Mitochondrial Import Receptors Tom20 and Tom22 Have Chaperone-like Activity*

Masato Yano‡, Kazutoyo Terada, and Masataka Mori‡

From the Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan

Most mitochondrial preproteins are synthesized in the cytosol with N-terminal signal sequences (presequences) or internal targeting signals. Generally, preproteins with presequences are initially recognized by Tom20 (translocase of the outer membrane) and, subsequently, by Tom22, whereas hydrophobic preproteins with internal targeting signals are first recognized by Tom70. Recent studies suggest that Tom70 associates with molecular chaperones, thereby maintaining their substrate preproteins in an import-competent state. However, such a function has not been reported for other Tom component(s). Here, we investigated a role for Tom20 in preventing substrate preproteins from aggregating. In vitro binding assays showed that Tom20 binds to guanidinium chloride unfolded substrate proteins regardless of the presence or absence of presequences. This suggests that Tom20 functions as a receptor not only for presequences but also for mature portions exposed in unfolded preproteins. Aggregation suppression assays on citrate synthase showed that the cytosolic domain of Tom20 has a chaperone-like activity to prevent this protein from aggregating. This activity was inhibited by a presequence peptide, suggesting that the binding site of Tom20 for presequence is identical or close to the active site for the chaperone-like activity. The cytosolic domain of Tom22 also showed a similar activity for citrate synthase, whereas Tom70 did not. These results suggest that the cytosolic domains of Tom20 and Tom22 function to maintain their substrate preproteins unfolded and prevent them from aggregating on the mitochondrial surface.

Received for publication, October 24, 2003, and in revised form, December 10, 2003
Published, JBC Papers in Press, December 29, 2003, DOI 10.1074/jbc.M311710200

‡ To whom correspondence may be addressed. Tel.: 81-96-373-5143; Fax: 81-96-373-5145; E-mail: myano@gpo.kumamoto-u.ac.jp or masa@gpo.kumamoto-u.ac.jp

‡ This work was supported by Grants-in-aid 14780550 (to M. Y.) and 14037257 (to M. M.) from the Ministry of Education, Science, Technology, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: TOM, translocase of the outer membrane of mitochondria; AIP, arylhydrocarbon receptor interacting protein; CS, citrate synthase; GFP, green fluorescent protein; GST, glutathione S-transferase; OTC, mature form of ornithine transcarbamylase; pOTC, precursor form of OTC; TPR, tetra-tricopeptide repeat; IMS, intermembrane space.
In the present study, we report that the cytotoxic domain of Tom20 serves as a preprotein receptor that binds not only to presequences but also to the unfolded mature portion of preproteins. An aggregation suppression assay indicated that Tom20 has a chaperone-like activity to prevent a substrate protein, citrate synthase (CS), from thermal aggregation. Furthermore, the cytotoxic domain of Tom22 showed a similar activity, whereas that of Tom70 did not. These results suggest that the cytotoxic domains of Tom20 and Tom22, but not Tom70, maintain their substrate preproteins unfolded on the mitochondrial surface and prevent them from aggregation.

EXPERIMENTAL PROCEDURES

Materials—Mitochondrial CS (EC 4.1.3.7) from pig heart was obtained from Sigma. The PTH-(69–84) peptide (EADKADNVYLTKAKSQ), corresponding to the region 69–84 of human parathyroid hormone, was purchased from Peptide Institute Inc. (Osaka, Japan). The presequence peptide (MLFLNRILLNAAFRNGHNFMVRNFRC-GQFLQ), corresponding to the presequence of human pOTC, wascommercially synthesized. The anti-porcine CS antibody was purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). Anti-green fluorescent protein (GFP) antiserum was prepared as described previously (27).

Construction of Plasmids—Human Tom70 cDNA (GenBank™ accession number NM_014820) (12, 13) was excised and cloned into pQE30 (Qiagen). The resulting plasmid, pQE30-His6-cTom70, expresses the N-terminally histidine-tagged cytotoxic domain of human Tom70 (residues 95–608). cDNA for pOTC-GFP (28) and GFP were PCR-amplified and cloned into pET-30a (+) (Novagen, Darmstadt, Germany). The resulting plasmids, pET-30a (+) pOTC-GFP-His6 and pET-30a (+) GFP-His6, express C-terminally histidine-tagged pOTC-GFP and GFP, respectively.

Expression and Purification of Proteins—Glutathione S-transferase (GST)-fused human Tom20 and Tom22 proteins were expressed and purified as described (14, 27). To obtain the cytotoxic domain of human Tom70, the pQE30-His6-cTom70 plasmid was transformed into S. glutamicum (Qiagen), and the expressed protein was purified, as the native protein, by metal chelation chromatography. To obtain pOTC-GFP-His8 and GFP-His8 proteins, the pET-30a (+) pOTC-GFP-His8 or pET-30a (+) GFP-His8 plasmid was transformed into S. glutamicum cells, respectively. The expressed proteins were purified by metal chelation chromatography under denaturing conditions in the presence of 6 M guanidinium chloride.

