Tom70p is targeted and inserted into the mitochondrial outer membrane in the N<sub>in</sub>-C<sub>cyto</sub> orientation, via an NH<sub>2</sub>-terminal signal anchor sequence. The signal anchor is comprised of two domains: an NH<sub>2</sub>-terminal hydrophilic region which is positively charged (amino acids 1–10) followed by the predicted transmembrane segment (amino acids 11–29). Substitution of the NH<sub>2</sub>-terminal domain with a matrix-targeting signal caused the signal anchor to adopt the reverse orientation in the outer membrane (N<sub>cyto</sub>-C<sub>in</sub>) or, if presented to mitoplasts, to arrest protein translocation at the inner membrane without insertion. Physically separating the transmembrane segment from the matrix-targeting signal by moving it downstream within the protein resulted in a failure to arrest in either membrane, and consequently the protein was imported to the matrix. However, if the mean hydrophobicity of the Tom70p transmembrane segment was increased in these constructs, the protein inserted into the inner membrane with an N<sub>in</sub>-C<sub>out</sub> orientation. Therefore we have determined conditions that allow the Tom70p transmembrane domain to insert in either membrane, pass through both membranes, or arrest without insertion in the inner membrane. These results identify the mean hydrophobicity of potential transmembrane domains within bitopic proteins as an important determinant for insertion into the mitochondrial inner membrane.
space-sorting signals. In one model the hydrophobic stretch is suggested to arrest translocation during unidirectional import within the inner membrane translocation machinery; processing of the precursor on either side of the membrane then liberates the mature protein into the intermembrane space (29). Another model proposes that following removal of the NH$_2$-terminus in the matrix, the hydrophobic domain redirects the precursor protein back from the matrix compartment to the intermembrane space (31). It has been consistently observed, however, that the hydrophobic domain, while capable of translocation arrest within the inner membrane import machinery, does not integrate into the surrounding bilayer. Clearly, this domain is functionally different from the structurally analogous regions in signal anchor and stop-transfer sequences.

Here we have addressed the question of how apparently similar stretches of hydrophobic amino acids within precursor proteins can function to target proteins to different locations within the mitochondrion. To do so, we have determined conditions and modifications that result in the transmembrane segment of a signal anchor sequence being inserted into either the outer or inner membrane, passing across both membranes without being arrested, or being arrested across the inner membrane without inserting into the bilayer. This recapitulation of targeting of the hydrophobic domain to different compartments was found to depend on several factors: 1) its net hydrophobicity, 2) whether or not it is permitted to pass across the outer membrane, 3) its distance from a matrix-targeting signal, and 4) the relative strength of the matrix-targeting signal.

MATERIALS AND METHODS

General Procedures—Previous articles describe the routine procedures used in this study (Refs. 17 and 32 and the references cited therein). These include in vitro transcription of pSP64 plasmids, translation of the resulting mRNA in rabbit reticulocyte lysate in the presence of $[^{35}$S]methionine, purification of mitochondria from rat heart and of mitoplasts from rat liver, protein import in vitro, and analysis of import products by SDS-polyacrylamide gel electrophoresis and fluorography.

Mitochondrial Import—Reaction mixtures contained 10% (v/v) rabbit reticulocyte lysate translation products labeled with $[^{35}$S]methionine, mitochondria or mitoplasts (0.5 mg protein/ml), 0.125 M sucrose, 32 mM KCl, 0.8 mM magnesium acetate, 9.0 mM Hepes, pH 7.5, 0.5 mM dithiothreitol, 0.5 mM ATP, 2.5 mM sodium succinate, 0.04 mM ADP, and 1.0 mM potassium phosphate, pH 7.5. Some reaction mixtures also contained 1.0 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP) as indicated in the figure legends. The import reaction mixtures were incubated at 4 or 30 °C for 30 min. For post-trypsin zone (CCCP) as indicated in the figure legends. The import reaction products by SDS-polyacrylamide gel electrophoresis and fluorography.

