Sepharose. The immunoprecipitates were analyzed on a 12% polyacrylamide minigel (Bio-Rad Laboratories, Richmond, CA) which was soaked in "Amplify" (Amersham Braunschweig, Germany) before exposure to x-ray film.

Results

Improvements in the Assay Methodology

Plasmids bearing hybrid CAT genes, expressed using the promoter and RNA processing signals of the procyclic acidic repetitive protein (PARP) genes (7, 29), were electroporated into *T. brucei*. After an overnight incubation, cells were broken and the compartmentation of CAT protein measured by differential centrifugation. In our original procedure, which involved shaking the trypanosomes with glass beads, 30–40% of CAT-PGK and 50% of GPDH, a glycosomal enzyme, were found in the microbody pellet. The CAT-PGK in the microbody pellet comigrated with GPDH in sucrose gradient centrifugation (11), confirming its association with glycosomes.

Low concentrations of digitonin permeabilize the plasma membrane but leave intracellular membranes intact. Using digitonin-permeabilized cells, we found that microbody compartmentation was much better preserved than after physical breakage. ~80% of CAT-PGK (see Table II) or GPDH (not shown) now remained in the glycosomal fraction. The association of two fusion proteins (CAT-SSL and CAT-NRWSSL) with glycosomes, rather than aggregates or some other undefined digitonin-impermeable compartment, was confirmed by sucrose gradient centrifugation (not shown). Tables I and II list results from assays using both methodologies.

Targeting by COOH-terminal Tripeptides

To test which variants of the -SKL signal were active in trypanosomes, plasmids encoding CAT bearing a variety of SKL-like COOH-terminal sequences were electroporated into *T. brucei* and the compartmentation of CAT activity tested the next day. Results are shown in Table I, together with the published compartmentation of the same hybrids in peroxisomes of primate cells. COOH-terminal tripeptides that are known to occur on glycosomal enzymes are marked. All variants tested were active in causing glycosomal association in trypanosomes, although some differences in targeting efficiency were detected. In primate cells, the COOH-terminal leucine appears essential for high-efficiency targeting (14); substitution by methionine results in a significantly reduced targeting efficiency (35). In contrast, SKM is as efficient as, or more efficient than, SKL in directing CAT to glycosomes.

Dissection of the PGK COOH-terminal Signal

Results of deletion and mutation of the PGK COOH-terminus are shown in Table II. From the deletions it was clear that the most important portion was once again the

Table I. Function of SKL Variant Signals

COOH-terminal extension	Percentage in glycosomes: Glass beads Mean	Glass beads No.	Digitonin Mean	Digitonin No.	Summary* T	Summary* M
None	3	(11)	5	(5)	−	−
SKL	16	(3)			+	+
·AKL	35	(2)	22	(2)	+	+
CKL	27	(3)	67	(2)	++	+
SRL	25	(2)	50	(2)	++	+
·SSL	16	(3)	22	(2)	+	−
·SHL	10	(2)	40	(2)	+	+
·SKM	27	(3)	48	(2)	+	±
·ARL						+

Compartmentation of CAT hybrid proteins bearing variants of the SKL entry signal in trypanosome glycosomes. Values for glass bead and digitonin fractionation are given as:

$$\frac{\text{CAT activity in glycosomal pellet}}{\text{CAT activity in pellet + supernatant}}$$

Results are expressed as the mean value, with the number of experiments (*No.*) indicated in parentheses.

* Summary shows overall conclusions. For trypanosomes (*T*): (++) 20–100% glycosomal localization by glass-bead method and 50–100% by digitonin method; (+) 9–20% glycosomal localization by glass bead method and 20–50% by digitonin method; and (−) <9 and 20% glycosomal localization by glass-bead and digitonin methods, respectively.

Mammalian values (*M*) for extent of peroxisomal localization as judged by immunofluorescence (35): (+) Efficient peroxisomal localization; (±) inefficient peroxisomal localization; (−) no detectable peroxisomal localization; and (·) COOH-terminal peptides found on sequenced trypanosomatid glycosomal enzyme genes.

