Targeting of passenger protein domains to multiple intracellular membranes

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The role of passenger domains in protein targeting was examined by fusing previously characterized targeting motifs to different protein sequences. To compare the targeting requirements for a variety of subcellular compartments, targeting of the fusion proteins was examined for endoplasmic reticulum, mitochondria and peroxisomes *in vitro* and in yeast. Although most passenger domains were only partially passive to translocation, motif-dependent targeting via motifs positioned at either end of one passenger domain (gPA) was demonstrated for all of the subcellular compartments tested. The data presented extend earlier suggestions that translocation competence is an intrinsic property of the passenger protein. However, the properties that determine protein targeting are not mutually exclusive for the compartments tested. Therefore, although the primary determinant of specificity is the targeting motif, our results suggest that translocation competence of the targeted protein augments the fidelity of transport.

INTRODUCTION

The delivery of proteins to different subcellular locations is mediated by short conserved protein sequence motifs that are recognized by components of the cellular targeting machinery. In many cases the conservation of sequence within a class of motifs is limited to patterns of charge and/or hydrophobicity. As a consequence, it has been suggested that the context of presentation is important for efficient recognition of the motif (Roberts et all., 1987; Andrews et al., 1988).

Several other lines of evidence suggest that context of presentation may be one aspect of a general mechanism that maintains the specificity of cellular targeting systems by placing constraints on the polypeptides being transported. The observation that deletion of the propeptide from preproapoA-I reduces the efficiency of co-translational translocation and processing is also consistent with this notion (Folz and Gordon, 1987). More striking is the demonstration that, without a functional signal peptide, 30% of wild-type translocation efficiency is achieved for the cell wall acid phosphatase across the yeast endoplasmic reticulum (ER) membrane (Silve et al., 1990). Furthermore, a signal sequence is not required for recognition and export of secretory proteins in *prlA* mutants of *Escherichia coli* (Derman et al., 1993). As the other known components of the secretory pathway are required for export of these proteins, features in the mature protein must be recognized by the translocation machinery (Derman et al., 1993). Finally, the insertion of membrane proteins into the ER membrane of *Saccharomyces cerevisiae* can be inhibited by C-terminal sequences (Green and Walter, 1992).

The observation that many different targeting motifs tend to be located at one of the two ends of the protein is consistent with the context of presentation contributing to recognition of the motif. Included in this class of targeting elements are those specifying localization to the ER, mitochondria, peroxisomes, chloroplasts and other subcellular membranes (Verner and Schatz, 1988). Well-characterized exceptions to the N- or C-terminal location of targeting motifs include nuclear localization sequences (Roberts et al., 1987; Silver, 1991) and peptide sequences that target cytosolic proteins to lysosomes for proteolysis (Dice, 1990). In the case of nuclear localization sequences, the effect of protein context on recognition of the targeting motif can be dramatic (Roberts et al., 1987). In contrast with nuclear localization elements and the N-terminal motifs described above, the effect of context on C-terminal elements remains to be rigorously defined.

A variety of protein domains have been used previously to identify and characterize targeting signals, including (but not limited to) alkaline phosphatase, dihydrofolate reductase, β-galactosidase, β-lactamase, chloramphenicol acetyltransferase, invertase and globin (Yost et al., 1983; Hurt et al., 1984; Lingappa et al., 1984; Manoil and Beckwith, 1986; Zerial et al., 1986; Haeuptle et al., 1989; Broome-Smith et al., 1990). However, some of these protein domains can be directed to only a subset of organelles (Kaiser and Botstein, 1990; Aitchison et al., 1991; Distel et al., 1992; Kragler et al., 1993). Furthermore, at least one cytosolic protein (mouse dihydrofolate reductase) has been shown to contain a cryptic mitochondrial import sequence (Hurt and Schatz, 1987).

To begin to dissect regulatory mechanisms for subcellular targeting systems we have examined the subcellular localization of three protein domains when fused to well-characterized targeting motifs. We report here that a protein domain previously characterized as being passive to signals fused to the N-terminus is not necessarily passive to signals at other positions. Furthermore, a passenger domain derived from the extracellular portion of *Staphylococcus aureus* Protein A is not passive to translocation across the ER membrane. However, a relatively small change converted this non-translocatable domain into one passive for import into multiple organelles both *in vitro* and *in vivo*. Moreover this domain, termed gPA, is passive to signals positioned at either the N- or C-terminus. We have used this domain to demonstrate that the C-terminal SKL sequence previously shown to be necessary for peroxisomal import in the yeast *S. cerevisiae* is also sufficient for targeting to peroxisomes *in vivo*. This result contrasts sharply with those obtained previously for targeting in yeast via SLK peptides fused to less rigorously defined passenger domains.

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; AOX, acyl-CoA oxidase; ER, endoplasmic reticulum; SRP, signal-recognition particle; UCP, uncoupling protein; YNB, yeast nitrogen base.

EXPERIMENTAL

Materials

Restriction endonucleases were purchased from New England Biolabs. SP6 RNA polymerase was from Epicenter Technologies. The RNAase inhibitor RNA guard was from Pharmacia LKB. [^{35}S]Methionine was from DuPont–New England Nuclear.

Recombinant DNA constructs

The PA passenger domain consists of amino acids 23–271 (the IgG-binding domains) of the mature Protein A from *Staph. aureus* (Uhlen et al., 1984). This segment does not include the N-terminal signal sequence or the membrane-binding domain of Protein A and therefore the protein behaves like a soluble cytoplasmic polypeptide (Nilsson et al., 1985). The gPA passenger contains the complete PA domain fused to 27 amino acids from the N-terminus of a domain of chimpanzee α-globin, which was previously modified to contain a glycosylation site (Perara and Lingappa, 1985). All constructs were cloned behind the SP6 RNA polymerase promoter in our standard cloning vector pSPUTK (Falcone and Andrews, 1991).

