

Biogenesis of Mitochondrial Inner Membrane Proteins*

Kostas Tokatlidis‡ and Gottfried Schatz§¶

From the ‡School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom and §Biozentrum, University of Basel, CH-4056 Basel, Switzerland

The Problem

The mitochondrial inner membrane of most organisms studied so far contains about a dozen proteins made by the mitochondrial genetic system and on the order of 10^2 proteins imported from the cytoplasm. This review briefly describes the two major pathways by which proteins made inside or outside the mitochondria are inserted into the inner membrane. These pathways were discovered only during the past few years; one of them involves a novel family of chaperone-like proteins in the mitochondrial intermembrane space.

Import of proteins from the cytoplasm into mitochondria occurs by distinct routes that are dictated by the properties and final destination of each protein within the mitochondria (1–3). However, most of the different routes described until recently have been variants of the general “matrix pathway.” In this pathway, a protein carrying a positively charged amphiphilic helix at its N terminus is bound by cytosolic chaperones, delivered to an array of import receptors on the mitochondrial surface, and then transported across two distinct, hetero-oligomeric protein translocation channels, the TOM complex in the outer membrane and the TIM23 complex in the inner membrane. During transport, the two complexes are transiently linked by the translocating precursor chain (4, 5). Transport across the TOM complex appears to be driven by binding of the basic N-terminal “presequence” to a series of acidic receptor domains of increasing avidity (“acid chain hypothesis”) (6, 7); insertion of the presequence into the TIM23 complex is driven electrophoretically by the potential across the inner membrane; and complete transport of the precursor into the matrix is driven by an ATP-powered import motor attached to the inner mouth of the TIM23 complex. Proteins destined for compartments other than the matrix can diverge from the general matrix pathway at different points (8–10). Some proteins destined for the outer membrane can arrest in the TOM complex and then escape into the outer membrane (11); and some intermembrane space proteins can arrest in the TIM23 complex and then be proteolytically released into the intermembrane space (8).

A few years ago it seemed likely that a variation of this general matrix pathway would also sort proteins to the inner membrane. According to this view, a protein destined for the inner membrane would arrest in the TIM23 complex and escape “sidewise” into the phospholipid bilayer of the inner mem-

brane. So far, however, no inner membrane protein has been shown to follow such a pathway. Arrest in the TIM23 complex has only been found for some proteins destined for the intermembrane space.

Arrest in the TIM23 Complex

The TIM23 complex functioning in the matrix pathway consists of the two integral membrane proteins Tim17p and Tim23p, which together form a gated, protein-conducting channel across the mitochondrial inner membrane. Most precursors enter this channel with their N-terminal matrix signal first, presumably in an unfolded conformation. A few precursors appear to contain an internal matrix signal and may insert into the channel as a loop (12). As the channel diameter of the TIM23 complex is at most 20 Å (13), it is not clear how this channel can accommodate a looped polypeptide chain.