COOH-terminal peptide, -SSL. However, -SSL by itself had a rather poor targeting capacity, working better in combination with three upstream amino acids. -SSL does not target proteins to mammalian peroxisomes to any significant extent (14). It is noteworthy that within the context of the COOH-terminal hexapeptide of the gPGK signal, a basic amino acid in the signal was not essential for targeting: tyrosine or glycine, but not aspartic acid, could be substituted for the arginine residue. At the first amino acid of the tripeptide, the spectrum of acceptable degeneracy was similar to that for mammalian cells (14, 35): glutamate, leucine, and threonine were not tolerated but alanine was functional. Requirements for the second and third amino acids were however clearly more relaxed in trypanosomes than in mammalian cells: serine, asparagine, tyrosine, and even aspartic acid could be substituted at the second position (within the context of the COOH-terminal hexapeptide) whereas in monkey cells only basic residues were functional; and a COOH-terminal tyrosine or methionine could be substituted for the terminal leucine.

Immunolocalization of CAT in Trypanosomes

Our experiments so far had shown glycosomal association of CAT-PGK and its derivatives, but we could not rule out the possibility that the association was an external attachment to the glycosomal membrane, which could have occurred during cell breakage. We therefore examined the localization of CAT and CAT-PGK in trypanosomes by immunofluores-

Table II. Analysis of T. brucei g-PGK Signal

COOH-terminal extension	Percentage in glycosomes: Glass beads Mean	Glass beads No.	Digitonin Mean	Digitonin No.	Summary
None	3	(11)	5	(5)	−
SAVVSYASAGTGTLSNRWSSL	35	(11)	81	(5)	++
SAVVSYASAGTGTLSNR	6	(4)	9	(2)	−
SAVVSYASAGTGTLSN	4	(3)	6	(2)	−
SAVVSYASAGTGTLSNDWSSL	11	(5)	5	(2)	−
SAVVSYASAGTGTLSNHWSSL	27	(4)	66	(2)	++
SAVVSYASAGTGTLSNYWSSL	24	(15)	54	(2)	++
NRWSSL	32	(14)	77	(2)	++
WRWSSL	45	(13)	82	(2)	++
NGWSSL	28	(4)	59	(2)	++
NRNSSL	38	(3)	67	(2)	++
NRWASL	45	(13)	69	(2)	++
NRWESL	2	(3)	10	(2)	−
NRWLSL	2	(3)	19	(2)	−
NRWTSL	3	(3)	17	(3)	−
NRWSNL	30	(14)	75	(2)	++
NRWSDL	25	(13)	37	(2)	+
NRWSHL	31	(4)	84	(2)	++
NRWSYL	40	(3)	37	(2)	+
NRWSSN	5	(3)	14	(3)	−
NRWSSD	4	(3)	10	(3)	−
NRWSSH	6	(3)	18	(3)	−
NRWSSY	24	(3)	27	(3)	+
SSL	16	(3)	22	(2)	+

Compartmentation of CAT hybrid proteins bearing variants of the PGK entry signal in trypanosome glycosomes. Mutated amino acids are shown in bold type. Values are calculated as for Table I.

