Interactions of the Human Mitochondrial Protein Import Receptor, hTom20, with Precursor Proteins in Vitro Reveal Pleiotropic Specificities and Different Receptor Domain Requirements*

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Tom20 is part of a multiple component, dynamic complex that functions to import specific cytosolic proteins into or through the outer membrane of the mitochondrion. To analyze the contribution of Tom20 to precursor protein recognition, the cytosolic domain of the human mitochondrial import receptor, hTom20, has been expressed as a fusion protein with glutathione S-transferase and conditions established to measure specific interactions of the receptor component with precursor proteins in vitro. Reconstitution of receptor binding from purified components revealed that a prototypic matrix-destined precursor protein, pODHFR, interacts with Tom20 by a mechanism that is dependent on an active matrix targeting signal but does not require cytosolic components or ATP. Binding was influenced by both salt concentration and detergent. The effect of salt or detergent, however, varied for different precursor proteins. In particular, detergent selectively enhanced binding of pODHFR to receptor, possibly because of induced changes in the structure of the signal sequence. Finally, mutations were introduced into hTom20 which had a dramatic effect on binding of some precursor proteins but not on others. Taken together, the results suggest that hTom20 recognizes and physically interacts with precursor proteins bearing a diverse array of topogenic sequences and that such pleiotropic specificity for these precursor proteins may involve different domains within the receptor molecule.

The majority of mitochondrial proteins are synthesized on cytoplasmic polysomes and subsequently imported to a specific subcompartiment within the organelle (for review, see Refs. 1–4). In most cases, delivery to mitochondria requires correct interactions between the precursor protein and proteinaceous receptors on the mitochondrial surface. Extensive studies in yeast and Neurospora have identified this receptor as a heterooligomeric complex composed of Tom20, Tom22, Tom37, and Tom70/71–72. The proteins Tom20 and Tom22 have been postulated to recognize incoming precursor proteins that harbor NH2-terminal cleavable targeting signals (5, 6), whereas the Tom37/70 complex is believed to import proteins that are presented to mitochondria as a complex with MSF (mitochondrial import stimulation factor) (7–9).

Tom20 was initially identified as a potential receptor because antibodies against this protein blocked in vitro import of precursor proteins (10–12). Subsequent analysis by electron microscopy revealed an exclusive location of Tom20 to the outer membrane (10, 11), and antibody labeling and protease treatment studies of both in vivo and in vitro imported Tom20 demonstrated that although a segment of the protein is embedded in membrane, the bulk of the polypeptide faces the cytosol (10–12). Functional studies showed that interference of Tom20 action either by antibody inhibition in vitro (10–18) or by genetic ablation in vivo (11, 19–21) resulted in a decreased level of import of a large variety of mitochondrial precursor proteins. To date, however, biochemical analyses of direct interaction of precursor proteins with purified Tom20 have not been reported.

We have recently identified and partially characterized the human homolog of Tom20 and discovered several interesting features that may distinguish the receptor from its fungal counterparts. First, antibodies against hTom20 inhibit import of uncoupling protein (UCP)1 whereas a close relative of UCP, ADP/ATP carrier, is refractory to such inhibition in the fungal context (10). Also, hTom20 exhibits quality control properties and guards the outer membrane import machinery against unscheduled bypass import mediated by cryptic import signals (22). To gain biochemical insight into these and other properties of hTom20, we have expressed its cytosolic domain as a fusion protein with GST and analyzed direct interactions between the immobilized hTom20 domain and various types of precursor proteins. Our results show that hTom20 interacts directly with a diverse array of precursor proteins, perhaps involving different domain requirements for different classes of precursor protein. Also, the lack of interaction of hTom20 with a precursor protein bearing a cryptic matrix targeting signal suggests that quality control by hTom20 may arise because hTom20 physically blocks the precursor from gaining access to downstream components of the import machinery rather than by a mechanism that involves direct physical interaction with the cryptic signal sequence.

MATERIALS AND METHODS

Biochemical Procedures—Previous articles (23–25) describe the routine procedures used in this study for in vitro transcription and translation, SDS-PAGE, Western blot analysis, fluorography, and quantitation of radioactive bands.

Plasmid Construction—The plasmid pGST-D30hTom20 was con-
Sepharose beads was incubated with 2 mM 15 min, dried, and exposed to film to assess levels of 35S-labeled translocation Blue R-250 (Bio-Rad) to assess relative amounts of eluted GST and were separated by 12% SDS-PAGE and stained with Coomassie Bril-

containing 10 mM reduced glutathione. The proteins (15 μl of acetate, 15% glycerol, pH 7.5). Cells were lysed at 4 °C by 5 s bursts at setting 6.0 from a small probe sonicator (Sonic Dismembrator, Fisher Scientific), insoluble debris was removed by centrifugation for 15 min at 17,000 × g, and the supernatant was incubated for 1 h at room temperature with 4 ml of a 50% slurry of glutathione-Sepharose 4B (Pharmacia) that had been equilibrated in 0.4 × KMH-G. The Sepharose was washed twice with 40 ml of 0.4 × KMH-G and then resuspended in 6 ml of 0.4 × KMH-G. GST fusion protein was eluted from an aliquot of the Sepharose by incubation for 30 min at room temperature in 50 mM Tris, pH 8, containing 10 mM reduced glutathione, and the protein concentration was determined using Bio-Rad reagent. Unbound Sepharose 4B was added to equalize the amount of protein/input Sepha-

rose matrix.