pSPgPA

The coding region for glycoglobin was cloned into pSPUTK as an *Nco*I–*Eco*RI fragment. To facilitate subsequent cloning steps, the region encoding the IgG-binding domains of Protein A (from PRIT 2T; Pharmacia) were subcloned into the multiple cloning site of pSPUTK to generate the plasmid pSPUTKPA. The coding region for the IgG-binding domains of Protein A (contained in a *Bgl*II–*Pvu*I fragment of pSPUTKPA) was subsequently cloned into the *Bam*HI site of the glycoglobin gene and the *Pvu*I site within the β-lactamase gene in pSPUTK. To facilitate further plasmid construction, a *Bam*HI 8-mer linker was inserted into the *Nru*I site at the 3′ end of the Protein A-coding region directly 5′ of the stop codon. For most coding regions, termination occurs at a TAA codon 21 codons 3′ of this *Bam*HI site. The resulting passenger protein was designated glycoglobin Protein A (gPA). A map of the plasmid encoding gPA (pSPMP366) is illustrated in Figure 1. Diagrams of the subsequent related plasmids indicating the restriction sites used to assemble the different coding regions are also shown (Figure 1). To re-create an in-frame stop codon at the *Bam*HI site, a *Xba*I 8-mer linker was inserted into the site after restriction endonuclease digestion with *Bam*HI and end-repair with the Klenow fragment of DNA polymerase I. This adds the following peptide to the end of the Protein A-coding region before the STOP codon: Ser-Arg-Ile-Leu.

pSPSpgPA

The coding region for the glycoglobin sequence contains a *Bss*HII cleavage site near the 5′ end. Therefore the coding region of gPA was inserted as a *Bss*HII–*Bam*HI fragment into a construct containing the signal sequence of bovine preprolactin fused to the 5′ end of glycoglobin (pSPtp462; Andrews et al., 1992). This manipulation replaced most of the glycoglobin-coding sequence with the corresponding region of gPA.

pSPSlSTgPA

A plasmid containing the coding region of the IgM stop-transfer sequence fused to the 5′ end of glycoglobin has been described elsewhere (Rothman et al., 1988). The coding region of this plasmid is flanked by *Nco*I and *Pst*I cleavage sites at the 5′ and 3′ ends respectively. Therefore the sequence encoding the IgM transmembrane domain fused to glycoglobin was added to a plasmid (pSPMP469) that encodes the lactamase signal sequence with *Nco*I and *Pst*I cleavage sites at the 3′ end. The resulting plasmid (pSPMP473) encodes the lactamase signal sequence fused to the N-terminus of the IgM stop-transfer and glycoglobin sequences. The glycoglobin-coding region was then replaced with one encoding gPA (as above for pSPSpgPA) by inserting the PA domain into pSPMP473 after digestion with *Bss*HII and *Pvu*I.

pSPpOCTgPA

The gPA domain was inserted as a *Bgl*II–*Eco*RI fragment into pSP019 (Nguyen and Shore, 1987), a recombinant plasmid containing the coding region for rat preornithine carbamoyltransferase (pOCT). The resulting plasmid codes for the 32 amino acids of the pOCT mitochondrial presequence and 29 amino acids of the mature protein followed by the gPA domain.

pSPgPASKL

The plasmid pSPgPA was digested with *Bam*HI, and pairs of complementary synthetic oligonucleotides encoding Ile-Leu-Ser-Lys-Leu-STOP or Ile-Leu-STOP containing GATC 5′ overhangs were inserted, resulting in the plasmids pSPgPASKL and pSPgPASTOP respectively. For expression in yeast, coding sequences for gPASTOP and gPASKL were inserted into pSG522 (a gift from Dr. S. Subramani, University of California, San Diego, CA, U.S.A.). This plasmid encoding *S. cerevisiae* 3-oxoacyl-CoA thiolase regulated by the acyl-CoA oxidase (AOX) promoter (Dmochowska, et al., 1990) was constructed in pRS315 (Sikorski and Hieter, 1989) and is described in detail elsewhere (Glover et al., 1994). The plasmid pRS315 is a Bluescript-based vector containing the *S. cerevisiae LEU2* gene which complements leucine auxotrophy as well as *CEN6* and *ARSH4* sequences to permit autonomous replication in yeast. The plasmid pSG522 was digested with *Xba*I, end-repaired with Klenow fragment of DNA polymerase and digested with *Nco*I to remove the entire thiolase-coding region. Both pSPgPASKL and pSPgPASTOP were digested with *Eco*RV and *Nco*I and the coding regions for the gPA fusion proteins were inserted into the prepared yeast expression vector to yield pgPASKL-AOX and pgPASTOP-AOX respectively. These plasmids were then used to express the fusion proteins gPASKL and gPASTOP in yeast.

pSPgPACb5

The coding region of the hydrophobic C-terminus of rat cytochrome b_5 (35 amino acids) was excised from a PGEM1 plasmid containing the complete coding sequence (a gift from Dr. David Meyer, University of California, Los Angeles, CA, U.S.A.) as a *Bcl*I–*Pvu*I fragment. The ends generated by *Bam*HI and *Bcl*I are complementary, therefore the coding region for the cytochrome b_5 hydrophobic domain was inserted directly into pSPgPA after digestion with *Bam*HI and *Pvu*I.

pSPSpPA

A plasmid containing the coding region for the signal peptide of bovine preprolactin and the codon for the first amino acid of prolactin as part of a *Sal*I site has been described elsewhere (Andrews et al., 1988). To insert the IgG-binding domains of Protein A into this plasmid, *Sal*I and *Xho*I restriction sites were added to the 5′ end of the sequences encoding the IgG-binding domains of Protein A. Then both plasmids were digested with *Sal*I and *Eco*RI and the region coding for the IgG-binding domains of Protein A was inserted into the vector containing the