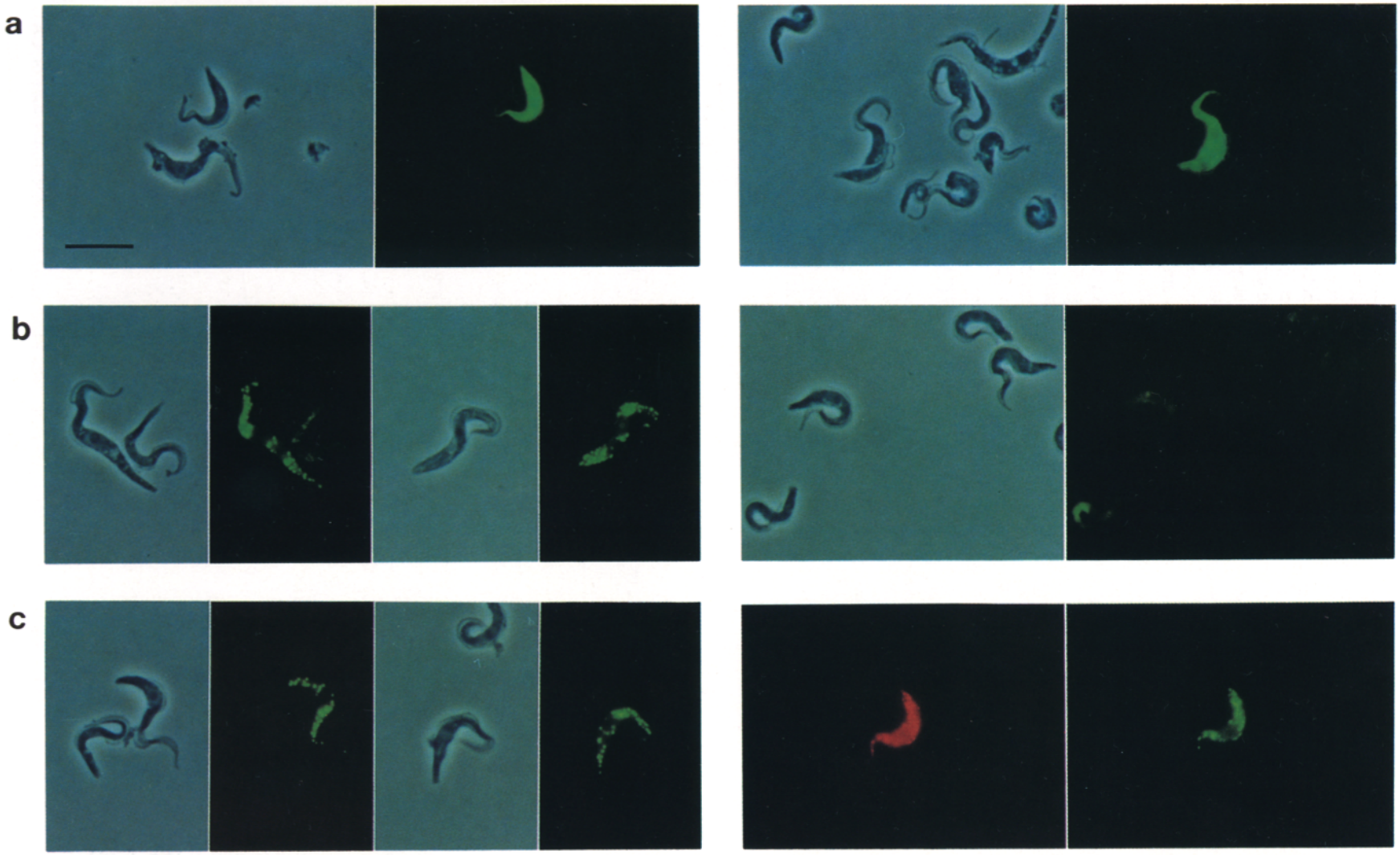

Figure 2. Immunofluorescence staining of trypanosomes expressing CAT or CAT-PGK after transient transfection. Trypanosomes were photographed under phase illumination (*left*, *a–e*) and for fluorescein (*a–f*) or rhodamine (*f*) immunofluorescence after permeabilization with Triton or digitonin and staining using anti-CAT (*a*, *c*, *d*, and *f*) or anti-aldolase (*b*, *e*, and *f*) antibodies. All parasites express aldolase, which serves as a positive control and is located in the glycosomes. (*a*) CAT, Triton-permeabilized; (*b*) aldolase control, Triton-permeabilized; (*c*) CAT-PGK, Triton-permeabilized; (*d*) CAT, digitonin-permeabilized; (*e*) aldolase control, digitonin-permeabilized; and (*f*) Triton-permeabilized, double immunofluorescence for aldolase (*red*) and CAT-PGK (*green*). Bar, 10 μm.