FIG. 1. Fusion protein constructs. Standard recombinant DNA and polymerase chain reaction methodology were employed to construct pSP64 plasmids encoding various fusion proteins containing the following NH$_2$-terminal domains fused to amino acids 4–186 of mouse DHFR: 1) pOMD29 (Li and Shore (3)), amino acids 1–29 of yTom70p followed by Gly; 2) pO-SA 36 (formerly pO-OMD; Li and Shore (20)), amino acids 1–36 of rat pOCT, Gly, amino acids 11–29 of yTom70p, and Gly-Pro; 3) pO-DHFR (Skerjanc et al. (34)), amino acids 1–36 of pOCT followed by Gly-Arg; 4) pO-SA 141, amino acids 1–141 of pOCT, amino acids 11–29 of yTom70p, followed by Gly; 5) pO-141A is pO-SA 141 lacking amino acids 11–29 of yTom70p; 6) pO-SA 242, as in number 4 except that the NH$_2$ terminus contains amino acids 1–242 of pOCT; 7) pO-242A is pO-SA 242 lacking amino acids 11–29 of yTom70p. All constructs were verified by nucleotide sequence analysis. Helix, matrix-targeting signal of pOCT; shaded box, the signal anchor sequence of yTom70p (amino acids 11–29); boxed DHFR, amino acids 4–186 of DHFR.

NH$_2$-terminal signal anchor domain of Tom70p (amino acids 1–29) fused to dihydrofolate reductase (DHFR) and is targeted and inserted into the outer mitochondrial membrane in the N$_{cyt}$-C$_{mito}$ orientation (Fig. 5A) (3, 4, 20). Replacement of the extreme hydrophilic NH$_2$-terminus of the pOMD29 signal anchor with the matrix-targeting signal of pOCT created pO-SA 36 (formerly pO-OMD), which inserts into the outer membrane in an orientation opposite that of pOMD29, i.e. N$_{mito}$-C$_{cyt}$ (Fig. 5A) (17, 20). Deletion of the predicted transmembrane portion (amino acids 11–29) of the pOMD29 signal anchor abolishes the ability of the protein to target mammalian mitochondria in vitro (4), whereas the pO-SA 36 fusion construct containing the pOCT matrix-targeting signal but lacking the Tom70p transmembrane segment (i.e. pO-DHFR) is efficiently imported to the matrix (34, 35) (Fig. 5A).

A Downstream Signal Anchor Transmembrane Segment Does Not Arrest Transport of a Matrix-destined Protein—In pO-SA 36, the transmembrane portion of the Tom70p signal anchor is contiguous to the pOCT matrix-targeting signal. To investigate the consequences of physically separating these domains, a spacer region was introduced by replacing pOCT targeting signal of pOCT; shaded box, the signal anchor sequence of yTom70p (amino acids 11–29); boxed DHFR, amino acids 4–186 of DHFR.

A downstream signal anchor transmembrane segment does not arrest transport of a matrix-destined protein. In pO-SA 36, the transmembrane portion of the Tom70p signal anchor is contiguous to the pOCT matrix-targeting signal. To investigate the consequences of physically separating these domains, a spacer region was introduced by replacing pOCT targeting signal of pOCT; shaded box, the signal anchor sequence of yTom70p (amino acids 11–29); boxed DHFR, amino acids 4–186 of DHFR.
Alkali-resistant fraction was obtained following import reaction (Post-Trypsin).

Mitoplasts were used in place of intact mitochondria. The 35S-precursor proteins, as indicated (p), were incubated with intact mitochondria in the presence (lanes 2–4) or absence (lanes 2–4) of CCCP or in the presence (lanes 6 and 7) or absence (lanes 2–5, 8, and 9) of 1.0 μM CCCP. In lanes 5 and 6 the mitoplasts were subjected to treatment with protease at the end of the import reaction (Post-Trypsin), whereas the alkali-resistant fraction was analyzed in lanes 8 and 9 (Alkali). p and m refer to precursor and processed (mature) polypeptides, respectively, and are designated by arrows.