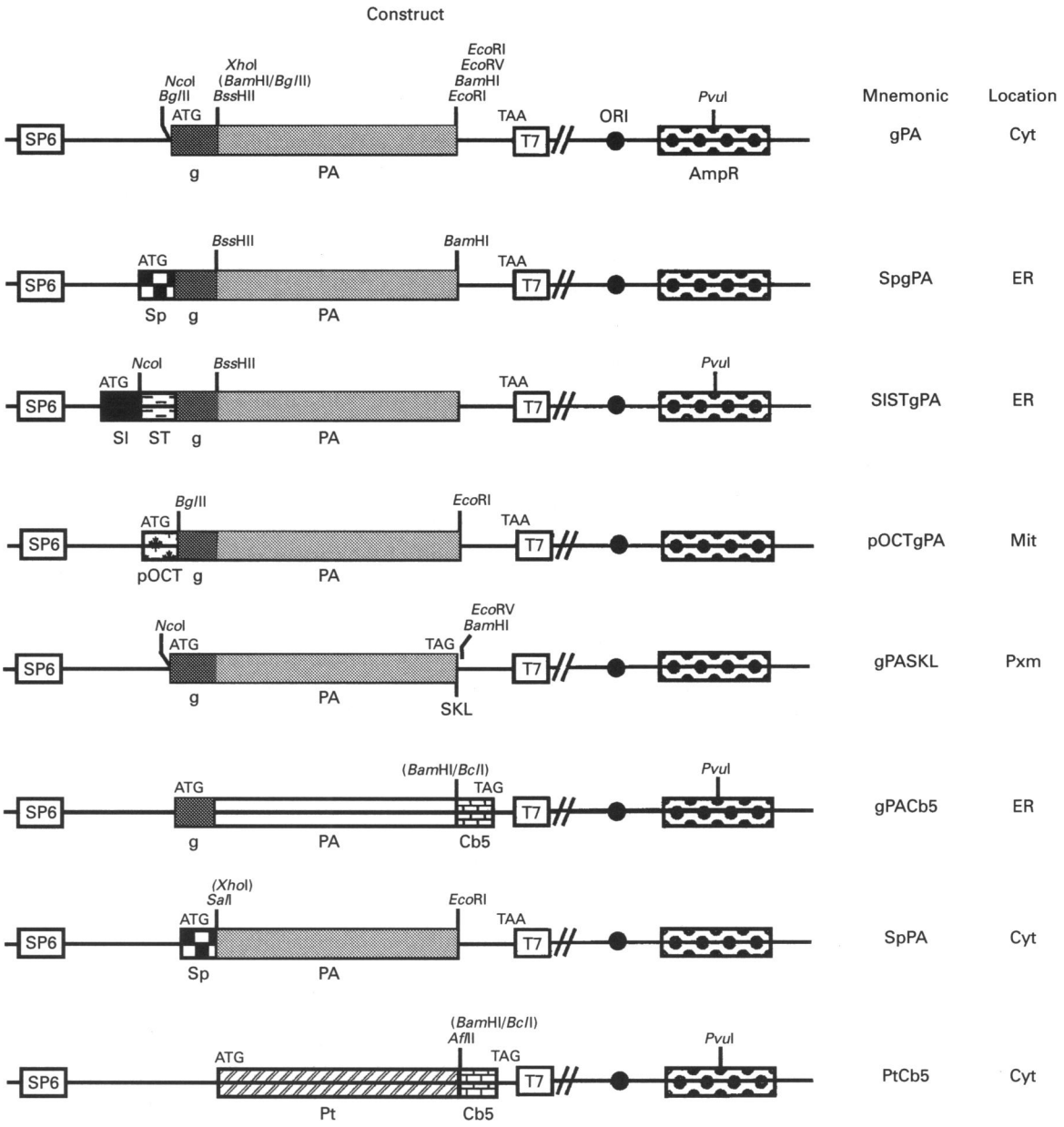

Figure 1 Schematic representations of the plasmids and fusion proteins

The individual subdomains of each molecule are represented by boxes. The restriction sites and other plasmid sequences relevant to the construction of the plasmids are indicated above the diagrams. The name of each coding region is listed below the diagram. The mnemonic for and subcellular location of each fusion protein are given to the right of the diagrams. The designation of the subdomains is: g, 27 amino acids derived from a globin passenger domain; PA, IgG-binding domains of Protein A; Sp, secretory signal sequence from preprolactin; Sl, lactamase signal sequence; ST, IgM stop-transfer domain; pOCT, mitochondrial import sequence from ornithine carbamoyltransferase; Cb5, C-terminal membrane-insertion sequence from cytochrome b_5; Pt, amino acids 58–199 of prolactin. Plasmid designations: SP6 and T7, promoter sequences for SP6 and T7 polymerases respectively; ORI, origin of replication; ATG, start codon; TAA and TAG, termination codons; restriction sites indicated in parentheses were lost during the manipulations. Additional designations: Cyt, cytosol; ER, endoplasmic reticulum; Mit, mitochondria; Pxm, peroxisomes.

preprolactin signal to generate the plasmid pSPMP305. To co-ordinate the reading frames of the preprolactin-signal sequence and the IgG-binding domains, the plasmid pSPMP305 was cut with *Xho*I, end-repaired with the Klenow fragment of polymerase and religated.

pSPPtCb5

The plasmid containing the Pt passenger (amino acids 58–199 of prolactin) has been described elsewhere (Rothman et al., 1988; Andrews et al., 1992). A *Bam*HI 10-mer linker was inserted into the *Afl*II site within the coding region of Pt after digestion with *Afl*II and end-repair with Klenow fragment of DNA polymerase. This allowed fusion of the Pt coding region with that for the hydrophobic C-terminus of rat cytochrome b_5 (as above for pSPgPACb5) at amino acid 188 of prolactin.

The fusion junctions and inserted oligonucleotides in all plasmids were sequenced with $Vent_R$ DNA polymerase (New England Biolabs) which was used according to instructions provided by the manufacturer. Complete details regarding the

construction of any of the plasmids are available from the authors.

Membranes and organelles

Salt-extracted canine pancreas microsomal membranes were prepared as described (Walter and Blobel, 1983). Rat heart mitochondria (at 0.75 mg/ml protein) were prepared immediately before use from neonatal rats (5–10-day-old Sprague–Dawley rats) as described by Argan et al. (1983). The P100 membrane fraction of Rat-2 cell was prepared as described previously (Andrews et al., 1993).

