In Vivo Import of Firefly Luciferase into the Glycosomes of *Trypanosoma brucei* and Mutational Analysis of the C-Terminal Targeting Signal

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The compartmentalization of glycolytic enzymes into specialized organelles, the glycosomes, allows the bloodstream form of *Trypanosoma brucei* to rely solely on glycolysis for its energy production. The biogenesis of glycosomes in these parasites has been studied intensively as a potential target for chemotherapy. We have adapted the recently developed methods for stable transformation of *T. brucei* to the in vivo analysis of glycosomal protein import. Firefly luciferase, a peroxisomal protein in the lantern of the insect, was expressed in stable transformants of the procyclic form of *T. brucei*, where it was found to accumulate inside the glycosomes. Mutational analysis of the peroxisomal targeting signal serine-lysine-leucine (SKL) located at the C-terminus of luciferase showed that replacement of the serine residue (Serine548) with a small neutral amino acid (A, C, G, H, N, P, T) still resulted in an import efficiency of 50–100% of the wild-type luciferase. Lysine549 could be substituted with an amino acid capable of hydrogen bonding (H, M, N, Q, R, S), whereas the C-terminal leucine550 could be replaced with a subset of hydrophobic amino acids (I, M, Y). Thus, a peroxisome-like C-terminal SKL-dependent targeting mechanism may function in *T. brucei* to import luciferase into the glycosomes. However, a few significant differences exist between the glycosomal targeting signals identified here and the tripeptide sequences that direct proteins to mammalian or yeast peroxisomes.

INTRODUCTION

The study of the parasitic kinetoplastida, *Trypanosoma brucei*, has revealed a number of novel biochemical processes. The compartmentalization of several glycolytic enzymes into specialized membrane-bound organelles, the glycosomes (Opperdoes and Borst, 1977), is a feature unique to all kinetoplastida and has been studied particularly well in the long-slender bloodstream form of *T. brucei* (Fairlamb and Opperdoes, 1986; Opperdoes, 1987). Because of the absence of a functional tricarboxylic acid cycle and a mitochondrial electron transport chain, the *T. brucei* bloodstream forms depend solely on glycolysis for their energy supply during rapid cell divisions once every 6–8 h. The high concentrations of seven glycolytic enzymes and two glycerol-metabolizing enzymes inside the glycosome allow glycolysis to proceed at a rate 50 times higher than that found in

the mammalian host (Fairlamb and Opperdoes, 1986). This high glycolytic rate, which effectively compensates for the relatively poor energy yield from glycolysis, is apparently essential for the rapid growth and survival of *T. brucei* in the infected mammalian blood. We have been investigating the mechanism and targeting signals responsible for the import of glycolytic enzymes into this unique organelle, with the anticipation that a disruption of glycosomal protein import may result in an effective anti-trypanosomal chemotherapy.

An evolutionary relationship between glycosomes and peroxisomes was proposed based on their structural and functional resemblance (Borst, 1986; Michels and Opperdoes, 1991) and the fact that, like peroxisomal proteins in higher eukaryotes, glycosomal proteins are encoded by nuclear genes, synthesized on free polyribosomes, and imported into the glycosomes without

proteolytic modifications (Hart *et al.*, 1987). Most peroxisomal proteins contain at their C-terminals the tripeptide serine-lysine-leucine (SKL) or a closely analogous tripeptide, which is necessary for the import of these proteins into peroxisomes (Gould *et al.*, 1989). An antibody specific for the C-terminal SKL tripeptide recognized six as yet unidentified glycosomal proteins from *T. brucei*, some of which are apparently minor components of the glycosome (Keller *et al.*, 1991). Of the nine major glycosomal enzymes whose sequences have been determined to date, three end in a variant of the SKL tripeptide sequence. The glycosomal glyceraldehyde-3-phosphate dehydrogenases (gGAPDH) from *T. brucei* and *T. cruzi* have been found to contain AKL and ARL, respectively, at their C-terminals (Michels *et al.*, 1986; Kendall *et al.*, 1990), and *T. brucei* phosphoglucose isomerase (PGI) ends in SHL (Marchand *et al.*, 1989). Most glycosomal proteins, including *T. brucei* gGAPDH, for which the three-dimensional structure has been deduced, also contain two surface clusters of positive charges that are not found on cytosolic isoenzymes and that have been thought to play a role in the import of these proteins (Wierenga *et al.*, 1987). It is thus not clear whether the tripeptide sequences mentioned above are either required or sufficient for the import of proteins into the glycosomes of *T. brucei*. To test whether SKL can target a cytoplasmic protein to the glycosome, Fung and Clayton (1991) have added an SKL-tripeptide to the C-terminus of chloramphenicol acetyltransferase (CAT), and this protein was expressed transiently in *T. brucei*. Some of the CAT activity indeed copurified with glycosomal markers on a sucrose density gradient, but the inefficiency of the transient transformation system did not allow electron microscopic verification that the modified CAT protein was actually imported into the matrix of *T. brucei* glycosomes.

