Protein Sorting between Mitochondrial Membranes Specified by Position of the Stop-Transfer Domain

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Abstract. Recently, we fused a matrix-targeting signal to a large fragment of vesicular stomatitis virus G protein, which contains near its COOH-terminus a well-characterized endoplasmic reticulum (ER) stop-transfer sequence; the hybrid G protein was sorted to the inner mitochondrial membrane (Nguyen, M., and G. C. Shore. 1987. J. Biol. Chem. 262:3929–3931). Here, we show that the 19 amino acid G stop-transfer domain functions in an identical fashion when inserted toward the COOH-terminus of an otherwise normal matrix precursor protein, pre-ornithine carbamyl transferase; after import, the mutant protein was found anchored in the inner membrane via the stop-transfer sequence, with its NH₂ terminus facing the matrix and its short COOH-terminal tail located in the intermembrane space. However, when the G stop-transfer sequence was placed near the NH₂ terminus, the protein was inserted into the outer membrane, in the reverse orientation (NH₂ terminus facing out, with a large COOH-terminal fragment located in the intermembrane space). These observations for mitochondrial topogenesis can be explained by a simple extension of existing models for ER sorting.

Materials and Methods

Earlier publications describe the procedures used for transcription-translation of recombinant plasmids (25), import of translation products by purified rat heart mitochondria in vitro (26), analysis of products by SDS-PAGE (25), and NH₂-terminal radiosequence analysis of electroeluted polypeptides (26). Additional details, as well as the procedures used for the construction of recombinant plasmids, are given in the figure legends.

Results

In an earlier study (27), it was shown that a hybrid protein, containing the signal sequence of rat pre-ornithine carbamyl transferase (pOCT) fused to a large COOH-terminal frag-
Figure 1. Construction of pSPOGS and pSPOGP encoding pOCT precursor derivatives carrying the VSV G stop-transfer sequence. (A) pSP09 is derived from pSP64 and contains a DNA insert coding for a functional full length copy of rat liver pOCT. (B) pSPOGS encodes pOCT containing the VSV G stop-transfer sequence positioned between amino acids 319 and 321 of the pOCT sequence. The derived polypeptide precursor is designated pOCT-GST319. (C) pSPOGP encodes pOCT-GST35, which is pOCT containing the VSV G stop-transfer sequence positioned between amino acids 35 and 37 of pOCT. Slashes, pOCT signal sequence; solid region, VSV G stop-transfer sequence. Also shown is the single-letter amino acid code for the VSV G stop-transfer domain (boxed) and the flanking pOCT sequences; amino acids that were added as a consequence of DNA manipulation are indicated by an asterisk. The downward arrow in C denotes the pOCT signal processing site between amino acids 32 and 33. To construct pSPOGS (B), pSP09 was digested to completion with SacII and a single copy of a SacII adapter composed of two complementary oligodeoxyribonucleotides, 5'-TTTCCATGGATCCCGGGC-3' and 5'-CCGGATCCATGGAAAGC-3', and containing an NcoI site and a unique Smal site, was inserted and ligated. A portion of the adapter was then deleted by cutting with PvuII to completion with Smal followed by a partial digestion with NcoI; the deleted fragment was replaced with a second synthetic double-stranded DNA fragment composed of 5'-CATGGTTCTCTATGGCTTTTTTTTATTTATGGTTAATCATTGGACTATTCTTGGT'-3' and 5'-GAACCA AGAATAGTCCAATGATTAACCC TATGATAAAGAA...O DEVFFY S P L S ML[SASFFFZI G LI I G LF L V LIG G SLV F PEAE...KP V O S O V M LISIASFFFZII G LIIGLFLVLILKGRDLLTLK...]