Cell-free transcription and translation

Transcription of SP6 plasmids was as described (Gurevich et al., 1991). Transcription-linked translation reactions (10 μl) were performed as described previously (Andrews, 1989) in a rabbit reticulocyte lysate system containing 10% (v/v) transcription reaction mixture and 10 μCi of [^{35}S]methionine in translation buffer (10 mM Hepes, 100 mM potassium acetate, 2 mM $MgCl_2$, pH 7.5). Wheat germ translation reactions and translation arrest assays were performed as described previously (Andrews, 1989). Endoglycosidase H digestions were performed using a maltose-binding protein–endoglycosidase H fusion protein purchased from New England Biolabs. Digestion conditions were as specified by the manufacturer. Protease protection and sedimentation assays were performed as described (Andrews, 1989). Where indicated, Protein A-containing constructs were precipitated using IgG–Sepharose (Pharmacia). For extraction with Na_2CO_3, microsomes were collected from a translation reaction mixture by centrifugation through a sucrose cushion (0.5 M sucrose in translation buffer) in an airfuge (Beckman Instruments) for 10 min at 138 kPA in an A-100 30° rotor (120000 *g*) at 4 °C. After resuspension of the pellets in 25 μl of translation buffer, 75 μl of 133 mM Na_2CO_3 was added (final pH 11.5) and samples were incubated at 0 °C for 30 min. Collection of extracted membranes and soluble proteins was as described previously (Andrews et al., 1992). Mitochondria (75 μg of protein) were added to samples containing 3 μl of the translation reaction mixture and 47 μl of reticulocyte lysate in the presence or absence of 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma) followed by incubation at 30 °C for 60 min. Proteolysis of mitochondria-import-reaction mixtures and Na_2CO_3 extraction of mitochondria were performed as described above for microsomes, except that, after the import reaction, mitochondria were collected by centrifugation for 5 min at 12000 *g* at 4 °C and washed twice with mitochondria resuspension buffer (250 mM sucrose, 10 mM Hepes, 2 mM K_2HPO_4, 5 mM sodium succinate, 1 mM dithiothreitol, 1 mM ATP, 80 μM ADP, pH 7.2). The final mitochondrial pellet was resuspended in 100 μl of 0.1 M Na_2CO_3 (pH 11.5) and processed as described above.

Expression in yeast

S. cerevisiae strain DL-1 (matα, leu2, ura3, his3) was used for expression of the recombinant proteins. Exponential-phase cells were washed three times in water and resuspended in a minimal volume of 1 M sorbitol. Plasmid DNA was introduced by electroporation as described (Becker and Guarente, 1991). Immediately after electroporation, cells were transferred into 0.1 ml of 1 M sorbitol and plated on to selective medium. Transformants were selected and maintained on YNBD agar + his + ura [0.67% yeast nitrogen base (YNB) without amino acids, 2% (w/v) glucose, 20 μg/ml histidine and 20 μg/ml uracil]. For induction, 10 ml of an overnight culture grown in YNBD agar was pelleted, washed in water, and used to inoculate 500 ml of SCIM [0.67% YNB, 0.5% yeast extract, 0.5% peptone, 0.5% (w/v) Tween 40, 0.1% glucose, 0.1% (w/v) oleic acid] containing histidine and uracil as above (Erdmann et al., 1989). Cells were grown for 20 h, then harvested and fractionated as previously described (Lewin et al., 1990). Samples of postnuclear supernatant, 20000 *g* supernatant, 20000 *g* pellet, all equivalent to 1% of the total cell fraction, and 5 μg each of mitochondrial and peroxisomal proteins, were separated by SDS/PAGE and transferred electrophoretically to nitrocellulose. The membrane was probed with alkaline phosphatase-conjugated rabbit IgG and the blot was developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as recommended by the manufacturer (BRL).

RESULTS

Diagrams of the various plasmids and fusion proteins used in this study are presented in Figure 1 along with the subcellular localization determined for each of the proteins. The names of the subdomains that make up the fusion proteins are indicated beneath the appropriate diagrams in Figure 1. As a convenient nomenclature, the names assigned to the various subdomains are listed in the order that they occur in the fusion proteins. Therefore the molecule designated SpgPA contains the preprolactin signal sequence (Sp), the globin-derived sequence (g) and the IgG-binding domain of Protein A (PA) fused in that order.

Targeting to ER

To examine translocation of the fusion proteins across the ER membrane, plasmids encoding the fusion proteins were transcribed *in vitro* using SP6 polymerase followed by translation of the transcription products in a reticulocyte lysate protein-synthesizing system. Translations were carried out in the presence or absence of canine pancreatic microsomal membranes. Translocation of the ER membrane was most conveniently assayed by the appearance of signal-cleaved and glycosylated products. The gPA passenger contains five potential glycosylation sites (one in the globin domain and four in the PA domain). Translocation of the passenger domains was confirmed by protection of signal-processed and/or glycosylated, but not unprocessed, molecules from exogenously added proteinase K, with protection abolished by solubilization of the membrane with non-ionic detergent. Digestion with endoglycosidase H was used to confirm that molecules with decreased mobilities in SDS/PAGE had been glycosylated. A polypeptide was judged integral to the membrane if it was not extracted from membranes by incubation in 0.1 M Na_2CO_3 (pH 11.5) (Fujika et al., 1982; Andrews, 1989).

The IgG-binding domain from Protein A (PA) has been shown to behave as a soluble cytoplasmic protein (Nilsson et al., 1985). To determine whether this domain is also passive to membrane translocation (as expected from its normally extracellular location), a plasmid was constructed encoding a molecule termed SpPA containing the signal sequence of preprolactin (Sp) fused to the N-terminus of PA. Expression of SpPA in reticulocyte lysate resulted in a molecule that was degraded by protease and therefore not translocated into the lumen of the ER (Figure 2a, lanes 1–4).