To examine the possibility that the transmembrane portion of the Tom70p signal anchor in pO-SA 141 arrested import of the polypeptide across the inner membrane but failed to permit release from the translocation pore into the surrounding lipid bilayer, import of pO-SA 141 was examined in mitoplasts and compared with various control polypeptides. The generation of mitoplasts (32) was monitored by the release of the intermembrane space marker, sulfite oxidase, which was over 90% complete as judged by Western blot analysis (not shown). As shown in Fig. 3 (top panel), pO-SA 141 was imported and processed (lane 4), and the processed form of the molecule demonstrated Δψ-dependent resistance to exogenous trypsin (compare lanes 5 and 6 with lane 4) indicating complete translocation of the polypeptide chain to the soluble matrix compartment. Consistent with this conclusion, both pO-DHFR and pO-SA 141 were also protected from trypsin following import into intact mitochondria and subsequent hypotonic shock of the organelle to disrupt the outer membrane (20) (not shown). As expected (see also Fig. 2), the imported product in mitoplasts was extracted by alkali (lane 9). Very similar import results were obtained for pO-141Δ and pO-DHFR (Fig. 3, panels 2 and 3) and for pO-SA 242 and pO-SA 242 (not shown). Finally, pO-SA 141 was completely degraded by trypsin following incubation with intact mitochondria in the presence of CCCP (not shown), indicating that pO-SA 141 did not insert into the outer membrane even in the absence of an electrochemical potential across the inner membrane.

Previous studies have documented import and insertion of pOMD29 into the inner membrane of mitoplasts in the N_in-C_out orientation (3) (Fig. 5B). As shown in Fig. 3, panel 5, this
results in acquisition of resistance to extraction by alkali (compare lanes 4 and 9) but leaves the bulk of the polypeptide exposed at the surface of mitoplasts where it is susceptible to degradation by exogenous trypsin (compare lanes 4 and 5). Thus, the transmembrane segment of Tom70p is competent for insertion into the inner membrane in the context of pOMD29 but not in the context of pO-SA 141 or pO-SA 242. However, of particular interest were the findings with pO-SA 36, in which the Tom70p transmembrane segment is immediately adjacent to the pOCT matrix-targeting signal. Import and processing of pO-SA 36 into intact mitochondria was performed as described under “Materials and Methods.” Conditions and analyses were the same as in Fig. 2, as is the nomenclature.

Increasing the Hydrophobicity of the Tom70p Signal Anchor Transmembrane Segment—In a previous study (22), mutations were introduced into the transmembrane segment of the Tom70p signal anchor, in which alanines at positions 14, 15, 17, and 18 were converted to isoleucine. These changes did not affect the ability of the signal anchor in the context of pOMD29 to select and insert into the mitochondrial outer membrane in vitro (22). Here, the identical changes were introduced into the Tom70p hydrophobic domain of pO-SA 141, to create pO-SA 141-I4 (Fig. 4A). They resulted in the mean hydrophobicity of this segment increasing from 1.17 to 1.74 (Fig. 4A) employing the hydrophobic scale of Kyte and Doolittle (36) (Secondary Structure Prediction, Prosis Program, Hitachi Software Engineering Co., Ltd.). B, standard import of pO-SA 141-I4 and pO-DHFR into intact mitochondria was performed as described under “Materials and Methods.” Conditions and analyses were the same as in Fig. 2, as is the nomenclature.

Increasing the Hydrophobicity of the yTom70p Domain of pO-SA 141 that Permit Insertion into the Mitochondrial Inner Membrane. A, the amino acid sequence (single letter code) of the transmembrane portion of the yTom70p signal anchor (amino acids 11–29) is shown together with mutations of alanine residues at positions 14, 15, 17, and 18 to isoleucine. These mutations were made in pO-SA 141 to create pO-SA 141-I4 and result in the predicted hydrophobic index of the Tom70p transmembrane domain increasing from 1.17 to 1.74, as determined using the Secondary Structure Prediction, Prosis Program (Hitachi Software Engineering Co., Ltd.). B, standard import of pO-SA 141-I4 and pO-DHFR into intact mitochondria was performed as described under “Materials and Methods.” Conditions and analyses were the same as in Fig. 2, as is the nomenclature.

FIG. 4. Mutations in the yTom70p domain of pO-SA 141 that permit insertion into the mitochondrial inner membrane. A, the amino acid sequence (single letter code) of the transmembrane portion of the yTom70p signal anchor (amino acids 11–29) is shown together with mutations of alanine residues at positions 14, 15, 17, and 18 to isoleucine. These mutations were made in pO-SA 141 to create pO-SA 141-I4 and result in the predicted hydrophobic index of the Tom70p transmembrane domain increasing from 1.17 to 1.74, as determined using the Secondary Structure Prediction, Prosis Program (Hitachi Software Engineering Co., Ltd.). B, standard import of pO-SA 141-I4 and pO-DHFR into intact mitochondria was performed as described under “Materials and Methods.” Conditions and analyses were the same as in Fig. 2, as is the nomenclature.