In Vitro Binding Assay—Purified GST-fused proteins were absorbed on glutathione-Sepharose beads in binding buffer (20 mM Hepes-KOH, pH 7.4, 50 mM KCl, 1 mM MgCl2, and 0.1 mg/ml bovine serum albumin). A reticulocyte lysate containing 35S-labeled proteins was mixed with the beads in the binding buffer (total 300 μl). Binding reaction was performed for 30 min at 25 °C with gentle shaking. Unbound proteins were removed by centrifugation in an Ultrafree-MC centrifugal filter unit (Millipore Corp., Bedford, MA), and the retained beads were washed once with binding buffer. Bound proteins were eluted by adding elution buffer A (50 mM Tris-HCl, pH 8, and 15 mM glutathione), and the eluate was subjected to SDS-PAGE. Radioactivity in the gels was visualized and quantified using a FUJIX BAS2000 image plate analyzer (Fuji Film Co., Tokyo, Japan). Elution of proteins was checked by staining with Coomassie Brilliant Blue R-250.

RESULTS

The Cytotoxic Domain of Tom20 Binds to the Presequence and the Unfolded Mature Portion of Preproteins—To examine whether Tom20 serves as a receptor only for the presequence or for the whole preprotein, a GST-capture binding assay was performed (Fig. 1). We first confirmed our previous observation (27) that Tom20 binds to a presequence (Fig. 1A). Reticulocyte lysates containing in vitro translated proteins were incubated with GST fusions prebound with glutathione-Sepharose beads, and GST fusions and the bound proteins were then eluted with reduced glutathione. pOTC-GFP is a protein in which the presequence of pOTC is fused with GFP (28). Tom20-(25–145) is a GST-fused protein in which GST was N-terminally fused with residues 25–145 of Tom20 (see also Fig. 2A). When in vitro translated pOTC-GFP and GFP were subjected to the binding assay, pOTC-GFP bound to Tom20, whereas GFP did not. This result, together with our previous observations (27), indicate that Tom20 binds to the presequence portion of pOTC-GFP but not to the GFP domain. We showed previously that the GFP domain of pOTC-GFP is stably folded when it is translated in rabbit reticulocyte lysate (27). Therefore, Tom20 apparently does not bind to the folded form of GFP.

We then asked whether Tom20 can bind to the unfolded form of GFP. Purified GFP and pOTC-GFP denatured in 6 M guanidinium chloride were subjected to the binding assay (Fig. 1B). Surprisingly, unfolded GFP as well as unfolded pOTC-GFP
bound to Tom20. This suggests that Tom20 binds to the unfolded GFP polypeptide regardless of the presence or absence of the presequence portion. Thus, Tom20 appears to have a specificity for unfolded proteins.

To confirm this, we used another protein, citrate synthase (Fig. 1C). CS is a mitochondrial protein that is initially synthesized as a precursor form with an N-terminal presequence. The precursor form of CS is imported into the mitochondria and processed into the mature form. Therefore, purified CS from pig heart has no presequence. When native and unfolded forms of purified CS were subjected to the binding assay, the denatured form of CS bound to Tom20, whereas the native form of the enzyme did not. This suggests that Tom20 can recognize the unfolded CS but not the folded enzyme. Taken together with the observation regarding GFP, Tom20 has a general specificity for unfolded proteins. These results suggest that Tom20 serves as a receptor for unfolded preproteins on the mitochondrial surface.

**Tom20 Has a Chaperone-like Activity**—The observation that Tom20 binds to unfolded proteins suggests that Tom20 has a chaperone activity. Therefore, we examined whether Tom20 can prevent a substrate protein from aggregation by using an aggregation suppression assay (Fig. 2). Native CS was incubated with Tom20-(25–145) (Fig. 2A) at 43 °C, and the aggregation of CS was monitored by measuring the light scattering (Fig. 2B). When CS alone was incubated, it began to aggregate after 4 min and reached a plateau at 15 min. Aggregation of CS was not affected by the addition of GST. In contrast, when CS was incubated with equimolar Tom20 mutants in the absence of CS (open square) was also monitored. C, aggregation of CS (0.15 μM) was performed in the presence of 0.5 μM GST-fused Tom20 mutants at 43 °C for 10 min. Aggregated proteins were recovered by centrifugation, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. % Input represents the percentage of input CS.