We have adapted the newly developed methods for stable expression of the neomycin phosphotransferase (*neo*) gene in *T. brucei* (Lee and Van der Ploeg, 1990; ten Asbroek *et al.*, 1990; Eid and Sollner-Webb, 1991) for the analysis of glycosomal protein import. In this study, firefly luciferase, which is targeted to the peroxisomes by means of its C-terminal SKL sequence (Keller *et al.*, 1987; Gould *et al.*, 1989), was stably coexpressed with *neo* or with the hygromycin phosphotransferase gene (*hyg*) in *T. brucei*. By site-directed mutagenesis, we substituted each of the three amino acids in SKL and tested the resulting 60 mutants for their ability to be imported into glycosomes in vivo.

MATERIALS AND METHODS

Construction of Transformation Vectors

The transformation vector pTSA-NEO2 (Figure 1) contains a multicloning site 40 base pair (bp) downstream of a polypyrimidine tract and adjacent AG dinucleotide that constitutes the putative procyclic acidic repetitive protein (PARP) splice signal and acceptor site, followed

by a second such splice signal preceding the *neo* gene. A *T. brucei* $\beta\alpha$ -tubulin intergenic region of 640 bp is located downstream of the *neo* gene. This sequence contains a unique *Mlu* I restriction endonuclease site that allows linearization of the vector DNA and insertion of the entire plasmid into a tubulin locus of *T. brucei* by homologous recombination (Lee and Van der Ploeg, 1990). To construct pTSA-NEO2, the multicloning site of pBluescript (Stratagene, San Diego, CA) was purified as a 90 bp *Apa* I to *Sst* I fragment, blunt-ended with T4 polymerase (Sambrook *et al.*, 1989), and inserted into the blunt-ended *Hind*III site of pJP25 (Sherman *et al.*, 1991) between the splice acceptor site and the CAT gene, resulting in the plasmid pJP25.MCS1. The orientation of the insert is *Xho* I to *Sst* II. A 3-kilobase (kb) *Sca* I to *Aat* II fragment containing the PARP splice acceptor signal followed by the *neo* gene, the *T. brucei* $\beta\alpha$ -tubulin intergenic region, and SV40 sequences containing the small t antigen intron and a polyadenylation signal was cut from the plasmid BNSp-Neo-T (Lee and Van der Ploeg, 1990) and ligated into the plasmid pJP25.MCS1 cut with *Xba* I in the multicloning site, blunt-ended with Klenow fragment, and cut with *Aat* II. The *Bam*HI site located downstream of the tubulin intergenic region of pTSA-NEO2 was removed by partial *Bam*HI digest followed by filling in with Klenow and ligation. This resulted in the vector pTSA-NEO3, which now has a unique *Bam*HI site in the multicloning region. To produce the vector pTSA-HYG1, the hygromycin phosphotransferase gene was cut from the vector pALT-Hyg (Lee and Van der Ploeg, 1991) with *Bam*HI, blunt-ended with T4 DNA polymerase, gel-purified, and inserted into the vector pTSA-NEO3 from which the *neo* gene had been removed with *Sac* II and *Bst* I, blunt-ending with T4 DNA polymerase. The protein coding region of the firefly luciferase gene was purified on a 2.1-kb *Nco* I to *Ssp* I fragment from the plasmid T7 BiP-Luc (Macejak *et al.*, 1990), filled in with Klenow to restore the ATG initiation codon of luciferase at the *Nco* I site, and ligated into the *Eco*RV site of pTSA-NEO2, resulting in the plasmid pLUN100 (Figure 1), with the ATG of luciferase 72 bp downstream from the splice acceptor site. The luciferase gene was later cut from pLUN100 with *Xho* I and *Sma* I and inserted into pTSA-HYG1 cut with the same two enzymes to result in the plasmid pLUH1 that encodes wild-type luciferase and hygromycin resistance.