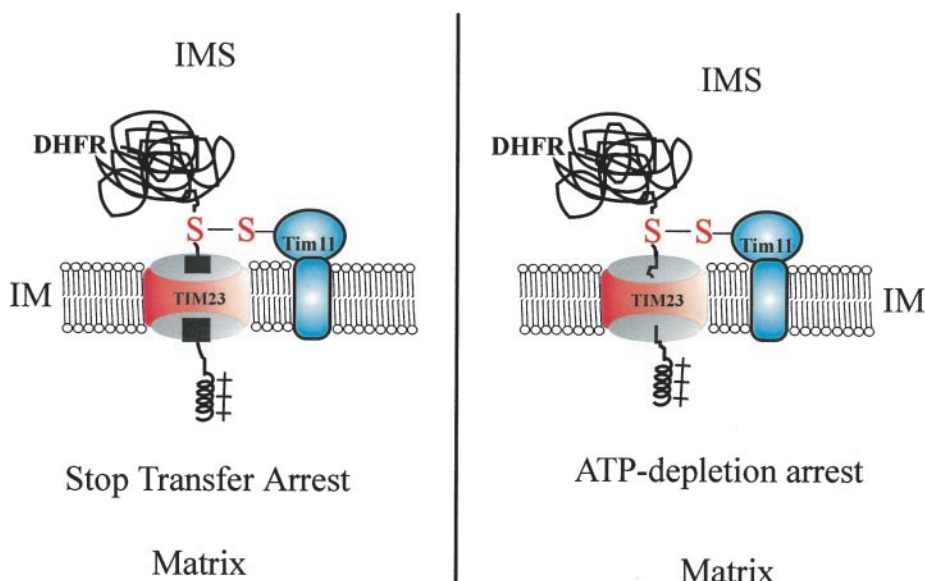
Proteins targeted to the aqueous intermembrane space by arrest in the TIM23 complex carry a matrix signal followed by an uncharged “sorting signal” (14). We have proposed that the matrix signal directs the precursor into the TIM23 complex, that the sorting signal causes it to become stuck there, and that the mature protein is released from the stuck translocation intermediate into the intermembrane space by proteolytic cleavage on the outer face of the inner membrane (“stop transfer” model; Refs. 8 and 14). The alternative “conservative sorting” model proposed that the entire bipartite signal enters the matrix and then loops back into the inner membrane to direct re-export of the entire precursor chain into the intermembrane space (15, 16). Both models invoked the same proteolytic cleavage of the stuck translocation intermediate but predicted a different membrane environment of the intermediate’s sorting signal. The stop transfer model predicted a similar environment as for a precursor *en route* to the matrix, whereas the conservative sorting predicted a location in a distinct re-export system. To test these predictions, we generated the translocation intermediate of an intermembrane space-targeted precursor that had already reached the intermembrane space but had not yet been proteolytically released from its anchor site in the inner membrane. Next to its sorting signal, the intermediate carried a unique cysteine that could be cross-linked with up to 80% efficiency to an 11-kDa inner membrane protein, which we termed Tim11p (17). The unusually high cross-linking efficiency argued against the possibility that the sorting signal of the translocation intermediate looped through the matrix. Tim11p was also efficiently cross-linked to the translocation intermediate of a matrix-targeted precursor whose complete import into the matrix was arrested by depleting the matrix of ATP. As reported by us (17), Tim11 is neither a sorting signal receptor nor part of the TIM23 complex. Subsequent work (18, 19)¹ identified it as a protein associated with the F_1F_0 -ATPase complex. Proximity of the ATPase complex to a protein import channel has also been observed in chloroplasts (20). Although a role of Tim11p in protein sorting is still uncertain, the protein has served as a unique topological marker to show that the molecular environment of the sorting signal during the final step of protein sorting to the intermembrane space is very similar or identical to that of a precursor *en route* to the matrix (Fig. 1).

* This minireview will be reprinted in the 1999 Minireview Compendium, which will be available in December, 1999. Work on this topic was supported by The Royal Society, The Wellcome Trust (to K. T.), the Swiss National Science Foundation, the European Economic Community, the Human Frontiers Science Program, and the Louis-Jeantet Foundation (to G. S.).

¶ To whom correspondence should be addressed. Tel.: 41 61 267 2150; Fax: 41 61 267 2148; E-mail: schatz@ubaclu.unibas.ch.

¹ C. Brunner, K. Tokatlidis, and G. Schatz, unpublished data.

FIG. 1. Arrest in the TIM23 complex for protein sorting to the intermembrane space. Disulfide cross-linking (in red) shows that the translocation intermediate arrested by its stop transfer signal (black bar) is in the same or a very similar molecular environment as a matrix-targeted precursor arrested by lack of ATP in the matrix. IM, inner membrane; IMS, intermembrane space; DHFR, dihydrofolate reductase fused to either an intermembrane space targeting signal (left) or to a matrix targeting signal (right).



A Separate "Insertion Complex" in the Inner Membrane

Models explaining protein import into the inner membrane through docked TOM and TIM23 complexes followed by arrest of the precursor in the inner membrane have always faced a major problem; docking of the two protein transport channels is thought to be effected by interaction of a precursor's N-terminal basic matrix signal with an acidic Tim23 domain on the outer face of the inner membrane, but many polytopic inner membrane proteins lack a basic targeting signal at their N terminus. Instead, they contain one or several internal signals that remain poorly characterized (21, 22). Among the most abundant of such proteins are the metabolite transporters, a protein family that has 34 members in *Saccharomyces cerevisiae* and includes the ADP/ATP transporter (AAC). How can these insoluble proteins cross the aqueous intermembrane space if there is no mechanism that induces docking between the TOM and the TIM23 complexes? These precursors could negotiate the aqueous divide between the two membranes with the help of soluble chaperones, but efforts to detect chaperones in the intermembrane space had been unsuccessful.