It has been reported that the amino acids (approx. 20) immediately C-terminal to the site of signal-sequence cleavage

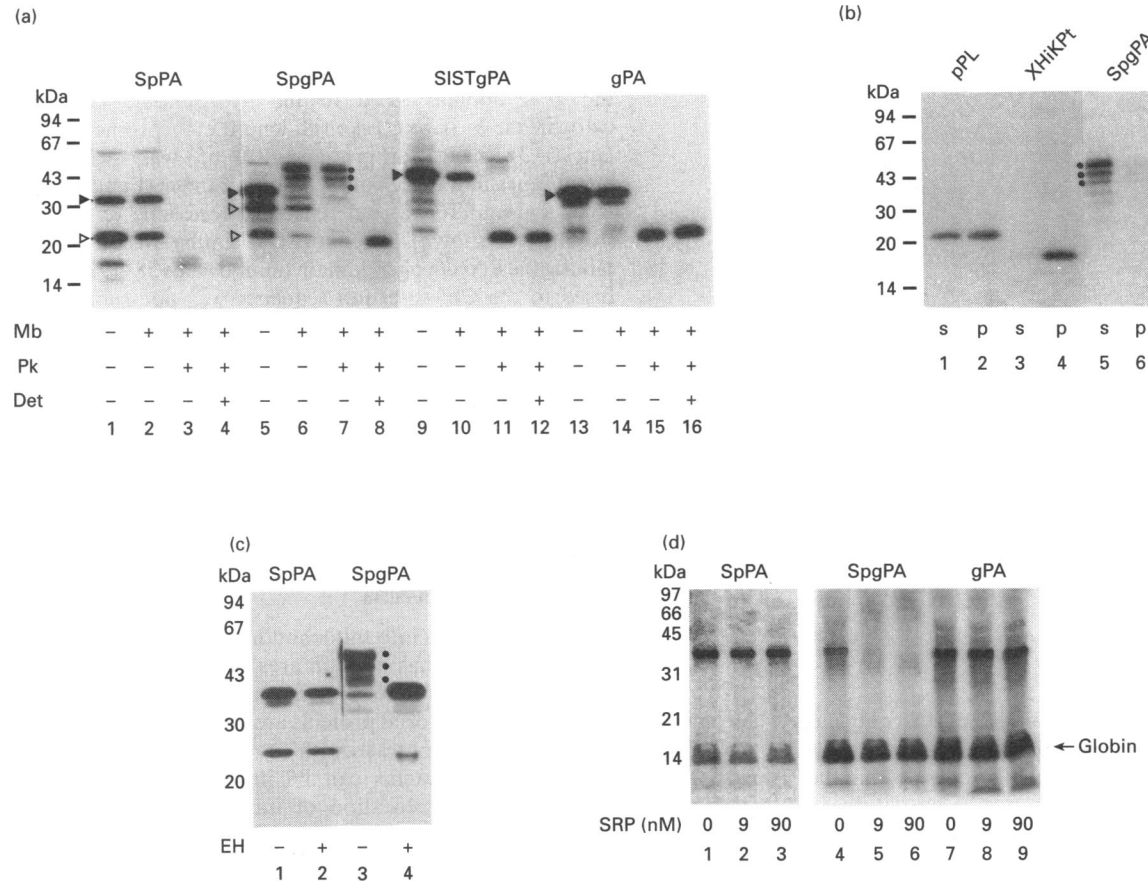

Figure 2 Targeting of fusion proteins to ER

(**a**) Co-translational targeting of fusion proteins with N-terminal targeting elements. SP6 polymerase transcription products for each of the molecules were translated in reticulocyte lysate in the presence or absence of canine pancreatic rough microsomes (Mb) as indicated. Translation products were subjected to proteolysis with proteinase K (Pk). As a control, the detergent Triton X-100 (Det) was added to 1% (v/v) to solubilize the microsomes in some reactions. Proteins were precipitated with IgG–Sepharose before analysis by SDS/PAGE. Full-length translation products are marked with an arrowhead to the left. Open arrowheads indicate translation products with migration positions consistent with the initiation of translation at an internal methionine. Glycosylated products of SpgPA are marked with a dot. (**b**) Membrane integration of fusion proteins with N-terminal targeting elements. SP6 polymerase transcription products for each of the molecules were translated in reticulocyte lysate in the presence of canine pancreatic rough microsomes. To assay membrane integration, membranes were separated from cytosolic proteins in the reticulocyte lysate by centrifugation, resuspended in translation buffer and adjusted to pH 11.5 with Na_2CO_3. After 30 min at 0 °C, lumenal contents (s) were separated from integral membrane proteins (p) by centrifugation. Glycosylated products of SpgPA are marked with a dot. (**c**) Digestion of carbohydrate with endoglycosidase H. SP6 polymerase transcription products for each of the molecules were translated in reticulocyte lysate in the presence of canine pancreatic rough microsomes. After precipitation with IgG–Sepharose, the translation products were digested with endoglycosidase H (EH) as indicated. Glycosylated products of SpgPA are marked with a dot. (**d**) SPR arrest of translation of the fusion proteins in wheat-germ extract. SP6 polymerase transcription products for each of the molecules, indicated above the panels, were translated together with the cytoplasmic control molecule globin, in wheat-germ extract in the presence or absence of SPRs as indicated. The migration position of globin, used as a signal-sequence negative control, is indicated. The migration positions of molecular-mass markers (in kDa) are indicated.

can alter the efficiency of co-translational translocation by affecting various signal–receptor interactions (Andrews et al., 1988). Therefore we replaced this region of SpPA with 27 residues from the N-terminus of a modified version of chimpanzee α-globin, a protein previously characterized as being passive to ER translocation (Andrews et al., 1992), to create the protein domain gPA.

When the Sp signal peptide was fused to the N-terminus of gPA, the resulting fusion protein (SpgPA) was translocated across the ER membrane. As expected the translocated product was signal-cleaved, glycosylated and protected from exogenous proteinase K (Figure 2a, lanes 5–8). In contrast, the gPA domain alone was not translocated across the ER membrane and remained accessible to proteases (Figure 2a, lanes 13–16). Moreover, translocation of SpgPA resulted in molecules that did not remain associated with membranes after extraction with Na_2CO_3, demonstrating that they were released into the lumen of the ER (Figure 2b, lanes 5 and 6). To confirm that the additional species observed for SpgPA (Figure 2a, lane 6, dots) resulted from glycosylation, the molecules were digested with endoglycosidase H. As expected digestion with the enzyme removed the carbohydrate from the translocated gPA molecules as demonstrated by the decrease in apparent molecular mass observed for these molecules (Figure 2c).