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The re-
In vitro import and processing of pOCT and pOCT-GST319. pSPO19 and pSPOGS were linearized with EcoRI, transcribed, and the resulting mRNAs translated in a rabbit reticulocyte lysate system containing [35S]methionine, as described in reference 25. Translation products (pOCT and pOCT-GST319, respectively) were incubated with purified rat heart mitochondria (0.5 mg protein/ml) using the import conditions described in references 26 and 27; mitochondria were recovered after import by centrifugation at 1,200 g for 5 min, dissolved in SDS sample buffer, and analyzed by SDS-PAGE and fluorography. For protease treatments, import incubation was cooled to 4°C, maintained for 20 min in the presence of 100 µg/ml proteinase K, adjusted to 2 mM phenylmethylsulfonyl fluoride, and incubated an additional 10 min before recovering the mitochondria by centrifugation. For alkali extractions, mitochondria (50 µg protein) were recovered after import, resuspended in 150 µl of 0.1 M Na2CO3, pH 11.5, sonicated to disperse completely the pellet, and incubated on ice for 30 min (8); the insoluble membrane fraction was then recovered by centrifugation in an airfuge operating for 10 min at 100,000 g. (A) Lanes 1 and 2, translation products obtained with no mRNA added (lane 1) or pSPOGS mRNA (lane 2); lanes 3-6, incubation of pOCT-GST319 with mitochondria; lane 3, mitochondrial pellet; lane 4, mitochondrial membranes after extraction of mitochondria with 0.1 M Na2CO3, pH 11.5 (ALKALI); lane 5, treatment of mitochondria after import with exogenous proteinase K (PROT.K); lane 6, as in lane 5 except that import was performed in the presence of 1.0 µM carbonylcyanide m-chlorophenyl hydrazone (CCCP) to collapse the transbilayer electrochemical potential across the mitochondrial inner membrane. (B) Lanes 7-10, import of pOCT, with conditions the same as for lanes 3-6, as indicated in the figure; only half of the import reaction was analyzed in lane 7. (C) Lanes 11-13, mitochondrial pellets after import of pOCT-GST319; lane 11, alkali extraction as in lane 4; lane 12, after import, mitochondria were treated with exogenous protease K in the presence of 0.2 mg/ml digitonin (DIG, 0.2), followed by extraction with 0.1 M Na2CO3, pH 11.5; lane 13, proteinase K digestion was performed in the presence of 0.5 mg/ml digitonin (DIG, 0.5). Precursor and processed forms of pOCT and pOCT-GST319 are indicated by p and m, respectively; m’, protease-clipped m; 37, a 37-kD intermediate product, which arises during processing of pOCT in vitro.

centration of digitonin, protease K gained access to the matrix and degraded completely both the hybrid protein (Fig. 2, lane 13) and OCT (not shown, see also reference 27). Complete degradation of pOCT-GST319 by protease K was also observed in the absence of digitonin, but where import had been prevented by CCCP, thus indicating that the digitonin effect was at the level of membrane permeabilization rather than simply facilitating degradation of an otherwise resistant protein. We conclude from these observations, therefore, that processed pOCT-GST319 is anchored at the inner membrane in a transmembrane orientation via the G stop-transfer domain, with its NH2 terminus facing the matrix and its COOH-terminal tail facing the intermembrane space. In all respects, pOCT-GST319 functions like the pOCT-VSV G hybrid described earlier (reference 27). Interestingly, however, the sequences flanking the G stop-transfer domain in the two molecules are rather different; whereas the stop-transfer in G is flanked immediately on either side with basic residues (13, 32), the flanking residues in pOCT-GST319 are neutral (or at some distance away, negatively charged) (Fig. 1 B). Clearly, therefore, it is the stop-transfer sequence per se, rather than contributions from the background polypeptide, that specifies translocation-arrest at the inner membrane.

Additionally, we have found that pOCT-GST319 was anchored in the inner membrane after import even in the presence of an inhibitor (26) that prevents proteolytic cleavage of the signal sequence (not shown). Processing, therefore, does not appear to be required to activate the sorting domain (i.e., the stop-transfer sequence). This is in marked contrast to the situation for Rieske Fe/S-protein in Neurospora, where the matrix targeting signal must be removed to allow the contiguous sorting domain to direct translocation to the intermembrane space (14).