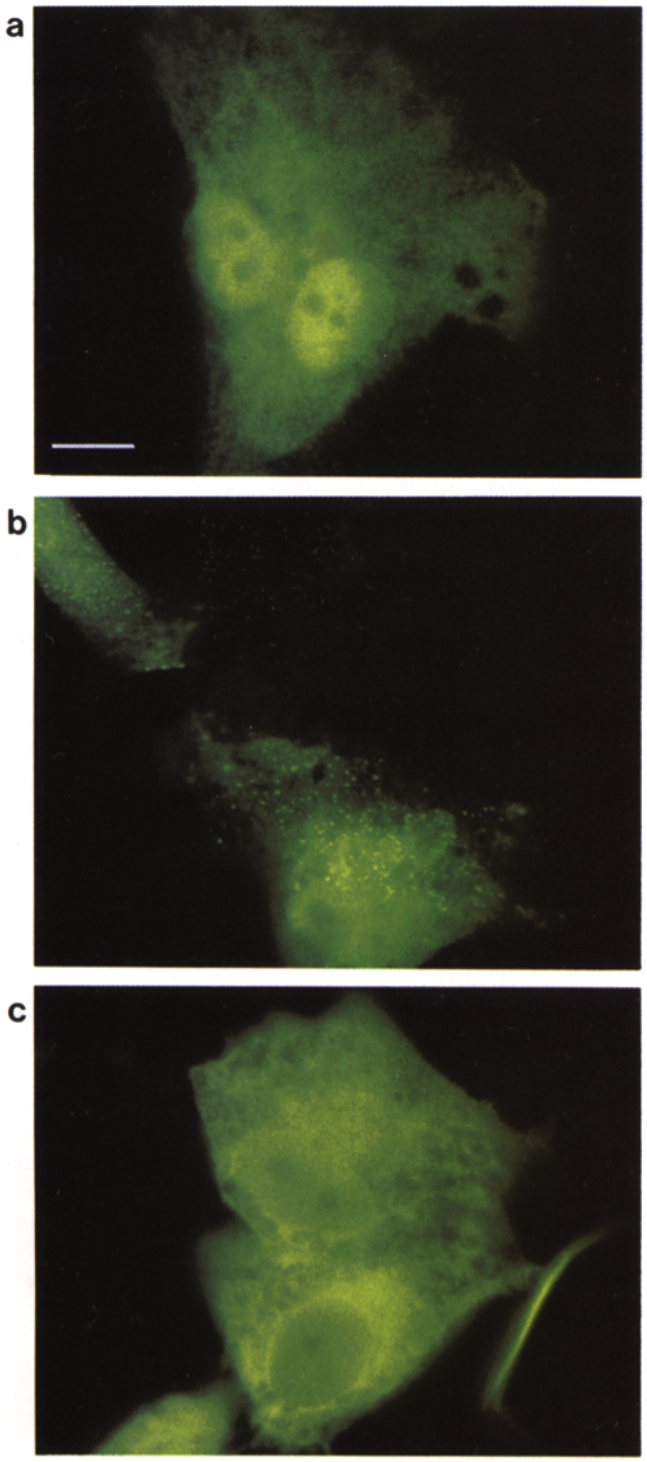

Figure 3. Immunofluorescence staining of CV-1 cells expressing CAT or CAT hybrid proteins. (*a*) CAT; (*b*) CAT-SLK; and (*c*) CAT-PGK. Bar, 10 μm.

cence. To develop the methodology, we used trypanosomes that had been selected permanently to express either CAT or CAT-PGK (see Materials and Methods). ~5% of these trypanosomes were expressing CAT or CAT-PGK. (The cells were not cloned, and the structure of the retained plasmid DNA has not been examined, so we do not know if the heterogeneity of expression was due to deletion of the CAT-coding sequences, or has some other explanation.) When we examined transiently transfected trypanosomes, we found that ~1% of the cells expressed CAT or CAT-PGK and the fluorescence was considerably brighter than in the permanently expressing cells. Results for these transiently transfected cells are therefore illustrated in Fig. 2. The cells were examined by immunofluorescence after fixation and permeabilization with Triton X-100, which permeabilizes the plasma membrane and the glycosomal membrane. Trypanosomes expressing CAT fluoresced throughout the cytoplasm (Fig. 2 *a*), whereas those expressing CAT-PGK showed bright fluorescent dots (Fig. 2 *c*) in a similar pattern to the positive glycosomal enzyme control, trypanosome aldolase (Fig. 2 *b*). Using rhodamine fluorescence, the punctate pattern of aldolase was more difficult to see but could still be detected at the narrow end of the trypanosomes (Fig. 2 *f*). Double staining of CAT-PGK (fluorescein) and aldolase (rhodamine) confirmed that CAT-PGK and aldolase colocalize (Fig. 2 *f*). The compartmentation of several of the other CAT fusion proteins was also checked by immunofluorescence (not shown) and the results correlated with those obtained by cell fractionation. In trypanosomes expressing CAT-SSL the pattern was consistent with partial import (not shown).

To confirm that the CAT-PGK had been imported across the glycosomal membrane, we once again took advantage of the differential sensitivity of the plasma membrane and glycosomal membrane to digitonin. After cell permeabilization with digitonin, the CAT fluorescence pattern was indistinguishable from that seen after Triton permeabilization (Fig. 2 *d*). As expected, aldolase fluorescence was extremely weak (Fig. 2 *e*), presumably because the glycosomal membrane was not permeabilized by digitonin and the aldolase was not accessible to the antibodies. CAT-PGK was completely undetectable (not shown), confirming that it too was shielded from antibodies by a digitonin-resistant membrane.