Mutagenesis of Luciferase

To mutate the peroxisomal targeting signal of luciferase, the luciferase gene was cut from pLUN100 using the flanking *Xho* I and *Sma* I restriction sites, introduced into the Bluescript vector for production of single-stranded DNA (+ strand), and annealed to the 5' phosphorylated degenerate oligonucleotides 5'GTTACATT-TACAATTT(CG)(ACGT)(ACGT)CTTCCGCCCTT, 5'CAGTTAC-ATTTTACA(CG)(ACGT)(ACGT)GGACTTTCCGCCC, or 5'AATACAGTTACATTTTA(CG)(ACGT)(ACGT)TTTGGACTTTCCGC, which allowed for all possible amino acid substitutions at either the serine548, lysine549, or leucine550 position of the luciferase protein. Mutated plasmids were recovered according to the Amersham System Version 2 (Amersham, Arlington Heights, IL) and mutants identified by double-stranded DNA sequencing of the C-terminus of the luciferase gene using the primer luc1: 5'GGAGGAGTTGTGTTTGTGG. The mutations were occasionally verified by sequencing the opposite strand using the primer SV1: 5'TCTGTAGGTAGTTTGTCC. A low amount of readthrough transcription and translation of the luciferase gene was found in transformed *Escherichia coli* cells, which could be detected by adding *E. coli* cells directly to the luciferase assay buffer supplemented with 1% Triton-X100. The presence of luciferase activity could thus be used to rule out at an early stage the possibility of second site mutations that introduce a frame shift. With the above mutagenesis primers, 17 of all 20 possible substitutions were obtained from sequencing 85 to 150 plasmids each. The remaining nine mutants were obtained by mutagenesis with four additional primers of the required specificities. The mutated luciferase genes were cut from pBluescript with *Xho* I and *Sma* I and inserted into pTSA-HYG1 cut with the same two enzymes.

DNA Transformation of *T. brucei*

Plasmid DNA was generally prepared by CsCl density gradient purification (Sambrook *et al.*, 1989). In some cases and for the preparation of all plasmids bearing a mutant luciferase gene, a medium scale procedure was applied that starts with a 5-ml alkaline lysate from cells grown in 50 ml Terrific Broth (Sambrook *et al.*, 1989), followed by RNAase digestion, two extractions with 5 ml chloroform, precipitation with an equal volume of isopropanol, two ethanol precipitations, and a final precipitation with 0.4 M NaCl and 6.5% PEG8000. The identity of each mutation was again verified by double-stranded DNA sequencing of the actual DNA preparation used in the transformation experiment. Fifty to 200 μ g plasmid DNA was linearized at the *Mlu* I site in the tubulin intergenic region, phenol and chloroform extracted, ethanol precipitated, completely dried under vacuum, and suspended in 50 μ l electroporation buffer (132 mM NaCl, 8 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.5 mM MgOAc_2 , 90 μ M CaOAc_2 , 5 mM *cis*-aconitate, 5 mM L-citrate, pH 7.5). The DNA sample was mixed with $3\text{--}5 \times 10^7$ *T. brucei* TREU667 cells washed once in 5 ml cold electroporation buffer, and resuspended in 400 μ l of same on ice. Electroporation was performed with a Gene Pulser apparatus (Bio-Rad, Richmond, CA) by applying three consecutive pulses of 12 500 V/cm and 25 μ F capacitance at 4°C through a pulse controller (Bio-Rad) set at 400 Ω , resulting in 60–95% cell death over a 24-h period of incubation in 5 ml Cunningham medium (Cunningham, 1977) supplemented with 5 mM *cis*-aconitate and 5 mM citrate at 27°C. The total time the cells spent in electroporation buffer was kept to 30 min or less. G418 (GIBCO, Grand Island, NY) or hygromycin B (Calbiochem, La Jolla, CA) was added to 25 μ g/ml of culture 3 d after electroporation and increased to 50 μ g/ml over the next 5 d in the case of G418, at which time the culture medium was replaced and incubation continued in the presence of 10–15 μ g/ml G418 or hygromycin. Drug-resistant cells generally appeared 3–6 wk after transformation. For the molecular analysis of insertion events and transcription, cell lines were cloned in the absence of drug by limiting dilution in 96-well microtiter plates in 50% conditioned Cunningham medium supplemented with 5 mM *cis*-aconitate and 5 mM citrate, followed by undisturbed growth for 2–3 wk at 27°C. Conditioned medium was obtained from TREU667 cells grown to $\sim 5 \times 10^6$ cells/ml and passed through a 0.2- μ m filter. The plating efficiency of this procedure approached 100% in the case of wild-type TREU667 but was somewhat lower for transformed cells expressing high amounts of luciferase.

Digitonin Treatment of Transformed Cells

For the experiment shown in Figure 4, transformants were diluted with wild-type cells to yield $\sim 5 \times 10^7$ cells (200 μ g protein), washed once in cold phosphate-buffered saline, pH 7.5, and suspended in 200 μ l cold HEDSK buffer (Sommer *et al.*, 1990), containing 25 mM HEPES (pH 7.8) 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, 50 mM KoAc, and 10 μ g/ml leupeptin or 50 μ g/ml phenylmethylsulfonyl fluoride. Digitonin was dissolved at increasing concentrations in dimethylformamide and added to the cold cell suspension in a 1- to 4- μ l volume. The mixture was incubated at 37°C for 2 min, vortexed for 10 s, and microfuged at 4°C for 10 min after removing an aliquot for determination of total enzyme activity. The pellet was resuspended in the same buffer. TX-100 and NaCl were added to all fractions to a final concentration of 0.1% and 0.15 M, respectively, before measuring enzyme activities. For the analysis of luciferase mutants, $\sim 3 \times 10^6$ transformed cells were treated as above with 2.5 μ g digitonin, and the luciferase activity was determined in the pellet and supernatant fractions.