SpPA and SpgPA vary by less than 30 amino acids, yet their translocation properties are profoundly different. To determine the specific translocation defect in SpPA, we assayed the interaction of the Sp signal sequence with signal-recognition particle (SRP). In wheat-germ extract, binding of SRP to ribosomes translating nascent secretory proteins results in an arrest of translation. This translation arrest was not observed for the cytoplasmic control molecule globin (Figure 2d) but is known to be particularly profound for preprolactin (Andrews, 1989). In translation reactions, addition of 9 nM SRP was

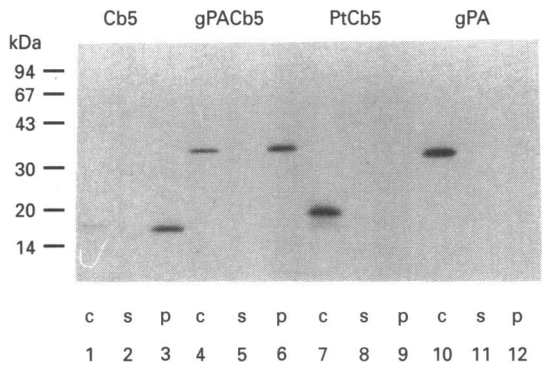

Figure 3 Post-translational targeting of fusion proteins with C-terminal targeting elements

The fusion proteins analysed are indicated above the panels. SP6 polymerase transcription products for each of the molecules were translated in reticulocyte lysate in the presence of canine pancreatic rough microsomes. To assay membrane integration, membranes were separated from cytosolic proteins (c) in the reticulocyte lysate by centrifugation, resuspended in translation buffer and adjusted to pH 11.5 with Na_2CO_3. After 30 min at 0 °C, lumenal contents (s) were separated from integral membrane proteins (p) by centrifugation. The migration positions of molecular-mass markers (in kDa) are indicated at the side.

sufficient to arrest translation of SpgPA completely whereas up to 90 nM SRP had negligible effect on the translation of SpPA (Figure 2d). We conclude from these results that the PA passenger domain is translocation defective because it does not permit co-translational recognition of the Sp signal sequence by SRP.

To demonstrate that the gPA domain could also be anchored on the cytoplasmic face of the ER membrane, a plasmid was constructed encoding a fusion protein containing the signal peptide of β-lactamase (Sl) followed by the IgM stop-transfer sequence (ST) and the gPA passenger. As expected, SlSTgPA was anchored with the majority of the protein located on the cytoplasmic side of the membrane where it was accessible to proteinase K (Figure 2a, lanes 9–12). In addition, both SlSTgPA and the previously published integral membrane control molecule XHiKPt [containing a transmembrane domain derived from IgM fused to the Pt passenger domain (amino acids 58–199 of bovine prolactin) (Andrews et al., 1992)] remained associated with the ER membranes after extraction with Na_2CO_3 (pH 11.5) (Figure 2b, lanes 7 and 8 and 3 and 4 respectively), confirming that these molecules integrated into the ER membrane. As a positive control for ER translocation, we used the well-studied protein preprolactin. On translocation of preprolactin, the signal peptide is cleaved and the resulting mature form of the protein is located in the lumen of the ER where it is protected from added proteases (Andrews et al., 1989, 1992). However, prolactin is a soluble protein therefore it can be extracted from microsomes with Na_2CO_3 (pH 11.5) (Figure 2b, lanes 1 and 2). However, for this particular molecule the extraction is not complete. Although the reason for incomplete extraction of prolactin is not known, this behaviour has been described previously for prolactin-containing molecules (Andrews et al., 1992).

An alternative mechanism for anchoring a protein on the ER membrane is via a C-terminal insertion sequence. Although several proteins contain putative insertion sequences (Kutay et al., 1993), membrane association has been analysed in detail only for cytochrome b_5 (Takagaki et al., 1983; Arinc et al., 1987; Holloway and Mantsch, 1989). Cytochrome b_5 is synthesized on cytoplasmic polysomes and post-translationally inserted into the cytoplasmic face of the ER membrane. The molecule is oriented with the majority of the protein on the cytoplasmic side.

To determine whether the gPA passenger domain can be targeted post-translationally to the ER membrane via a C-terminal insertion sequence, a plasmid was constructed encoding the gPA domain fused to the hydrophobic C-terminus of cytochrome b_5 (Cb5). Like full-length cytochrome b_5 (Figure 3, lanes 1–3), this fusion protein (gPACb5) was anchored on ER membranes in a carbonate-resistant manner (Figure 3, compare lanes 4–6 and 10–12) but remained accessible to proteinase K (results not shown). In contrast, a similar fusion protein containing the Pt passenger domain (amino acids 58–199 of prolactin) fused to the Cb5-insertion sequence was not anchored on ER membranes (Figure 3, lanes 7–9). The Pt domain was previously characterized as being passive to ER translocation with both N-terminal signal and signal-anchor sequences (Andrews et al., 1988, 1992; Rothman et al., 1988). These results indicate that the gPA domain is passive to ER-specific targeting elements at either end of the molecule whereas the Pt domain is not passive to motifs at the C-terminus.

Targeting to mitochondria

Import of proteins into mitochondria and the topology adopted by these proteins has been an area of intense study (Neupert et al., 1990). Similarly to proteins targeted to the ER, nuclear-encoded mitochondrial proteins are initially synthesized as larger preproteins. In contrast with translocation of ER proteins, mitochondrial proteins can be imported post-translationally. Furthermore, translocation of inner-mitochondrial-membrane proteins and matrix proteins requires an electrochemical gradient across the inner mitochondrial membrane.

To assay the gPA passenger for import into mitochondria, a plasmid was constructed encoding a well-characterized mitochondrial transit peptide fused to the N-terminus of gPA. The resulting fusion protein (pOCTgPA) contained the N-terminal signal peptide and 29 amino acids from the mitochondrial matrix protein preornithine carbamoyltransferase fused to gPA. As a positive control we used the uncoupling protein from brown adipose tissue (UCP) (Ridley et al., 1986). UCP is an inner-mitochondrial-membrane protein (Figure 4a, lanes 1–6) that lacks a cleavable transit peptide. As expected for an integral membrane protein, UCP was not extracted with Na_2CO_3 (Figure 4a, lanes 5 and 6).