Activity of the Trypanosomal PGK Signal in Primate Cells

The results so far indicated that there were both similarities and differences in targeting between trypanosome glycosomes and mammalian peroxisomes. To test whether the PGK signal functions in primate cells, we expressed the CAT-PGK hybrid in monkey cells under control of the SV-40 virus early promoter. CAT and CAT fusions were detected by immunofluorescence, using CAT alone as a negative control and CAT-SKL as positive control. Results are shown in Fig. 3. As already published (14) CAT-SKL was concentrated in bright dots (the peroxisomes) whereas CAT alone was spread all over the cytoplasm. The distribution of CAT-PGK was very similar to that of CAT. To confirm that the CAT, CAT-SKL, and CAT-PGK were made as expected, in vivo-labeled proteins were immunoprecipitated and examined by gel electrophoresis (Fig. 4). There was a clear difference in gel mobility between the three proteins; surprisingly, even the difference between CAT and CAT-SKL could be detected. A very small proportion of the precipitated CAT-PGK appeared to have been somewhat degraded, but we judge that this would be insufficient to change the immunofluorescence pattern. In these experiments, the amount of CAT-PGK was approximately three times that of CAT. Reduction of the amount of CAT-PGK (by reducing the

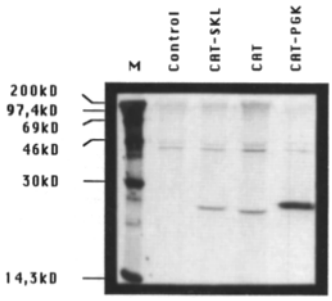

Figure 4. Synthesis of CAT, CAT-SKL, and CAT-PGK in transfected CV-1 cells. Immunoprecipitated proteins from [^{35}S]methionine-labeled cells were separated by SDS-PAGE.

amount of input DNA) did not influence the immunofluorescence pattern (not shown), indicating that the cytoplasmic localization was not a consequence of saturation of putative peroxisomal import receptors.

Discussion

Specificity of Glycosomal Targeting

The results described above clearly show that the microbody targeting signal for *T. brucei* PGK resides within the COOH-terminal 6 amino acids, and that this targeting signal does not function in monkey cells. A basic residue is not required for glycosomal targeting. It has been previously suggested, based on sequence and ultrastructure comparisons (38) and results of in vitro import assays (9, 30), that internal sequences of glycosomal PGK, especially basic residues, may play a role in targeting. Our results do not support this, but we cannot rule out the possibility that the CAT fusion protein behaves differently from the intact PGK molecule. Luciferase, for example, has COOH-terminal SKL, but alterations elsewhere in the protein can result in its redirection to the cytoplasm (13, and see below).

As for many microbody proteins, the most important component of the glycosomal PGK targeting signal resides in the COOH-terminal tripeptide. However, the spectrum of variants that is functional differs markedly from that observed for mammalian cells. The first amino acid can be serine, alanine, or cysteine, as for peroxisomes. However, requirements at the second position are very relaxed (we have not yet found anything that doesn't work) and the third leucine can be replaced by tyrosine or methionine without much effect on glycosomal targeting. The ability of -SKM to function as a targeting signal is consistent with the finding of COOH-terminal SKM in *Leishmania mexicana* glyceraldehyde phosphate dehydrogenase (16). In monkey cells, -SKM has only weak activity (35).

We have found that some of the SKL variants, including -SKL itself, do not work as efficiently in trypanosomes as the PGK glycosomal targeting signal. Although -SSL is functional, it is clearly "helped" by the upstream amino acids. In this context, it is notable that of the glycosomal proteins so far sequenced, glyceraldehyde phosphate dehydrogenases share with PGK the basic amino acid at position -5 relative to the COOH terminus (Table III). Glucosephosphate isomerase, however, has an acidic residue at this position; this was not tolerated upstream of -SSL but appears not to affect the function of -SHL. Perhaps the trypanosomatids compensate for the relaxed specificity for -SKL-like signals by having more stringent context requirements. Even in mammalian peroxisomes, the SKL signal is context dependent. For example, deletion of the first 58 amino acids of luciferase, or insertion of four amino acids within the NH_2-terminus, can both abolish peroxisomal import despite the fact that the -SKL sequence is undisturbed (13). It is clear, too, that SKL-like signals are not the only possible microbody targeting signals. Rat ketoacylthiolase has a cleavable NH_2-terminal signal (34), which is homologous to the NH_2-termini of several other microbody proteins including trypanosome aldolase (5, 24); and the signal for the PGK "A" gene must reside somewhere other than at the COOH terminus, perhaps in the central insertion (22). Another probable entry signal is the 39-amino acid extension at the COOH terminus of the glycosomal PGK of *Crithidia fasciculata* (33): the final 6 amino acids are MVLASP. Results of preliminary experiments suggested that the *Crithidia* sequence was not capable of directing CAT into *T. brucei* glycosomes (H. Dörsam and C. Clayton, unpublished results), but as *Crithidia* and *T. brucei* are evolutionarily quite distant (4, 20) the meaning of this result is debatable.