Enzyme Assays

Luciferase activity was determined by diluting *T. brucei* cell lysates or digitonin-treated fractions with HEDSK buffer, supplemented with 0.1% TX-100 and 0.1 mg/ml bovine serum albumin, and by measuring the light emission in a liquid scintillation counter (model LS3801, Beckman, Fullerton, CA) within 12–15 s after addition of up to 10 μ l lysate to 100 μ l buffer containing 1 M glycine, pH 7.5, 0.1 M sodium

arsenate (Stanley and Williams, 1969), 5 mM MgCl_2 , 2 mM ATP, 0.1% TX-100 (Kricka and De Luca, 1982), and 0.1 mM D-luciferin. The amount of luciferase protein in the cell lysates was estimated by comparison with known amounts of purified luciferase (Sigma Chemical, St. Louis, MO) assayed at the same time.

Enzyme activities of the cytoplasmic marker glucose-6-phosphate dehydrogenase (G6PDH) and the glycosomal triosephosphate isomerase (TIM) were assayed as described in Bergmeyer (1974). Protein assays were performed by the Bradford (Bio-Rad) procedure, using IgG as a standard.

Immunoelectron Microscopy

Cryoultramicrotomy was performed according to Tokuyasu (1973). Immunolabeling of cryosections with rabbit antibody against firefly luciferase and thin plastic embedding of *T. brucei* cells was performed as described previously by Keller *et al.* (1984, 1991). The immunolabeled sections were observed without poststaining in a transmission electron microscope (model CM12, Philips Electronic Instruments, Mahwah, NJ) equipped with a 10- μ m-diameter objective aperture.

RESULTS

Simultaneous Expression of Luciferase and *neo* in *T. brucei* by Dicistronic Transcription

Transient transformation of trypanosomes with foreign DNA (Clayton *et al.*, 1990; Rudenko *et al.*, 1990; Jefferies *et al.*, 1991) occurs at relatively low frequencies, and we found this technique not to be suitable for the *in vivo* analysis of protein import in *T. brucei*. By targeted insertion of plasmid DNA into the genome, stable transformants of procyclic trypanosomes have been isolated that express the *neo* gene using either the promoter region from the *T. brucei* gene encoding PARP (Lee and Van der Ploeg, 1990) or from a *T. brucei* receptor RNA gene (Rudenko *et al.*, 1991). The *neo* gene could also be stably expressed without the addition of a promoter element by inserting it into an actively transcribed region, such as the tubulin or calmodulin locus (ten Asbroek *et al.*, 1990; Eid and Sollner-Webb, 1991). The presence of a strong promoter region in the plasmid DNA has the advantage that the same plasmid may be used for transient as well stable transformation assays. *Trans* splicing of polycistronic transcripts to produce individual mature mRNAs appears to be a favored mechanism for expression of tandemly repeated genes in trypanosomes (De Lange *et al.*, 1984; Parsons *et al.*, 1984; Agabian, 1990). To achieve stable expression of other genes in addition to *neo*, we reasoned that the presence of a splice acceptor signal in front of each gene should allow simultaneous expression of two plasmid-encoded genes transcribed from a single upstream PARP promoter. The vector pLUN100 thus contains the firefly luciferase gene and the *neo* gene arranged as shown in Figure 1.

Procyclic trypanosomes of *T. brucei* strain TREU667 were transformed by electroporation with pLUN100 DNA linearized at the *Mlu* I site, and exponentially growing cells were observed after 3–5 wk of incubation in the presence of G418, indicating stable expression of the *neo* gene. Lysates from the pLUN100 transformants

Figure 1. Vectors for stable transformation of *T. brucei* and expression of luciferase and *neo*. The plasmid pTSA-NEO2 contains the promoter and splice acceptor site from one of the *T. brucei* PARP genes, followed by a multicloning site (shown in black), a second splice signal preceding the *neo* gene, and the intergenic region of the *T. brucei* tubulin genes (Tubulin IR). A unique *Mlu* I site in the tubulin intergenic region is used to linearize the plasmid for targeted insertion into the tubulin gene cluster by selection of G418-resistant cells that stably express the *neo* gene after transformation. Simultaneous expression of luciferase was achieved by inserting the coding region from the firefly luciferase gene into the multicloning site of pTSA-NEO2, resulting in the plasmid pLUN100 with each gene preceded by a splice acceptor site.

were found to contain up to 5 μ g of enzymatically active luciferase per milligram of total cell protein during steady-state growth, easily detectable by visible light emission after addition of the substrates D-luciferin and ATP to the Triton X-100 lysed culture (Figure 2). The

level of luciferase expression in these transformants was up to 100 times higher than what could be achieved by transient expression of luciferase without the selection for G418-resistant cells. Selection with G418 after transformation of procyclic trypanosomes occasionally resulted in spontaneously resistant cell lines that did not incorporate the plasmid DNA. In agreement with Lee *et al.* (1991), we found that cells spontaneously resistant to hygromycin B appeared at a much lower frequency than observed for neomycin resistance. We therefore replaced the *neo* gene with the hygromycin resistance marker (*hyg*) (Lee *et al.*, 1991) to produce the vector pTSA-HYG1 that was subsequently used for expression of all luciferase mutants.