In mitochondrial import reactions, pOTCgPA was translocated across the mitochondrial membrane concomitant with an increase in migration during SDS/PAGE of the product. The change in migration of the protein is consistent with cleavage of the transit peptide (Figure 4a, compare lanes 7 and 8, upward pointing arrowhead). The processed protein but not the uncleaved preprotein was protease-resistant (Figure 4a, compare lanes 7 and 9). Moreover, translocation was dependent on the electrochemical gradient, as it was abolished by the addition of an uncoupling reagent (Figure 4a, lanes 8 and 10). Finally, the mature protein was not integral to the membrane, as it was extracted with Na_2CO_3 (Figure 4a, lanes 11 and 12). We conclude from these results that the gPA domain is passive to mitochondrial import.

Cytochrome b_5 has also been found associated with mitochondria via the hydrophobic insertion sequence at the C-terminus of the molecule. Therefore we examined the association of gPACb5 with mitochondria. Similarly to the results obtained with full-length cytochrome b_5 (results not shown), gPACb5 associated with mitochondria such that the majority of the mitochondrial-associated protein remained accessible to proteases (Figure 4b, lanes 1 and 2). Moreover, the hydrophobic

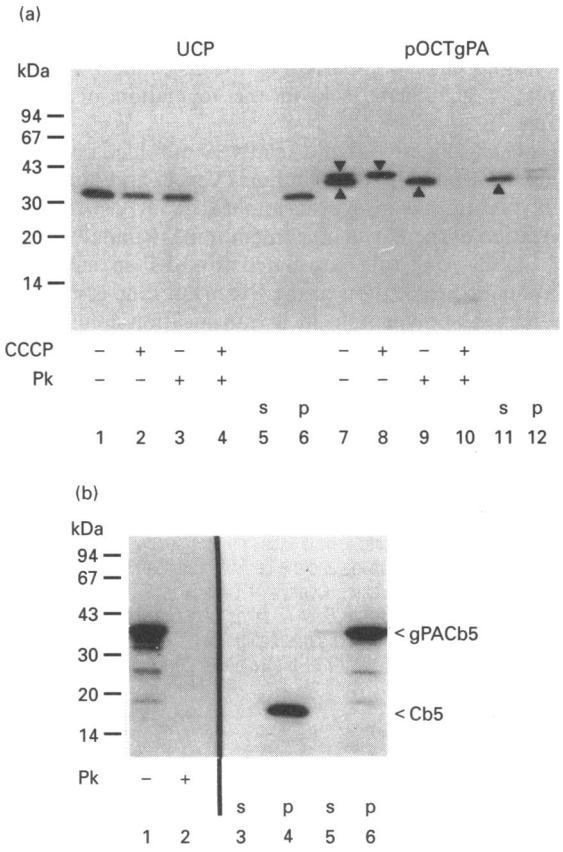

Figure 4 Targeting of fusion proteins to mitochondria

SP6 polymerase transcription products for each of the molecules were translated in reticulocyte lysate, translation was terminated by addition of cycloheximide and then mitochondria and additional reticulocyte lysate were added to the reaction. Import reactions were incubated for 1 h at 30 °C and mitochondria were collected from the reactions by centrifugation. The mitochondria were resuspended and subjected to proteolysis with proteinase K (Pk) where shown. As a control, the uncoupler CCCP was added to abolish the electrochemical gradient across the inner mitochondrial membrane. (**a**) Post-translational targeting of fusion proteins with N-terminal targeting elements: UCP, lanes 1–6; pOCTgPA, lanes 7–12. Downward pointing arrowheads indicate unprocessed full-length translation products of pOCTgPA. Upward pointing arrowheads mark processed (import-sequence-cleaved) OCTgPA. To assay membrane integration, mitochondria separated from the reticulocyte lysate by centrifugation were resuspended and adjusted to pH 11.5 with Na_2CO_3. After 30 min at 0 °C, integral membrane proteins (p) were separated from lumenal contents (s) by centrifugation. (**b**) Post-translational targeting of a fusion protein with a C-terminal targeting element. Translation products are shown, for gPACb5 in lanes 1 and 2 and 5 and 6 and for cytochrome b_5 (Cb5) in lanes 3 and 4. Proteinase K (Pk) was added to one reaction, lane 2. To assay membrane integration, membranes were separated from cytosolic proteins in the reticulocyte lysate by centrifugation, resuspended in translation buffer and adjusted to pH 11.5 with Na_2CO_3 (lanes 3–6). After 30 min at 0 °C, integral membrane proteins (p) were separated from lumenal contents (s) by centrifugation. Migration positions of the proteins are indicated on the right. The migration positions of molecular-mass markers (in kDa) are indicated on the left.

C-terminus of gPACb5 was tightly inserted into the mitochondrial membrane, as it was not extracted with Na_2CO_3 (Figure 4b, lanes 5 and 6).

Targeting to peroxisomes

The best-characterized peroxisomal targeting signal is a C-terminal tripeptide Ser-Lys-Leu (SKL) (Gould et al., 1987). The essential features of this motif appear to be Ser/Ala-Lys/Arg/His-Leu/Met/Ile (Gould et al., 1989). The SKL

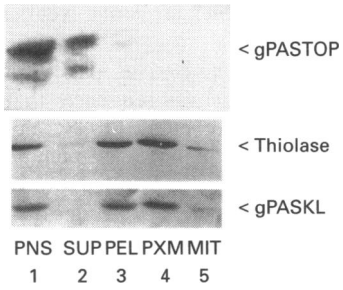

Figure 5 Targeting of gPA passenger to peroxisomes in a yeast via a C-terminal SKL motif

Oleic acid-induced yeast cells were spheroplasted and gently broken. The postnuclear supernatant (PNS) was separated into cytosolic (SUP) and organelle (PEL) fractions by centrifugation. The organelles were further fractionated into mitochondrial (MIT) and peroxisomal (PXM) fractions by density-gradient centrifugation on Nycodenz. Approx. 1% of the total fraction of PNS, SUP and PEL were analysed along with 5 μg each of MIT and PXM fractions. After separation by SDS/PAGE, the samples were transferred to nitrocellulose, which was then divided into thiolase and gPASKL panels. gPASTOP was analysed separately. The molecule analysed is indicated on the right. The gPA fusion proteins were detected using alkaline phosphatase-conjugated rabbit IgG. Thiolase was detected using rabbit anti-thiolase serum and alkaline phosphatase-conjugated goat anti-(rabbit IgG).

tripeptide has been shown to be both necessary and sufficient for targeting to peroxisomes in higher eukaryotes (Gould et al., 1989). However, in the yeast *S. cerevisiae* the tripeptide SKL is not sufficient for targeting of dihydrofolate reductase (Distel et al., 1992; Kragler et al., 1993) and neither is a related peptide (Ala-Lys-Ile) sufficient to target chloramphenicol acetyltransferase to peroxisomes (Aitchison et al., 1991).