We used immunofluorescence to assay peroxisomal import in mammalian cells and to confirm glycosomal import in trypanosomes. This assay is essentially qualitative and might not detect low-efficiency import (14). It is certainly possible that the discrepancies in targeting noted here are not as great as they appear. However, the difference in the be-

Table III. Known and Inferred Glycosomal Entry Signals

Protein	Organism	Location	Sequence	Reference
Glyceraldehyde phosphate dehydrogenase	*T. brucei*	COOH terminus	DRAAKL	(26)
Glyceraldehyde phosphate dehydrogenase	*T. cruzi*	COOH terminus	DRSARL	(19)
Glyceraldehyde phosphate dehydrogenase	*L. mexicana*	COOH terminus	AASSKM	(16)
Glyceraldehyde phosphate dehydrogenase	*T. borelli*	COOH terminus	KCHAKL	*
Glucosephosphate isomerase	*T. brucei*	COOH terminus	NELSHL	(25)
Glucosephosphate isomerase	*L. mexicana*	COOH terminus	NTRAHL	*
Phosphoglycerate kinase (C)	*T. brucei*	COOH terminus	NRWSSL	‡
Phosphoglycerate kinase (A)	*T. brucei*	Internal peptide?		(2)
Triosephosphate isomerase	*T. brucei*	Unknown		(32)
Fructose bisphosphate aldolase	*T. brucei*	NH_2 terminus or internal?		(5)
Phosphoglycerate kinase (C)	*C. fasciculata*	COOH terminus?		(33)

* P. Michels (ICP, Brussels), personal communication.
‡ This work.

havior of CAT-PGK between the two cell types is indisputable.

This is not the first time that an intracellular sorting signal has been shown to be related, but not identical, between different species. A signal for retrieval of proteins from the Golgi complex and retention in the ER is the COOH-terminal quadrapeptide KDEL or related sequences; but mammalian cells, plants and yeasts show varying specificities (8). Similarly, some COOH-terminal tripeptides will target proteins to peroxisomes in *Candida* species but not in *S. cerevisiae* (1). It will be interesting to see how the specificity is reflected in the corresponding peroxisomal and glycosomal import receptors. The differences in targeting specificity between monkeys and trypanosomes implies that it may be possible to design specific inhibitors of glycosomal targeting. Detailed characterization of the entry signals also gives us the information required to attempt a blockade of glycosomal import in vivo, and thereby to determine the extent to which trypanosomes are dependent on their unusual glycolytic compartmentation.

We are indebted to Christa Kuhn for CV-1 cells and guidance with their cultivation. We thank Anneloor ten Asbroek and Professor Piet Borst (Netherlands Cancer Institute) for pUC Tbneo 3; Mary Lee (Columbia University, New York) for bNsp-H-α and useful tips on trypanosome transformation; also Paul Michels (ICP, Brussels) and Jürg Sommer (University of California, San Francisco, CA) for communicating unpublished results including (Jürg Sommer) the fact that digitonin can be used for analysis of glycosomal targeting. We acknowledge Claudia Hartmann's technical assistance, Liz Wirtz for making the hygromycin-CAT-PGK plasmid, and Lennart Janz, Michael Hug, and John Flaspohler for useful discussions.

This work was supported by grant 900002 from the World Health Organization Special Program for Research and Training in Tropical Diseases (C. Clayton, J. Blattner, and H. Dörsam); a fellowship from the Netherlands Organization for Scientific Research (B. Swinkels) and National Institutes of Health grant DK41737 (S. Subramani).

Received for publication 26 May 1992 and in revised form 5 August 1992.

Note Added in Proof. Details of possible variants of the glycosomal targeting signal have also recently been reported by Sommer et al. (1992).