Southern blot analysis of individual cell lines cloned by limiting dilution revealed that between 10 and 30 plasmid copies had inserted in these strains, distributed over several distinct intergenic regions of the tubulin gene cluster. Transcription from the PARP promoter of the integrated pLUN100 plasmid is expected to result in RNA molecules containing both the luciferase and *neo* transcripts, which should then be *trans* spliced at the splice acceptor site in front of each gene to produce individual mature mRNAs. Using a *neo* and luciferase

TREU667/pLUN100 TREU667 Control

Figure 2. Expression of firefly luciferase in procyclic trypanosomes. TREU667/pLUN100 transformants and untransformed control cultures were grown to 10^7 cells/ml. Luciferin, ATP, and TX-100 were added to 0.1 mM, 2 mM, and 0.1%, respectively, and light emission was immediately photographed for 20 s on 400 ASA film at f1.4. The outline of the culture flasks was recorded by double exposure on the same frame using external illumination.

Figure 3. Northern blot analysis of pLUN100 transformants. RNA was prepared from TREU667/pLUN100 transformants by Guanidine-HCl extraction, separated into poly(A)⁺ and poly(A)⁻ fractions, and electrophoresed through a 1% agarose-formaldehyde gel (Sambrook *et al.*, 1989). Lanes 1, 5, and 9: 20 μ g total RNA from untransformed TREU667 cells; lanes 2, 6, and 10: 20 μ g total RNA from the TREU667/pLUN100 transformants; lanes 3, 7, and 11: 19 μ g of poly(A)⁻ RNA from pLUN100 transformants; lanes 4, 8, and 12: 1 μ g of poly(A)⁺ RNA from pLUN100 transformants. Lanes 1–4 of the corresponding nitrocellulose blot were probed with a β -tubulin HindIII-EcoRV fragment that recognizes a β -tubulin transcript of ~ 1.75 kb (Thomashow *et al.*, 1983). Lanes 5–8 were probed with the Pst I fragment of the *neo* gene (Beck *et al.*, 1982), and lanes 9–12 were probed with the Nco I-EcoRV fragment of the luciferase gene. All probes were labeled with [³²P]-dCTP by random priming (Bethesda Research Laboratories, Gaithersburg, MD). The size markers shown correspond to an RNA ladder (Bethesda Research Laboratories). Posthybridization washes of the blot were done in 0.1 $\times$ SSC at 68°C.

DNA probe, RNA molecules of ~ 2000 and 1800 nt, respectively, were found among the poly(A)⁺ RNA isolated from pLUN100 transformants on a Northern blot (Figure 3). Although we have not shown directly that the 39-nt leader sequence common to all *T. brucei* nuclear mRNA is attached to the 5' end of the plasmid-encoded messages, this leader sequence was found in a *neo* cDNA from a stable transformant of *T. brucei* strain 427-60 (Lee and Van der Ploeg, 1990). The uniform size of the luciferase mRNA indicates that poly(A) addition occurs at a distinct position 100 to 200 nt downstream of the luciferase gene, possibly at a cryptic site within a portion of the RNA transcribed from SV40 DNA (small t antigen intron), which was included on the fragment containing the luciferase gene used for construction of our vector. The size of the *neo* mRNA isolated from pLUN100 transformants suggests that the polyadenylation site within the tubulin intergenic region of the RNA is used in these transcripts. A primary transcript containing both genes could not be detected on this Northern blot, even after a 10 times longer exposure, which indicates that the processing events may occur

cotranscriptionally, as was suggested in the case of the *T. brucei* hsp70 gene (Huang and Van der Ploeg, 1991a).

Targeting of Luciferase to the Glycosome Requires the C-Terminal Tripeptide SKL

We used both digitonin permeabilization and immunoelectron microscopic analysis to localize the luciferase protein in transformed *T. brucei* cells. At low concentrations, the nonionic detergent digitonin selectively permeabilizes the cholesterol-containing plasma membrane of eukaryotic cells, while leaving many organellar membranes lacking cholesterol intact (Schulz, 1990). In *T. brucei*, the solubilization of glycosomal proteins occurs at 10 times higher digitonin concentrations than that at which cytoplasmic markers are released (Visser and Oppendoes, 1980). We incubated transformed cell lines expressing the wild-type luciferase or a mutant lacking the C-terminal SKL sequence (Δ SKL) with increasing amounts of digitonin and measured the luciferase activity released into the supernatant fractions after centrifugation (Figure 4). At the same time, we assayed for release of the cytosolic enzyme G6PDH and the glycosomal enzyme TIM. Approximately 0.5 mg digitonin was required per milligram total cell protein to solubilize 50% of the wild-type luciferase and the glycosomal marker TIM, whereas solubilization of the cytosolic G6PDH and the mutant luciferase was accomplished at 5–10 times lower concentrations of digitonin. These results demonstrate that the mutant luciferase lacking the C-terminal SKL-sequence remains in the cytoplasm of *T. brucei*, whereas the wild-type luciferase may indeed