Recent work has now uncovered chaperone-like proteins in the intermembrane space that are part of a novel machinery dedicated to importing inner membrane proteins. An important advance was the discovery that Tim22p, an inner membrane protein of yeast homologous to Tim23p, mediates import of carrier proteins into the inner membrane but not general protein import into the matrix (23). Genetic studies identified two partner subunits of Tim22p: a membrane-embedded 54-kDa protein termed Tim54p (24) and a peripheral 12-kDa protein termed Tim12p (25, 26). Tim12 had been discovered before (and named Mrs5p) as an extragenic suppressor of mutations blocking splicing of mitochondrial RNA (27). The suppressor screen had also netted the protein Mrs11p (28), a 10-kDa homolog of Tim12p later renamed Tim10p. Tim10p was located in the soluble intermembrane space whereas Tim12p was bound to the outer surface of the inner membrane. Each of these two proteins was essential for viability. As most of the known essential mitochondrial proteins participate in protein import (1–3, 29), these newly discovered proteins appeared to be parts of a novel protein import system (Fig. 2).

Tim10p exists in large molar excess over Tim12p. Most of it is found in a 70-kDa complex in the intermembrane space, and the remainder is located in a 300-kDa membrane-embedded complex (the so-called TIM22 complex) that contains all of

Tim12p, Tim22p, and Tim54p. A partner protein of Tim10p in the 70-kDa complex was found by searching for extragenic suppressors of a temperature-sensitive mutation in Tim10p. This screen netted a mutant allele of a 9-kDa protein (termed Tim9p) with the following properties. Its sequence was 25% identical to Tim10p; it was essential for viability; and most of it was associated with Tim10p in the 70-kDa intermembrane space complex. The properties of the chromatographically purified complex (30) and isotopic *in vivo* labeling studies (31) suggest that the most likely composition of the complex is (Tim9)₃(Tim10)₃.

A search of the yeast genome revealed two additional members of the Tim9p-Tim10p-Tim12p protein family: an 8-kDa protein termed Tim8p and a 13-kDa protein termed Tim13p (32, 33). Tim8p and Tim13p are present in the soluble intermembrane space as a separate 70-kDa complex that is distinct from the (Tim9)₃(Tim10)₃ complex. The Tim8p-Tim13p complex may also contain Tim9p, but Tim9 is only weakly bound and completely removed upon chromatographic purification of the complex. Neither Tim8p nor Tim13p is essential for viability. However, they appear to interact *in vivo* with Tim10p as deletion of either Tim8p or Tim13p is synthetically lethal with a temperature-sensitive mutation in Tim10p.

How do these proteins coordinate the insertion of the hydrophobic carriers? Inactivation or deletion of Tim10 blocks import of AAC and other multispinning inner membrane proteins across the TOM channel, suggesting that the 70-kDa (Tim9p)₃(Tim10p)₃ complex binds the incoming precursor and thereby pulls it into the intermembrane space. In contrast, inactivation or depletion of Tim12 allows import across the TOM channel and binding of the precursor to the inner membrane but prevents its correct potential-dependent insertion into the membrane. Inactivation of Tim12 thus has a similar effect as collapsing of the potential across the mitochondrial inner membrane. Cross-linking experiments have verified that Tim10 and Tim12 interact directly with the incoming precursor proteins. Inactivation or depletion of either Tim10 or Tim12 has little, if any, effect on the import of proteins into the matrix or of precursors with bipartite targeting signals into the intermembrane space (25, 26, 30, 34).

The soluble Tim9p-Tim10p complex is at least 20-fold more abundant than the membrane-associated TIM22 complex (31).¹ Why? One explanation would be that the two complexes have different affinities for precursors; the soluble Tim9p-Tim10p complex may be a low affinity chaperone-like system designed