References

1. Aitchison, J. D., W. W. Murray, and R. A. Rachubinski. 1991. The carboxy-terminal tripeptide alanine-lysine-isoleucine is essential for targeting *Candida tropicalis* trifunctional enzyme to yeast peroxisomes. *J. Biol. Chem.* 266:23197–23203.
2. Alexander, K., and M. Parsons. 1991. A phosphoglycerate kinase-like molecule localized to glycosomal microbodies: evidence that the topogenic signal is not at the C-terminus. *Mol. Biochem. Parasit.* 46:1–10.
3. Borst, P. 1989. Peroxisome biogenesis revisited. *Biochim. Biophys. Acta.* 1008:1–13.
4. Briones, M. R. S., K. Nelson, S. M. Beverley, H. T. Affonso, E. P. Camargo, and L. M. Floeter-Winter. 1992. *Leishmania tarentolae* taxonomic relatedness inferred from phylogenetic analysis of the small subunit ribosomal RNA gene. *Mol. Biochem. Parasit.* 53:121–128.
5. Clayton, C. E. 1985. Structure and regulated expression of genes encoding fructose biphosphate aldolase in *Trypanosoma brucei*. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2997–3003.
6. Clayton, C. E. 1987. Import of fructose bisphosphate aldolase into the glycosomes of *Trypanosoma brucei*. *J. Cell Biol.* 105:2649–2653.
7. Clayton, C. E., J. Fueri, and M. Mowatt. 1990. The procyclic surface protein of Trypanosoma brucei. *In* Molecular and Immunological Aspects of Parasitism. American Association for the Advancement of Science, New Orleans, LA. 55–64.
8. Denecke, J., R. de Rycke, and J. Botterman. 1992. Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2345–2355.
9. Dovey, H. F, M. Parsons, and C. C. Wang. 1988. Biogenesis of glycosomes of Trypanosoma brucei: an in vitro model of 3-phosphoglycerate kinase transport. *Proc. Natl. Acad. Sci. USA.* 85:2598–2602.
10. Fairlamb, A. H., and F. R. Opperdoes. 1986. Carbohydrate metabolism in African trypanosomes, with special reference to the glycosome. *In* Carbohydrate metabolism in cultured cells. M. J. Morgan, editor. Plenum publishing corporation, New York. 183–224.
11. Fung, K., and C. E. Clayton. 1991. Recognition of a peroxisomal tripeptide entry signal by the glycosomes of *Trypanosoma brucei*. *Mol. Biochem. Parasit.* 45:261–264.
12. Gibson, W. C., B. W. Swinkels, and P. Borst. 1988. Post-transcriptional control of the differential expression of phosphoglycerate kinase genes in *Trypanosoma brucei*. *J. Mol. Biol.* 201:315–325.
13. Gould, S. J., G. A. Keller, and S. Subramani. 1987. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol.* 105:2923–2931.
14. Gould, S. J., G. A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1403–1411.
15. Gould, S. J., G.-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani. 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:85–90.
16. Hannaert, V., M. Blaauw, L. Kohl, S. Allert, F. R. Opperdoes, and P. A. M. Michels. 1992. Molecular analysis of the cytosolic and glycosomal glyceraldehyde phosphate dehydrogenase in Leishmania mexicana. *Mol. Biochem. Parasit.* In press.
17. Keller, G.-A., S. Gould, M. Deluca, and S. Subramani. 1987. Firefly luciferase is targeted to peroxisomes in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 84:3264–3268.
18. Keller, G.-A., S. Krisans, S. J. Gould, J. N. Sommer, C. C. Wang, W. Schliebs, W. Kunau, S. Brody, and S. Subramani. 1991. Evolutionary conservation of a microbody targeting signal that targets proteins to peroxisomes, glyoxysomes, and glycosomes. *J. Cell Biol.* 114:893–904.
19. Kendall, G., A. F. Wilderspin, F. Ashall, M. A. Miles, and J. M. Kelly. 1990. Trypanosoma cruzi glycosomal glyceraldehyde-3-phosphate dehydrogenase does not conform to the "hotspot" topogenic signal model. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2751–2758.
20. Lake, J. A., V. de la Cruz, P. C. G. Ferreira, C. Morel, and L. Simpson. 1988. Evolution of parasitism: kinetoplastid protozoan history reconstructed from mitochondrial rRNA gene sequences. *Proc. Natl. Acad. Sci. USA.* 85:4779–4783.
21. Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.
22. LeBlancq, S. M., B. W. Swinkels, W. C. Gibson, and P. Borst. 1988. Evidence for gene conversion between the phosphoglycerate kinase genes of *Trypanosoma brucei*. *J. Mol. Biol.* 200:439–447.
23. Lee, M. G.-S., and L. H. T. Van der Ploeg. 1991. The hygromycin B-resistance gene as a selectable marker for stable transformation of *Trypanosoma brucei*. *Gene (Amst.).* 105:255–257.
24. Marchand, M., A. Poliszak, W. C. Gibson, R. K. Wierenga, F. R. Opperdoes, and P. A. M. Michels. 1988. Characterization of the genes for fructose bisphosphate aldolase in *Trypanosoma brucei*. *Mol. Biochem. Parasit.* 29:65–76.
25. Marchand, M., U. Kooystra, R. K. Wierenga, A.-M. Lambeir, J. Van Beeumen, F. R. Opperdoes, and P. A. Michels. 1989. Glucosephosphate isomerase from *Trypanosoma brucei*. Cloning and characterization of the gene and analysis of the enzyme. *Eur. J. Biochem.* 184:455–464.
26. Michels, P. A. M., M. Marchand, L. Kohl, S. Allert, R. K. Wierenga, and F. R. Opperdoes. 1991. The cytosolic and glycosomal isoenzymes of glyceraldehyde-3-phosphate dehydrogenase in *Trypanosoma brucei* have a distant evolutionary relationship. *Eur. J. Biochem.* 198:421–428.
27. Opperdoes, F. R. 1987. Compartmentation of carbohydrate metabolism in trypanosomes. *Annu. Rev. Microbiol.* 41:127–151.
28. Osinga, K. A., B. W. Swinkels, W. C. Gibson, P. Borst, G. H. Veeneman, J. H. Van Boom, P. Michels, and F. R. Opperdoes. 1985. Topogenesis of the microbody enzymes: a sequence comparison of the genes for the glycosomal (microbody) and cytosolic phosphoglycerate kinases of *Trypanosoma brucei*. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3811–3817.
29. Sherman, D., L. Janz, M. Hug, and C. E. Clayton. 1991. Anatomy of the procyclic acidic repetitive protein promoter of *Trypanosoma brucei*. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3379–3386.
30. Sommer, J. M., J. A. Thissen, M. Parsons, and C. C. Wang. 1990. Characterization of an in vitro assay for import of 3-phosphoglycerate kinase into the glycosomes of *Trypanosoma brucei*. *Mol. Cell Biol.* 10:4545–4554.