Figure 4. Digitonin solubilization of luciferase, glucose-6-phosphate dehydrogenase, and triosephosphate isomerase from transformed *T. brucei*. Procyclic TREU667 trypanosomes were transformed with a plasmid encoding wild-type luciferase or the mutant luciferase lacking the C-terminal SKL sequence (Δ SKL). The cells were treated with increasing concentrations of digitonin and centrifuged to remove insoluble material. Enzyme activities were assayed in the supernatant fractions and compared with the total activities found in the intact cells. $\blacktriangle$, G6PDH; $\circ$, TIM; $\square$, wild-type luciferase; $\blacksquare$, SKL deletion mutant.

be localized inside a membrane-bound organelle, probably the glycosome. To definitively establish the subcellular localization of luciferase, thin cryosections of a cell line expressing wild-type luciferase and the Δ SKL mutant were reacted with an antibody raised against firefly luciferase (Keller *et al.*, 1987) followed by secondary antibody conjugated to 10 nm colloidal gold particles. Wild-type luciferase produced in these cells was found almost exclusively inside the glycosomes (Figure 5A). A very small number of gold particles were also found in the cytosol, which may represent the enzyme en route to the glycosomes. In the cells that expressed the mutant luciferase lacking the C-terminal SKL tripeptide, no gold particles were found inside the glycosomes (Figure 5B). However, we occasionally observed some accumulation of mutant luciferase in the nucleus of transformed cells. This may be due to a weak nuclear targeting signal present in the luciferase protein, which could direct the protein to the nucleus in the absence of a strong glycosomal targeting signal. The C-

terminal sequence -RKIREILIKAKKGGKSKL of luciferase indeed bears some resemblance to the bipartite nuclear targeting signals found in many nuclear proteins (Dingwall and Laskey, 1991). The partial nuclear localization of the Δ SKL mutant (Figure 5B) was, however, not represented in the pelletable fraction after digitonin extraction. We thus conclude that any mutant luciferase present in the nucleus was released during the digitonin treatment and cell fractionation.

Tripeptide Variants that can Function as Glycosomal Import Signals in T. brucei

A limited number of conservative substitutions in the peroxisomal targeting signal of luciferase that still allow import into mammalian peroxisomes have been described (Gould *et al.*, 1989). To determine the exact glycosomal requirements for protein import, we have substituted each of the three amino acids of the SKL sequence for all other 19 amino acids or a termination

Figure 5. Cryosections of *T. brucei* procyclic cells expressing the wild-type or mutant firefly luciferase. Electron-dense gold particles represent the distribution of luciferase. (A) Wild-type luciferase is localized exclusively to the glycosomes (G). (B) Cytoplasmic distribution of a mutant luciferase protein lacking the C-terminal tripeptide (Δ SKL). Some mutant luciferase is also found in the nucleus (N). (C) Partial glycosomal localization of a luciferase mutant ending in YKL. (D) Distribution of a luciferase mutant ending in SKV. Bars, 0.2 μ m.

codon (Figure 6). The relative amounts of luciferase activity that were pelletable after digitonin extraction of transiently transformed cell cultures were used as an indicator of protein import into the glycosome. We have chosen this method because the trypanosomes are too small for fluorescent microscopic analysis of subcellular localization. It is a rapid and extremely sensitive technique, with high reproducibility and easy quantitation. Actual import of luciferase was verified by immunoelectron microscopy in several cases and agreed closely with the digitonin solubilization results. Our results indicate that the serine at position 548 of luciferase can be replaced by alanine, cysteine, and asparagine without significantly lowering the import efficiency (Figure 6). A substitution of S548 to proline, glycine, and histidine also appear to be well tolerated, with $\geq 50\%$ of the wild-type import. The lysine549 can be replaced by histidine and arginine, as found in the peroxisomal studies, but also by glutamine, asparagine, methionine, and serine. The amino acid requirement at the extreme C-terminus exhibited the most selectivity; only isoleucine, methionine, and tyrosine allowed wild-type levels of import into the glycosome. A surprisingly large number of mutations, but none of the three deletion mutants (termination codons), resulted in a partial digitonin solubilization of the luciferase activity. The percentage of luciferase distribution in such mutants remained the same both during transient transformation and when assayed in stable transformants. In the mutant S548Y, which was studied in more detail, the luciferase distribution remained constant at $38 \pm 7\%$ over at least a 50-fold range when assayed from 2 h after transformation to 48 h or when compared with a stable transformant with high specific activity. Two mutants in this class, S548Y and L550V, were analyzed by electron microscopy, which confirmed that the luciferase protein was distributed both inside the glycosome and in the cytoplasm (Figure 5, C and D). In none of our studies did we detect an accumulation of luciferase molecules at the perimeter of the glycosome.