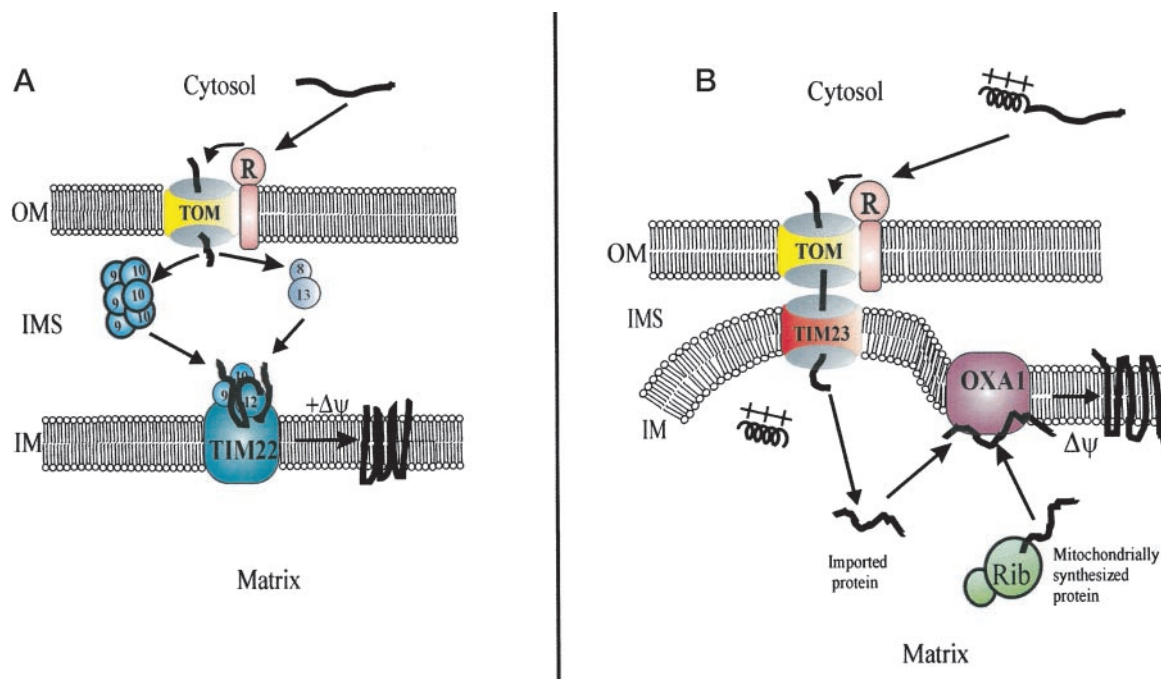


FIG. 2. **Different modes of insertion of proteins into the inner membrane.** A, insertion by the TIM22 pathway involving the 70-kDa $(\text{Tim}9)_3(\text{Tim}10)_3$ and $\text{Tim}8\text{p-Tim}13\text{p}$ complexes in the aqueous intermembrane space and the 300-kDa TIM22 complex in the inner membrane. The electrochemical potential $\Delta\psi$ facilitates insertion. B, insertion from the matrix involving the OXA1 complex. Both imported and mitochondrially synthesized proteins can be inserted via this pathway. The presence of matrix-targeted precursors that directs translocation via docking of the TOM to the TIM23 complex is shown as a positively charged helix. OM, outer membrane; IM, inner membrane; IMS, intermembrane space; R, receptor complex in the outer membrane; Rib, mitochondrial ribosomes.

to capture the appropriate precursors as these emerge from the TOM complex. In contrast, the TIM22 complex may have a high affinity for a precursor bound to a $(\text{Tim}9\text{p})_3(\text{Tim}10\text{p})_3$ complex. However, it is still unclear how $\text{Tim}9\text{p}$, $\text{Tim}10\text{p}$, and $\text{Tim}12\text{p}$ interact with the precursor. Do they recognize a specific sequence motif and, if so, which one? It has been suggested that $\text{Tim}10\text{p}$ and $\text{Tim}12\text{p}$ recognize a conserved region after the first transmembrane domain of mitochondrial metabolite carriers (26, 34). These carriers all have three similar repeats of approximately 100 amino acids each, which would argue for three distinct internal targeting signals. These signals might be recognized by three complementary motifs in the heterotrimeric $\text{Tim}9\text{p-Tim}10\text{p}$ complex, thereby allowing each complex to bind one precursor molecule. Alternatively, the $\text{Tim}9\text{p-Tim}10\text{p}$ complex might bind hydrophobic sequences with little, if any, sequence preference. This latter model could explain the observation that the $(\text{Tim}9)_3(\text{Tim}10)_3$ and $\text{Tim}8\text{p-Tim}13\text{p}$ complexes also mediate the import of polytopic inner membrane proteins that are unrelated to the metabolite carrier family (35).