**Tom20 and Tom22 Have Chaperone-like Activity**
Tom20 and Tom22 Have Chaperone-like Activity

DISCUSSION

Although the importance of cytosolic chaperones in mitochondrial protein import has been established (1–3), there are few studies on the chaperone-like function of the receptor proteins on the mitochondrial surface. In the present study, we found that the cytosolic domain of Tom20 binds to unfolded proteins regardless of the presence or absence of presequence (Fig. 1). This suggests that Tom20 functions as a receptor not only for presequences but also for the unfolded portions of mature preproteins. Although a number of mitochondrial preproteins are known to be maintained in an unfolded state on the mitochondrial surface, the extent to which preproteins are in a folded or unfolded state during their cytosolic transport and during translocation into mitochondria was not known. Our finding suggests that preproteins are maintained unfolded throughout the import process from the cytosol to the inside of mitochondria. On the other hand, some preproteins are transferred to the mitochondrial surface in a folded state. They are then imported into the mitochondria by sequential unfolding of the preprotein driven by the pulling force applied by mitochondrial Hsp70 (30). Also in this case, Tom20 and Tom22 may serve as receptors for partially unfolded portions of the preproteins.

The present results also suggest that Tom20 has a chaperone-like activity capable of suppressing the thermal aggregation of CS (Fig. 2). This activity may be important for maintaining mitochondria-targeted preproteins unfolded on the mitochondrial surface and suppressing their aggregation. Because preproteins that failed to be translocated into mitochondria are rapidly degraded (20, 31), the activity of Tom20 may increase the efficiency of preprotein import by preventing preproteins from degrading. The importance of this activity for the mitochondrial protein import was suggested by our recent find-
ing on AIP (20). The chaperone-like activity of AIP appears to stabilize matrix precursor proteins such as pOTC and facilitate its import into mitochondria. The importance of chaperones in protein transport is also illustrated by the role of trigger factor as a ribosome-associated protein that stabilizes the translocation-competent form of a secretory precursor protein in *Escherichia coli* (32, 33). Trigger factor exhibits chaperone-like activity to suppress the thermal aggregation of substrate proteins (34).

In the current studies, the active site of Tom20 responsible for chaperone-like activity was suggested to be identical or close to the binding site for a presequence peptide (Fig. 3). In general, chaperone proteins have a hydrophobic surface for binding to their unfolded substrate proteins. Because the binding groove of Tom20 for presequences is composed of hydrophobic residues (22), it is possible that the groove also serves as a binding site for unfolded proteins. More than one Tom20 molecule may sequentially bind to a single preprotein molecule to prevent it from aggregation. We suppose that a Tom20 molecule first binds to the presequence of a preprotein, and then another Tom20 molecule(s) binds to the mature portion. Then the preprotein, dissociated from the Tom20 molecules, is transferred to Tom22 and is translocated across the outer membrane through the general import pore. Mitochondrial preproteins may be strictly distinguished from other proteins by cooperative recognition by Tom20 and other receptors such as Tom22 and/or by sequential recognition of the multiple sites in presequences by these receptors.

Furthermore, we also found that the cytosolic domain of Tom22 has a chaperone-like activity (Fig. 4). Because preproteins initially recognized by Tom20 are subsequently trans-

![Figure 4](image-url)
performed at 43°C in the absence (open circle) or presence of 0.5 μM Tom70-(95–608) (closed square). The aggregation of 0.5 μM Tom70-(95–608) in the absence of CS (open square) was also monitored. B, aggregation of CS (0.15 μM) was performed at 43°C for 10 min in the absence or presence of 0.5 μM Tom70-(95–608). Aggregated proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. % Input represents the percentage of input CS.

Tom70-(95–608) does not suppress the thermal aggregation of CS. A, aggregation of CS (0.15 μM) was monitored at 43°C in the absence (open circle) or presence of 0.5 μM Tom70-(95–608) (closed square). The aggregation of 0.5 μM Tom70-(95–608) in the absence of CS (open square) was also monitored. B, aggregation of CS (0.15 μM) was performed at 43°C for 10 min in the absence or presence of 0.5 μM Tom70-(95–608). Aggregated proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. % Input represents the percentage of input CS.

Acknowledgments—We thank Dr. Nicholas J. Hoogenraad (La Trobe University, Bundoora, Australia) for Tom70 cDNA and for critical reading of the manuscript. We also thank colleagues of our laboratory (Kumamoto University) for discussions.