The luciferase protein is relatively sensitive to protease digestion both in vivo and in vitro. The half-life of luciferase in mammalian cells has been estimated at 3 h and in *E. coli* at 45 min (Thompson *et al.*, 1991). A disproportionately high turnover rate of luciferase in either the glycosome or the cytoplasm of *T. brucei* might affect the apparent import efficiency of our luciferase mutants. We thus compared the stability of glycosomally located (wild-type) with cytoplasmic (Δ SKL) luciferase in the presence of 50 $\mu\text{g}/\text{ml}$ cycloheximide, a concentration that we have shown previously to completely block protein synthesis of *T. brucei* strain TRUE667 under our culture conditions (Bass *et al.*, 1992). Interestingly, luciferase activity remained relatively stable in these cultures after addition of cycloheximide (Figure 7), with a half-life of both glycosomal and cytoplasmic luciferase activity exceeding 48 h. We thus concluded that the

Figure 6. Digitonin permeabilization of trypanosomes expressing mutant luciferase proteins. Luciferase assays were performed 2 d after transformation of procyclic TREU667 trypanosomes with linearized plasmids encoding wild-type or mutant luciferase. The percentage of luciferase activity recovered in the pellet fraction after digitonin extraction and centrifugation is shown for each mutant. Error bars correspond to $\pm$ SD of at least two independent transformation experiments with multiple assays each, and include the results from stable transformants where available. The right hand column indicates the localization of luciferase mutants tested in mammalian CV-1 cells, based on Gould *et al.* (1989), where P = peroxisomal localization and C = cytosolic localization, as determined by indirect immunofluorescence analysis. ^a, AKI also functions as the signal for import of hydratase-dehydrogenase-epimerase (HDE) into peroxisomes of *C. tropicalis* (Aitchison *et al.*, 1991) ^b, Not tested in CV-1 cells, but GKI can target HDE to the peroxisomes of *C. tropicalis*. ^c, Not tested in CV-1 cells, but AQI can target HDE to the peroxisomes of *C. tropicalis*.

Figure 7. In vivo half-life of glycosomal and cytoplasmic luciferase. Cycloheximide (CHX) was added at time 0 to exponentially growing stable transformants of *T. brucei* expressing wild-type or mutant (Δ SKL) luciferase. Aliquots of each cell culture were removed at the indicated time points and assayed for total activity of wild-type ($\square$) and mutant (Δ) luciferase without cycloheximide treatment and wild-type ($\blacksquare$) and mutant ($\blacktriangle$) luciferase in the presence of 50 μ g/ml cycloheximide. Error bars indicate $\pm$ SD of three experiments.

apparent subcellular localization of luciferase is unlikely affected by its turnover in exponentially growing cells.

DISCUSSION

We have constructed transformation vectors that allow the simultaneous stable expression of multiple foreign genes in *T. brucei*, with either *neo* or *hyg* as a selectable marker. Huang and Van der Ploeg (1991b) have independently made a similar construct that incorporates the PARP promoter and a *neo* and *cat* gene, each preceded by a splice acceptor site. Using our vectors, we have successfully expressed the CAT gene, mouse and *T. brucei* ornithine decarboxylase (Bass *et al.*, 1992), and firefly luciferase (this work) in procyclic trypanosomes.

Hypotheses on how proteins are targeted to the glycosome have been presented in the past (Wierenga *et al.*, 1987; Swinkels *et al.*, 1988). However, except for the transient expression of a CAT-SKL fusion protein (Fung and Clayton, 1991), none of them have been tested in a functional in vivo assay. Our results show unambiguously that when the cDNA encoding firefly luciferase is expressed in *T. brucei*, the protein is imported into the glycosomes. The SKL targeting signal that directs firefly luciferase to the peroxisomes in mammalian cells is also necessary for import of the enzyme into the glycosomes in *T. brucei*. Thus, those properties unique to the glycosomal enzymes, such as the surface clusters of positive charges (Wierenga *et al.*, 1987), may not be required for protein import into the glycosome, at least for those proteins that end in an

SKL-like targeting signal. The functional conservation of this protein import thus supports the previous evidence for a close evolutionary relationship between glycosomes and peroxisomes, despite the vastly different biochemical functions carried out in the two classes of microbodies (Borst, 1986; Michels and Opperdoes, 1991).