30a. Sommer, J. M., Q.-L. Cheng, G.-A. Keller, and C. C. Wang. 1992. In vivo import of firefly luciferase into the glycosomes of *Trypanosoma brucei* and mutational analysis of the C-terminal targeting signal. *Mol. Biol. Cell.* 3:749–759.

31. Subramani, S., R. Mulligan, and P. Berg. 1981. Expression of the complementary DNA for mouse dihydrofolate reductase in simian virus 40 vectors. *Mol. Cell Biol.* 1:854–864.
32. Swinkels, B. W., W. C. Gibson, K. A. Osinga, R. Kramer, G. H. Veeneman, J. H. van Boom, and P. Borst. 1986. Characterization of the gene for the microbody (glycosomal) triosephosphate isomerase of *Trypanosoma brucei*. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1291–1298.

33. Swinkels, B. W., R. Evers, and P. Borst. 1988. The topogenic signal of the glycosomal (microbody) phosphoglycerate kinase of *Crithidia fasciculata* resides in a carboxyterminal extension. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1159–1165.
34. Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski, and S. Subramani. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3255–3262.
35. Swinkels, B., S. J. Gould, and S. Subramani. 1992. Targeting efficiencies of various permutations of the concensus C-terminal tripeptide peroxisomal targeting signal. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 305: 133–136.
36. Swinkels, B. W., A. Loiseau, F. R. Opperdoes, and P. Borst. 1992. A phosphoglycerate kinase-related gene conserved between *Trypanosoma brucei* and *Crithidia fasciculata*. *Mol. Biochem. Parasitol.* 50:69–78.
37. ten Asbroek, A. L. M. A., M. Ouellette, and P. Borst. 1990. Targeted insertion of the neomycin phosphotransferase gene into the tubulin gene cluster of *Trypanosoma brucei*. *Nature (Lond.).* 348:174–175.
38. Wierenga, R. K., B. Swinkels, P. A. M. Michels, K. Osinga, O. Misset, J. van Beeumen, W. C. Gibson, J. P. M. Postma, P. Borst, F. R. Opperdoes, and W. G. J. Hol. 1987. Common elements on the surface of glycolytic enzymes from *Trypanosoma brucei* may serve as topogenic signals for import into glycosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:215–221.