REFERENCES

1. Mori, M., and Terada, K. (1998) Biochim. Biophys. Acta 1403, 12–27
2. Terada, K., Ohitsuuka, K., Inamoto, N., Yoneda, Y., and Mori, M. (1995) Mol. Cell. Biol. 15, 3708–3713
3. Terada, K., and Mori, M. (2000) J. Biol. Chem. 275, 24728–24734
4. Lithgow, T., Glick, B. S., and Schatz, G. (1995) Trends Biochem. Sci. 20, 98–101
5. Lill, R., and Neupert, W. (1996) Trends Biochem. Sci. 6, 56–61
6. Neupert, W. (1997) Annu. Rev. Biochem. 66, 863–917
7. Herrmann, J. M., and Neupert, W. (2000) Curr. Opin. Microbiol. 3, 210–214
8. Pfanner, N., and Geissler, A. (2001) Nat. Rev. Mol. Cell Biol. 2, 339–349
9. Hanson, B., Nuttal, S., and Mihara, K. (1996) Eur. J. Biochem. 235, 750–753
10. Goping, I. S., Millar, D. G., and Shore, G. C. (1995) FEBS Lett. 372, 45–50
11. Seki, N., Moczko, M., Nagase, T., and Zufall, N. (1995) FEBS Lett. 375, 367–370
12. Alvarez-Dolado, M., Gonzalez-Moreno, M., Valencia, A., Zenke, M., Bernal, J., and Munoz, A. (1999) J. Neurochem. 73, 2240–2249
13. Suzuki, H., Maeda, M., and Mihara, K. (2002) J. Cell Sci. 115, 1895–1905
14. Yano, M., Hoogenraad, N., Terada, K., and Mori, M. (2000) Mol. Cell. Biol. 20, 7205–7213
15. Sasaki, K., Suzuki, H., Tsunenoka, M., Maeda, M., Iwamoto, R., Hasuwa, H., Shida, S., Takahashi, T., Sakaguchi, M., Endo, T., Miura, Y., Mekada, E., and Mihara, K. (2000) J. Biol. Chem. 275, 31996–32002
16. Suzuki, H., Okazawa, Y., Komiya, T., Saeki, K., Mekada, E., Kitada, S., Ito, A., and Mihara, K. (2000) J. Biol. Chem. 275, 37930–37936
17. Johnston, A. J., Hoogenraad, J., Dougan, D. A., Truscott, K. N., Yano, M., Mori, M., Hoogenraad, N. J., and Ryan, M. T. (2002) J. Biol. Chem. 277, 42197–42204
18. Young, J. C., Hoogenraad, N. J., and Hartl, F. U. (2003) Cell 112, 41–50
19. Brix, J., Zeigler, G. A., Dietmeier, K., Schneider-Mergener, J., Schulz, G. E., and Pfanner, N. (2000) J. Mol. Biol. 303, 479–488
20. Yano, M., Terada, K., and Mori, M. (2003) J. Cell Biol. 163, 45–56
21. Komiya, T., Rospert, S., Schatz, G., and Mihara, K. (1997) EMBO J. 16, 4267–4275
22. Abe, Y., Shodai, T., Muto, T., Mihara, K., Torii, H., Nishikawa, S., Endo, T., and Kohda, D. (2000) Cell 100, 551–560
23. Obita, T., Muto, T., Endo, T., and Kohda, D. (2003) J. Mol. Biol. 328, 495–504
24. von Heijne, G., Steppuhn, J., and Herrmann, R. G. (1989) Eur. J. Biochem. 180, 535–545
25. Brix, J., Rudiger, S., Bukau, B., Schneider-Mergener, J., and Pfanner, N. (1999) J. Biol. Chem. 274, 16522–16530
26. Schleif, E., Silvius, J. R., and Shore, G. C. (1999) J. Cell Biol. 145, 973–978
27. Yano, M., Kanazawa, M., Terada, K., Takeya, M., Hoogenraad, N., and Mori, M. (1998) J. Biol. Chem. 273, 26844–26851
28. Yano, M., Kanazawa, M., Terada, K., Nanchain, H., Yamaizumi, M., Hanson, B., Hoogenraad, N., and Mori, M. (1997) J. Biol. Chem. 272, 8459–8465
29. Pirkl, F., Fischer, K., and Buchner, J. (2001) J. Biol. Chem. 276, 37034–37041
30. Neupert, W., and Brunner, M. (2002) Nat. Rev. Mol. Cell Biol. 3, 555–565
31. Wright, G., Terada, K., Yano, M., Sergeev, I., and Mori, M. (2001) Exp. Cell Res. 267, 107–117
32. Crooke, E., Guthrie, B., Lecker, S., Lill, R., and Wickner, W. (1988) Cell 54, 1003–1011
33. Lill, R., Crooke, E., Guthrie, B., and Wickner, W. (1988) Cell 54, 1013–1018
34. Maier, R., Schulz, C., and Schmid, F. X. (2001) J. Mol. Biol. 314, 1181–1190