The question whether a C-terminal tripeptide is sufficient for the import of any protein into peroxisomes or glycosomes has not been resolved clearly. An SKL-like sequence fused to the C-terminus of CAT can direct this protein to mammalian peroxisomes (Gould *et al.*, 1989) and probably to the glycosomes of *T. brucei* (Fung and Clayton, 1991). Similarly, a dihydrofolate reductase (DHFR)-SKL fusion protein is targeted to the mammalian peroxisome (Distel *et al.*, 1992). On the other hand, neither the DHFR-SKL fusion protein (Distel *et al.*, 1992) nor a CAT with a C-terminal AKI fusion protein (Aitchison *et al.*, 1991) appears to be peroxisomal when expressed in the yeast *Saccharomyces cerevisiae*. Thus, either *S. cerevisiae* requires additional signals for peroxisomal targeting or some posttranslational event, such as aggregation, proposed by Aitchison *et al.* (1991), prevents these fusion proteins from entering the yeast peroxisome.

We substituted each of the three amino acids in the C-terminal SKL sequence of luciferase and determined the effect of the resulting 60 mutations on the import of the protein into the glycosome. With the exception of AKI, which we recently have constructed and also found to support import of luciferase into the glycosome, we have not tested other double or triple mutant combinations of amino acid substitutions that might allow import. Many such multiple combinations, however, may constitute an effective targeting signal for luciferase. From our present results, we reached the following conclusions.

A much larger number of amino acid substitutions resulted in glycosomal import than was expected from previous studies on peroxisomes (Gould *et al.*, 1988, 1989). In addition to the basic amino acids histidine and arginine, several other amino acids capable of forming hydrogen bonds can substitute for the lysine549 residue in the import of luciferase into glycosomes. This finding may also apply to peroxisomal targeting, because a lysine to glutamine substitution in the C-terminal targeting signal of the hydratase-dehydrogenase-epimerase in the yeast *Candida tropicalis* did not affect the import of this protein into peroxisomes (Aitchison *et al.*, 1991). Thus, the C-terminal tripeptide ANL found in catalase of both human (Korneluk *et al.*, 1984) and rat (Furuta *et al.*, 1986) should be reevaluated as a potential targeting signal, despite the proposition by Gonzalez (1991) that conserved internal SKL-like sequences found in many catalases might function as targeting signals.

At the extreme C-terminus, the targeting signal of luciferase is not restricted to leucine. Isoleucine, me-

thionine, and tyrosine can effectively substitute for this amino acid in glycosomal import. Several examples of a methionine at this position were also found on peroxisomal proteins, most notably in the Japanese firefly luciferase ending in AKM (Masuda *et al.*, 1989) and isocitrate lyase in glyoxysomes of cotton (Turley *et al.*, 1990) and castor beans (Beeching and Northcote, 1987).

Two clear differences between the specificities of glycosomal versus mammalian peroxisome targeting signals emerged when we compared our results with the 20 mutants of luciferase tested in monkey kidney CV-1 cells (Gould *et al.*, 1989). First, a lysine549 to serine substitution in luciferase is imported into glycosomes, but the same mutation abolished the import of the protein into mammalian peroxisomes. Interestingly, *T. brucei* glycosomal phosphoglycerate kinase (gPGK) ends in the sequence SSL (Osinga *et al.*, 1985), making it the likely targeting signal. A serine in the central position of the C-terminal tripeptide is also found on several plant peroxisomal proteins, including a peroxidase enzyme in potato (Roberts *et al.*, 1988) and tomato (Roberts and Kolattukudy, 1989) ending in ASM and catalase of cotton (Ni *et al.*, 1990) and maize (Bethards *et al.*, 1990) ending in PSI and PSM, respectively. Second, replacement of leucine with isoleucine at the extreme C-terminus of luciferase did not affect the import of the protein into *T. brucei* glycosomes, whereas this mutation resulted in a cytoplasmic localization of luciferase in mammalian cells (Gould *et al.*, 1989).

It is likely that the tripeptide sequences AKL, ARL, and SHL found at the C-terminals of *T. brucei* and *T. cruzi* gGAPDH and *T. brucei* PGI, respectively, could function as the targeting signals for glycosomal import of these proteins. However, of the nine glycosomal enzymes sequences that are known, *T. brucei* fructose biphosphate aldolase (Clayton, 1985), *T. brucei* triosephosphate isomerase (Swinkels *et al.*, 1986), *T. brucei* phosphoenolpyruvate carboxykinase (Kueng *et al.*, 1989; Parsons and Smith, 1989), as well as a minor glycosomal phosphoglycerate kinase encoded by the A gene of the *T. brucei* PGK gene cluster (Alexander *et al.*, 1990), still do not contain an obvious C-terminal tripeptide sequence that might function in glycosomal import by our present findings. These proteins may rely on a somewhat different import mechanism. Using our newly constructed transformation vectors, we are now in a position to express those proteins at high levels in trypanosomes to investigate alternate and possibly unique mechanisms of protein targeting to glycosomes.

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