FIG. 5. Schematic of the location and orientation of the various fusion proteins following import into mitochondria (A) and mitoplasts (B). See text for discussion. Cyto, cytosol; OM, outer membrane; IMS, intermembrane space; IM, inner membrane; N, NH$_2$ terminus; stippled ovals, inner membrane translocation machinery; black cylinder, Tom70p signal anchor; hatched cylinder, Tom70p signal anchor with four alanines mutated to isoleucines which increases its hydrophobicity. Processing of the NH$_2$ terminus in the matrix is shown.
polypeptide has a single downstream transmembrane segment, this means that this bitopic-processed polypeptide spans the inner membrane once, leaving the COOH terminus in the intermembrane space. This was confirmed following import into mitoplasts, where subsequent digestion of processed pO-SA 141-I4 by trypsin yielded a polypeptide fragment whose size was consistent with an N_{in}-C_{out} orientation (data not shown).

Conclusions—In this study, we have examined the function of the Tom70p signal anchor transmembrane segment when placed in different contexts relative to a matrix-targeting signal. The results are summarized in Fig. 5. When the Tom70p transmembrane segment is contiguous to the pOCT matrix-targeting signal (pO-SA 36) and presented to intact mitochondria in vitro, it inserts into the outer membrane in the N_{cyto}-C_{in} orientation, which is opposite that observed for the native Tom70p signal anchor (pOMD29) (Fig. 5A). When this same polypeptide construct, pO-SA 36, is presented to mitoplasts, the transmembrane segment causes arrest of the polypeptide across the inner membrane in the N_{in}-C_{out} orientation, but it does not trigger insertion into the membrane lipid bilayer (Fig. 5B). If placed at some distance downstream of the matrix-targeting signal (pO-SA 141 and pO-SA 242) and presented to either intact mitochondria or mitoplasts, the transmembrane segment is no longer capable of arresting translocation or triggering insertion into either mitochondrial membrane, and the protein is translocated entirely to the matrix compartment. Increasing the net hydrophobicity of the Tom70p transmembrane segment within the context of this latter construct, however, results in its insertion into the inner membrane in the N_{in}-C_{out} orientation (pO-SA 141-I4). These findings are similar to those found for bacteria, where it has been shown that protein insertion into the cell membrane requires a threshold hydrophobicity for the transmembrane segment (37).

The observed outcomes that were specified by the hydrophobic domain within the various polypeptide constructs examined in this study closely mimic those specified by hydrophobic domains that exist within native proteins: signal anchor sequences that direct insertion into the outer membrane, stop-transfer sequences that specify insertion into the outer or inner membrane, and intermembrane sorting sequences that cause translocation arrest across the inner membrane. Therefore, help to identify characteristics of these closely related hydrophobic segments that are likely important for function. In particular, our findings suggest that insertion of a potential transmembrane segment into the inner membrane requires a relatively high net hydrophobicity when this segment is located at some distance downstream of a strong matrix-targeting sequence. In the absence of an outer membrane (i.e. mitoplasts), however, a transmembrane segment of lower hydrophobicity will either insert into the inner membrane if located adjacent to a weak matrix-targeting signal (i.e. the native Tom70p signal anchor) or it will only arrest translocation if adjacent to a strong matrix-targeting signal. The ability of a hydrophobic domain to insert into the inner membrane, therefore, likely depends on four inter-related factors: 1) its net hydrophobicity, 2) whether or not it is permitted to pass across the outer membrane, 3) its distance from a matrix-targeting signal, and 4) the relative strength of the matrix-targeting signal. How the constituent components of the outer and inner membrane translocation machineries discriminate between these different contexts and control protein sorting, however, is not known. Presumably, it involves a complex interplay between the dynamic and reversible interactions that can occur between the two import machineries and, in addition, may result from the different requirements that individual precursor proteins may have for ATP, the electrochemical potential, and chaperone interactions.