Import of pOCT-GST35

The fate of pOCT-GST35 after import is described in Fig. 3. After transcription-translation of the derived pSP64 plas-
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**Figure 3.** In vitro import of pOCT-GST35. Conditions for import, protease digestion, and alkaline (pH 11.5) extraction are given in Fig. 2. The procedure used for immunoprecipitation is described in references 1 and 25. Antiserum was raised (25) against a synthetic peptide corresponding to amino acids 1-27 of the pOCT signal sequence (10) coupled to hemocyanin by the glutaraldehyde method (31). (A) The two translation products of pSPOGP mRNA (p and * see text) were incubated with mitochondria under standard import conditions, and the mitochondria recovered by centrifugation. Lane 1, import products precipitated with monospecific antibody against OCT (ANTI-OCT); lane 2, import products precipitated with monospecific antibody against the pOCT signal peptide (ANTI-SP); lanes 3 and 4, before recovery of mitochondria, import mixtures were treated with proteinase K (PROT. K), the mitochondria were recovered, and import products precipitated with anti-SP (lane 3) or anti-OCT (lane 4); lane 5, total import products after digestion with exogenous proteinase K; lane 6, as in lane 5 except that mitochondria were extracted with 0.1 M Na2CO3, pH 11.5. p, full-length pOCT-GST35; *, pOCT-GST35 with a COOH-terminal truncation; p′, protease-clipped p. (B) Import incubations were treated with exogenous proteinase K in the presence of the indicated concentrations of digitonin; mitochondria were recovered, analyzed by SDS-PAGE and fluorography, and the relative levels of pOCT-GST35 (p or p′ in A) or of processed pOCT (m in Fig. 2 B) were determined by laser densitometric tracings of autoradiographs, and plotted against digitonin concentration. (●) processed pOCT; (○) pOCT-GST35.

We have found in this study that an ER stop-transfer sequence derived from VSV G is functional when placed in the context of an otherwise normal and well-characterized mitochondrial matrix precursor protein, pOCT (17, 18, 25, 26).
Figure 4. A protease-sensitive component on the surface of mitochondria is required for insertion of pOCT-GST35 into the OMM. Before import, mitochondria were treated with 40 μg trypsin (lane 3) after which inhibitors were added to the trypsin sample or with 40 μg trypsin preinhibited with aprotinin and phenylmethylsulfonyl fluoride (lanes 1 and 2), exactly as described in reference 32 (TRYPSIN, pre). Import was carried out as described in Fig. 3, after which the import mixtures were treated with (lanes 2 and 3) or without (lane 1) proteinase K (PROTEINASE K, post), as described in Fig. 2. Mitochondria were recovered and radioactive products visualized following SDS-PAGE and fluorography (only half of total product was applied to lane 1). p, p', and * are defined in Fig. 3.

inserted toward the NH₂ terminus (pOCT-GST35), the protein was delivered to the OMM; near the COOH terminus (pOCT-GST319), the G stop-transfer sequence resulted in translocation arrest at the IMM. Because pOCT-GST319 and pOCT-VSV G were both targeted to the inner membrane where they assumed the same transmembrane disposition strongly implies that it is the stop-transfer domain per se rather than contributions from immediate flanking sequences (which are quite different in the two proteins) that functions as the sorting signal. That pOCT-GST319 and pOCT-GST35 are delivered to different membranes, yet contain identical matrix-targeting and stop-transfer sequences, suggests that position of the two signals relative to one another may be an important determinant in mitochondrial membrane sorting.

pOCT-GST35 displays import characteristics typical of outer membrane proteins (7, 9, 16, 23); it is inserted into the membrane without cleavage of the signal sequence and without the requirement of an electrochemical potential. Furthermore, insertion was prevented by pretreatment of intact mitochondria with trypsin (Fig. 4), using the identical conditions that also prevented import of normal pOCT to the matrix (11). It would appear, therefore, that pOCT-GST35 follows a typical import pathway rather than simply partitioning into the membrane bilayer by an unrelated mechanism.