Insertion of Polytopic Proteins from the Matrix

The third insertion pathway for inner membrane proteins involves yet another large membrane-bound complex, the OXA1 complex. This complex is named after $\text{Oxa}1\text{p}$, its only subunit that has so far been identified. $\text{Oxa}1\text{p}$ is a polytopic inner membrane protein with five potential transmembrane regions. It is made in the cytoplasm with a transient N-terminal matrix targeting signal, imported into the matrix by the general matrix import pathway, cleaved to the mature protein, and then inserted from the matrix into the inner membrane. This insertion step requires a potential across the inner membrane (36) and resembles the Sec-independent, ΔpH -dependent insertion of polytopic proteins into the bacterial plasma membrane. No component of that bacterial pathway has as yet been identified. $\text{Oxa}1\text{p}$ is required for its own insertion and also mediates the insertion of mitochondrially synthesized polytopic

proteins into the inner membrane (37–39). $\text{Oxa}1\text{p}$ may thus be the central component of a protein insertion machinery that inserts hydrophobic transmembrane regions from the matrix into the inner membrane.

How does insertion mediated by the OXA1 complex compare with that mediated by the TIM22 complex? Both processes require a membrane potential and insert proteins according to the “positive inside” rule (40). However, whereas insertion by the TIM22 complex obviously occurs post-translationally, the mode of insertion mediated by the OXA1 complex is not yet clear. The matrix-located proteins $\text{mhsp}70$ ($\text{Ssc}1\text{p}$) and $\text{hsp}60$ have been reported to function as chaperones for mitochondrially encoded proteins (41, 42), but it remains to be shown that these chaperones deliver their cargo to the OXA1 complex. Insertion of some very hydrophobic mitochondrially made proteins by the OXA1 pathway may well occur co-translationally. As some features of this pathway are also found in bacteria (see above), it may reflect the evolutionary origin of mitochondria from bacterial ancestors. On the other hand, bacteria appear to lack recognizable homologs of the small Tim proteins of the mitochondrial intermembrane space. The TIM22 pathway may thus have originated late in the evolution of eukaryotic cells (Fig. 2).

A Human Disease Linked to Defective Protein Import into the Mitochondrial Inner Membrane

It was recently reported that DDP1, a small human protein whose loss causes a severe deafness-dystonia syndrome, is homologous to $\text{Tim}8\text{p}$ of yeast (32). As DDP1 was shown to be localized in mitochondria of mammalian cells and as isolated yeast mitochondria imported radiolabeled DDP1 into their intermembrane space, the human deafness-dystonia syndrome is a mitochondrial disease caused by a defective import system for inner membrane proteins. As the human genome contains several DDP1 homologs (32), a study of these proteins should identify additional human diseases of similar etiology.