To examine the possibility that these protein domains were not entirely passive to targeting to peroxisomes, we attached the SKL sequence to the C-terminus of the gPA passenger. To prevent the SKL-bearing fusion protein (gPASKL) from forming inclusion bodies, it was expressed at a relatively low level using pRS315 equipped with an *S. cerevisiae* acyl-CoA oxidase promoter. To assay for peroxisomal import by cell fractionation, the distribution of gPASKL was compared with that of the endogenous peroxisomal protein thiolase. The two polypeptides co-fractionate precisely, being found in similar relative amounts in the postnuclear supernatant 20000 *g* pellet and in fractions containing peroxisomes isolated from Nycodenz gradients (Figure 5). The fractions from the gradients that were enriched in mitochondria (Figure 5, fractions labelled MIT) contained only a small amount of the two proteins. In contrast, gPASTOP (lacking the Ser-Lys-Leu tripeptide) was recovered exclusively in the postnuclear supernatant with the bulk of the cytoplasmic proteins (Figure 5). Therefore the terminal tripeptide Ser-Lys-Leu is sufficient to target gPA to peroxisomes in *S. cerevisiae*.

DISCUSSION

We have shown that gPA is passive to targeting and membrane translocation via motifs at either the N- or C-terminus. The gPA domain appears to be a particularly useful passenger domain because it is passive to targeting to multiple subcellular membranes and expressed well both *in vitro* (reticulocyte lysate and wheat germ) and in yeast. Furthermore, the domain is easily detected in whole cell extracts because of the tight interaction between the Protein A domain and IgG. Although gPA behaves as a passive passenger in a variety of situations, it remains to be determined if the domain is passive in other contexts such as

transfected cells and for other targeting events such as nuclear import.

Proteins domains characterized as passive passengers have been used successfully to identify sequences that regulate membrane integration (Rothman et al., 1988; Yost et al., 1990; Andrews et al., 1992) and a novel element termed a pause-transfer sequence (Chuck et al., 1990). However, only a small number of protein domains have been rigorously characterized as passive passengers. Moreover, in contrast with gPA, those domains that have been shown to be passive have generally been examined only for a single targeting pathway (e.g. secretion).

Our results demonstrate that not all protein domains characterized as cytoplasmic are passive to translocation even if they are derived from a normally extracellular protein (Figure 2a, lanes 5–8). Moreover, they highlight the importance of the position of a putative targeting element within the translocated domain. For example, the Pt domain appears to be passive to ER targeting only via signals at the N-terminus (Figure 3, lanes 7–9).

Our demonstration that the C-terminal tripeptide Ser-Lys-Leu is sufficient for peroxisomal targeting in yeast contrasts sharply with earlier attempts using dihydrofolate reductase or chloramphenicol acetyltransferase (Aitchison et al., 1991; Distel et al., 1992; Kragler et al., 1993). Previously the Ser-Lys-Leu tripeptide has been shown to promote peroxisomal targeting only in cells from multicellular eukaryotes (Wendland and Subramani, 1993). The smallest peptides previously demonstrated to be sufficient for peroxisomal targeting in *S. cerevisiae* were the C-terminal six to seven residues of catalase, citrate synthase and luciferase (Kragler et al., 1993). Unlike the tripeptide sequence, these longer peptides were sufficient for targeting of dihydrofolate reductase (Kragler et al., 1993). Taken together these results suggest that the additional amino acids necessary for function of the targeting elements fused to dihydrofolate reductase may have been required for appropriate presentation of the canonical terminal tripeptide.

In vitro, the gPA passenger domain is also efficiently targeted by the C-terminal cytochrome b_5-insertion sequence. Therefore in reticulocyte lysate the gPA domain can be targeted to multiple organelles by motifs at either end of the molecule. Efficient recognition of two dissimilar C-terminal motifs (one positively charged and the other primarily hydrophobic) suggests that gPA folds such that the C-terminus is exposed.

Correlation of the translocation properties of different combinations of signal peptides and secretory proteins led to the suggestion that the translocated polypeptide contributes to the specificity of targeting pathways for secretion and mitochondrial import (Pugsley, 1990). Consistent with this model, both the mature part of secretory proteins and the cytoplasmic domain of an inner-membrane protein have been demonstrated to contribute to protein localization in *E. coli* (von Heijne et al., 1988; MacIntyre and Henning, 1990). Our data suggest that additional specificity provided by the translocated polypeptide may provide an explanation for the large number of random sequences that can substitute for the secretory signal sequence of yeast invertase (Kaiser et al., 1987). In particular, our demonstration that the passenger domains Pt and PA are not passive (to even co-translational translocation for PA) is also consistent with this model (Figures 2a, lanes 1–4, Figure 3, lanes 7–9).

Previous models predicted that the features of proteins necessary for efficient localization are specific for a particular target compartment (Pugsley, 1990). However, the facility with which gPA can be targeted to ER, mitochondria and peroxisomes argues that the essential features are not mutually exclusive for these subcellular compartments. Rather our results predict that the targeted domain contributes only translocation competence and that specificity is determined entirely by the targeting motif. Nevertheless, combined with a specific targeting motif, the additional specificity imparted by 'translocation competence' may play a significant role in the regulation of subcellular targeting.

It has been suggested that a relatively unfolded conformation is required for mitochondrial import (Verner and Lemire, 1989). Lack of folding has also been implicated in post-translational translocation of the *E. coli* inner membrane (Randall and Hardy, 1986). Finally, one role postulated for SRP in mediating co-translational translocation of the ER membrane is maintenance of the nascent polypeptide in a translocation-competent state (Sanz and Meyer, 1988). It is possible that the addition of 27 amino acids to the N-terminus of PA confers translocation competence by either preventing or significantly slowing down the folding of gPA. Experiments to address this issue are in progress.

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