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REFERENCES
1. Hannavy, K., Rospert, S., and Schatz, G. (1993) Curr. Opin. Cell Biol. 5, 694–700
2. Kiebler, M., Becker, K., Pfanner, N., and Neupert, W. (1993) J. Membr. Biol. 135, 191–207
3. Li, J.-M., and Shore, G. C. (1992) Biochim. Biophys. Acta 1106, 233–241
4. McBride, H. M., Millar, D. G., Li, J.-M., and Shore, G. C. (1992) J. Cell Biol. 119, 1451–1457
5. Shore, G. C., McBride, H. M., Millar, D. G., Steenaart, N. A. E., and Nguyen, M. (1995) Eur. J. Biochem. 227, 9–18
6. Nguyen, M., and Shore, G. C. (1987) J. Biol. Chem. 262, 3929–3931
7. Glauser, S. M., Miller, B. R., and Cumskey, M. G. (1990) Mol. Cell. Biol. 10, 1873–1881
8. Kaput, J., Goltz, S., and Blobel, G. (1982) J. Biol. Chem. 257, 15054–15058
9. van Loo, A. P. G. M., Brandli, A. W., and Schatz, G. (1986) Cell 44, 801–812
10. Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333
11. Hartl, F.-U., Pfanner, N., Nicholson, D. W., and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1–45
12. Smith, M. D., Petrik, M., Boucher, P. D., Barton, K. N., Carter, L., Reddy, G., Blachly-Dyson, E., Forte, M., Price, J., Verner, J., and McCauley, R. B. (1995) J. Biol. Chem. 270, 28331–28336
13. Liu, X., Bell, A. W., Freeman, K. B., and Shore, G. C. (1988) J. Cell Biol. 107, 595–599
14. Liu, X., Freeman, K. B., and Shore, G. C. (1990) J. Biol. Chem. 265, 9–12
15. Epaud, R. M., Liu, S. W., Argan, C., Gillespie, L. L., and Shore, G. C. (1986) J. Biol. Chem. 261, 10017–10020
16. Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., and Schatz, G. (1986) EMBO J. 5, 1327–1334
17. Steenaart, N. A. E., Silvius, J. R., and Shore, G. C. (1996) Biochemistry 35, 3764–3771
18. Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brutsch, H., and Schatz, G. (1990) EMBO J. 9, 3191–3200
19. Hase, T., Muller, U., Riezman, H., and Schatz, G. (1984) EMBO J. 3, 3157–3164
20. Li, J.-M., and Shore, G. C. (1992) Science 256, 1815–1817
21. Millar, D. G., and Shore, G. C. (1993) J. Biol. Chem. 268, 18403–18406
22. Millar, D. G., and Shore, G. C. (1994) J. Biol. Chem. 269, 12229–12232
23. Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J., and Shore, G. C. (1993) J. Biol. Chem. 268, 25265–25268
24. Millar, D. G., and Shore, G. C. (1996) J. Biol. Chem. 271, 25823–25829
25. Miller, B. R., and Cumskey, M. G. (1993) J. Cell Biol. 121, 1021–1029
26. Rose, J. K., and Gallione, C. J. (1981) J. Biol. Chem. 256, 519–528
27. Nguye, M., Argan, C., Sheffield, W. P., Bell, A. W., Shields, D., and Shore, G. (1987) J. Cell Biol. 104, 1155–1158
28. Nguyen, M., Bell, A. W., and Shore, G. C. (1988) J. Cell Biol. 106, 1499–1505
29. Glick, B. S., Beasley, E. M., and Schatz, G. (1992) Trends Biochem. Sci. 17, 455–459
30. Hahne, K., Haucke, V., Ramage, L., and Schatz, G. (1994) Cell 79, 829–839
31. Hartl, F.-U., and Neupert, W. (1990) Science 247, 930–938
32. McBride, H. M., Silvius, J. R., and Shore, G. C. (1995) Biochim. Biophys. Acta 1257, 162–168
33. Skerjanc, I. S., Sheffield, W. P., Randall, S. K., Silvius, J. R., and Shore, G. C. (1992) J. Biol. Chem. 267, 965–967
34. Heppel, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
35. Whitley, P., Saaf, A., Gafvelin, G., Johansson, M., Wallin, E., and von Heijne, G. (1995) Biochem. Soc. Trans. 23, 965–967
Alteration of a Mitochondrial Outer Membrane Signal Anchor Sequence That Permits Its Insertion into the Inner Membrane: CONTRIBUTION OF HYDROPHOBIC RESIDUES
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