REFERENCES

1. Neupert, W. (1997) *Annu. Rev. Biochem.* **66**, 863–917
2. Schatz, G. (1996) *J. Biol. Chem.* **271**, 31763–31766
3. Pfanner, N., Craig, E. A., and Hönlinger, A. (1997) *Annu. Rev. Cell Dev. Biol.* **13**, 25–51
4. Horst, M., Hilfiker-Rothenthal, S., Oppliger, W., and Schatz, G. (1995) *EMBO J.* **14**, 2293–2297
5. Berthold, J., Bauer, M., Schneider, H.-C., Klaus, C., Dietmeier, K., Neupert, W., and Brunner, M. (1995) *Cell* **81**, 1085–1093
6. Schatz, G. (1997) *Nature* **388**, 121–122
7. Dietmeier, K., Hönlinger, A., Bömer, U., Dekker, P. J., Eckerskorn, C., Lottspeich, F., Kübrich, M., and Pfanner, N. (1997) *Nature* **388**, 195–200
8. Glick, B. S., Brandt, A., Cunningham, K., Müller, S., Hallberg, R. L., and Schatz, G. (1992) *Cell* **69**, 809–822
9. Bömer, U., Meijer, M., Guiard, B., Dietmeier, K., Pfanner, N., and Rassow, J. (1997) *J. Biol. Chem.* **272**, 30439–30446
10. Haucke, V., Ocana, C. S., Hönlinger, A., Tokatlidis, K., Pfanner, N., and Schatz, G. (1997) *Mol. Cell. Biol.* **17**, 4024–4032
11. Hönlinger, A., Bömer, U., Alconada, A., Ecuerskorn, C., Lottspeich, F., Dietmeier, K., and Pfanner, N. (1996) *EMBO J.* **15**, 2125–2137
12. Fölsch, H., Guiard, B., Neupert, W., and Stuart, R. A. (1996) *EMBO J.* **15**, 479–487
13. Schwartz, M. P., and Matouschek, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.*, in press
14. Glick, B. S., Beasley, E. M., and Schatz, G. (1992) *Trends Biochem. Sci.* **17**, 453–459
15. Hartl, F.-U., Ostermann, J., Guiard, B., and Neupert, W. (1987) *Cell* **51**, 1027–1037
16. Hartl, F. U., and Neupert, W. (1990) *Science* **247**, 930–938
17. Tokatlidis, K., Junne, T., Moes, S., Schatz, G., Glick, B. S., and Kronidou, N. (1996) *Nature* **384**, 585–588
18. Arnold, I., Bauer, M. F., Brunner, M., Neupert, W., and Stuart, R. A. (1997) *FEBS Lett.* **411**, 195–200
19. Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schagger, H. (1998) *EMBO J.* **17**, 7170–7178
20. Scott, S. V., and Theg, S. M. (1996) *J. Cell Biol.* **132**, 63–75
21. Smagula, C., and Douglas, M. D. (1988) *J. Biol. Chem.* **263**, 6783–6790
22. Pfanner, N., Hoeben, P., Tropschug, M., and Neupert, W. (1987) *J. Biol. Chem.* **262**, 14851–14854
23. Sirrenberg, C., Bauer, M. F., Guiard, B., Neupert, W., and Brunner, M. (1996) *Nature* **384**, 582–585
24. Kerscher, O., Holder, J., Srinivasan, M., Leung, R. S., and Jensen, R. E. (1997) *J. Cell Biol.* **139**, 1663–1675
25. Koehler, C. M., Jarosch, E., Tokatlidis, K., Schmidt, K., Schweyen, R. J., and Schatz, G. (1998) *Science* **279**, 369–373
26. Sirrenberg, C., Endres, M., Fölsch, H., Stuart, R. A., Neupert, W., and Brunner, M. (1998) *Nature* **391**, 912–915
27. Jarosch, E., Tuller, G., Daum, G., Waldherr, M., Voskova, A., and Schweyen, R. J. (1996) *J. Biol. Chem.* **271**, 17219–17225
28. Jarosch, E., Rodel, G., and Schweyen, R. J. (1997) *Mol. Gen. Genet.* **255**, 157–165
29. Pfanner, N., Douglas, M. G., Endo, T., Hoogenraad, N. J., Jensen, R. E., Meijer, M., Neupert, W., Schatz, G., Schmitz, U. K., and Shore, G. C. (1996) *Trends Biochem. Sci.* **21**, 51–52
30. Koehler, C. M., Merchant, S., Oppliger, W., Schmid, K., Jarosch, E., Dolfini, L., Junne, T., Schatz, G., and Tokatlidis, K. (1998) *EMBO J.* **17**, 6477–6486
31. Adam, A., Endres, M., Sirrenberg, C., Lottspeich, F., Neupert, W., and Brunner, M. (1999) *EMBO J.* **18**, 313–319
32. Koehler, C. M., Leuenberger, D., Merchant, S., Renold, A., Junne, T., and Schatz, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 2141–2146
33. Kurz, M., Martin, H., Rassow, J., Pfanner, N., and Ryan, M. (1999) *Mol. Biol. Cell* **10**, 2461–2474
34. Endres, M., Neuper, W., and Brunner, M. (1999) *EMBO J.* **18**, 3214–3221
35. Leuenberger, D., Bally, N., Schatz, G., and Koehler, C. (1999) *EMBO J.* **18**, 4818–4822
36. Herrmann, J. M., Neupert, W., and Stuart, R. A. (1997) *EMBO J.* **16**, 2217–2226
37. Hell, K., Herrmann, J. M., Pratje, E., Neupert, W., and Stuart, R. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2250–2255
38. Hell, K., Herrmann, J., Pratje, E., Neupert, W., and Stuart, R. A. (1997) *FEBS Lett.* **418**, 367–370
39. He, S., and Fox, T. (1997) *Mol. Biol. Cell* **8**, 1449–1460
40. von Heijne, G. (1989) *Nature* **341**, 456–458
41. Herrmann, J. M., Stuart, R. A., Craig, E. A., and Neupert, W. (1994) *J. Cell Biol.* **127**, 893–902
42. Prasad, T. K., Hack, E., and Hallberg, R. L. (1990) *Mol. Cell. Biol.* **8**, 3979–3986