Because stop-transfer sequences presumably anchor proteins in membranes by simple hydrophobic interactions, a direct role of the lipid bilayer in decoding the G stop-transfer sequence as a specific sorting signal seems unlikely. Rather, our observations fulfill many of the predictions for a proteinaceous translocation apparatus carrying out such functions (3, 36), as do our observations that the pOCT signal peptide, though potentially amphiphilic (6), is incapable of penetrating membrane lipid bilayers (37). In further support of this notion is the finding that ADP/ATP translocase, an integral protein of the IMM, is initially imported via a water-accessible pathway (29). In Fig. 5, we have applied existing models developed for the ER to illustrate how simple positional information pertaining to stop-transfer signals might specify protein sorting between mitochondrial outer and inner membranes. The translocation machineries of the two membranes are shown as pore complexes (3, 36), components of which function in the recognition of matrix-targeting and stop-transfer signals. Two proposals are made. (a) When the protein translocation machinery encounters a stop-transfer domain, it releases this region of the polypeptide from the translocator into the surrounding lipid bilayer (3, 36) (perhaps at a gated interface between two subunits of the translocator, shown in face-view in the inset in A); if a distal strand of the polypeptide is simultaneously engaged in the translocator, it continues to be transported (e.g., A). (b) As the signal peptide emerges from the OMM translocator, it interacts with the translocator of the IMM and triggers physical interaction between the translocation machineries of the two membranes.

Figure 5. Protein sorting between mitochondrial membranes. (A) pOCT-GST35; (B) pOCT-GST319. Details are provided in the text.
membranes (presumably at a stable contact point which allows an incoming precursor polypeptide to span both membranes simultaneously, references 34 and 35); contact between the two renders the OMM translocator incapable of responding to a stop-transfer domain, while the IMM translocator is functional in this regard (B).

The available evidence suggests that precursor polypeptides are threaded into mitochondria NH₂-terminal first (34). In the case of pOCT-GST35, the signal sequence presumably engages the OMM translocator as an extended NH₂-terminal loop, thus leading to the observed orientation of the protein in the membrane (A). This is in distinct contrast to yeast OMM-70, which assumes a reverse orientation in the outer membrane (4), even though it shares with pOCT-GST35 a similar overall organization of its topogenic domains (NH₂-terminal signal sequence followed immediately by a stop-transfer sequence; reference 15). Analogous situations have been described for the ER; asialoglycoprotein receptor (38) and Sindbis virus PE (4), for example, both have internal uncleaved signal sequences, yet insert into the ER membrane in opposite transmembrane orientations. To account for these observations, together with findings for various mutants of opsin, Audigier et al. (2) have recently proposed that relatively strong interactions between the signal sequence and a cis-located signal-binding subunit of the ER translocation machinery prevents translocation of the NH₂ terminus (yielding an orientation in which the NH₂ terminus faces the cytoplasm), while a weaker interaction allows subsequent dissociation of the signal which can then transit (flip) across the membrane (2), yielding an orientation in which the NH₂ terminus faces the lumen. A similar explanation might account for the opposite orientations of pOCT-GST35 and OMM-70; according to such a model, the OMM-70 signal sequence disengages the signal-binding site at the surface of mitochondria and transmits the OMM before insertion of the stop-transfer sequence into the translocation machinery, while in the case of pOCT-GST35 the signal sequence disengages the cis binding site after insertion of the stop-transfer sequence (Fig. 5 A), and thus remains outside the organelle. If this model (2) is correct, the retention time of the signal sequence on the cis side of the translocation machinery might likewise govern the minimum distance between the signal sequence and the stop-transfer domain that permits passage of proteins to the inner membrane (Fig. 5 B).

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