For the binding assays, 10 μl of GST- or GST-Δ30hTom20-bound Sepharose beads was incubated with 2 μl of in vitro 35S-labeled translation product in a total volume of 50 μl containing 38 μl of 0.4 × KMH-G. The mixture was incubated for 20 min at room temperature with shaking. The Sepharose beads were then washed twice in 0.4 × KMH-G, and the protein was eluted with 20 μl of 50 mM Tris, pH 8, containing 10 mM reduced glutathione. The proteins (15 μl of elution) were separated by 12% SDS-PAGE and stained with Coomassie Bril-

liant Blue R-250 (Bio-Rad) to assess relative amounts of eluted GST and GST-Δ30hTom20. The gel was then soaked in Amplify (Amersham) for 15 min, dried, and exposed to film to assess levels of 35S-labeled translation product. The amount of radioactivity was quantitated by using a PhosphorImaging system (FUJIX BAS 2000 system).

RESULTS

pODHFR Interacts with Purified GST-Δ30hTom20—The fusion protein was produced in bacteria and purified by affinity binding to glutathione-Sepharose 4B (Fig. 1A). Assays were then developed to measure direct interactions of precursor proteins selectively with GST-Δ30hTom20. This was accomplished by comparing the amount of precursor protein that was released as a complex with either GST or GST-Δ30hTom20 following incubation with reduced glutathione, which competitively disrupts GST interaction with glutathione-Sepharose 4B. For each assay, it must be determined that similar amounts of GST- and GST-Δ30hTom20 are released from the Sepharose. This is easily determined by subsequent analysis of released complexes by SDS-PAGE and protein staining. Comparison of circular dichroism spectra of GST, Δ30hTom20, and GST-Δ30hTom20 are consistent with a properly folded hTom20 domain within the context of the fusion protein (data not shown).

In Fig. 1, GST- and GST-Δ30hTom20-Sepharose were incubated with reticulocyte lysate containing the transcription-translation product of pODHFR, a reporter protein in which the matrix targeting signal (MTS) of preprothymosin carboxyamid transferase has been fused to dihydrofolate reductase (26). Selective interaction of pODHFR was observed with GST-Δ30hTom20 but not with GST alone, as revealed by selective release with reduced glutathione (Fig. 1B, lanes 2 and 3). In contrast, the glutathione treatment liberated similar amounts of both GST and GST-Δ30hTom20 (Fig. 1C, lanes 2 and 3). Specificity of binding of pODHFR to GST-Δ30hTom20 was established by two criteria. First, interactions were not observed with β-lactamase (Fig. 1B, lanes 5 and 6), a bacterial precursor protein that is efficiently translocated into endoplasmic reticulum microsomes in vitro. Second, interaction of [35S]pODHFR

![Fig. 1. Binding of precursor proteins to GST and GST-Δ30hTom20 fusion proteins. Panel A](image-url) Coomassie Blue-stained gel detailing the purification of GST and GST-Δ30hTom20 fusion proteins. Cultures of TOPP2 (Stratagene) E. coli cells containing the plasmid pGEX-2T (Pharmacia) were grown in LB media containing 50 μg/ml ampicillin. At an _A_ 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentra-

tion of 1 mM, and the cultures were grown for another 90 min. The bacteria were sedi-

mented, and the resultant pellet was resuspended in 6 ml of 0.4 × KMH-G (10 mM Hepes, 32 mM KCl, 0.8 mM magnesium acetate, 15% glycerol, pH 7.5). Cells were lysed at 4 °C by 5 × 15-s bursts from a small probe sonicator (Sonic Dismembrator, Fisher Scientific), insoluble debris was removed by centrifugation for 15 min at 17,000 × g, and the supernatant was incubated for 1 h at room temperature with 4 ml of a 50% slurry of glutathione-Sepharose 4B (Pharmacia) that had been equilibrated in 0.4 × KMH-G. The Sepharose was washed twice with 40 ml of 0.4 × KMH-G and then resuspended in 6 ml of 0.4 × KMH-G. GST fusion protein was eluted from an aliquot of the Sepharose by incubation for 30 min at room temperature in 50 mM Tris, pH 8, containing 10 mM reduced glutathione, and the protein concentration was determined using Bio-Rad reagent. Unbound Sepharose 4B was added to equalize the amount of protein/input Sepharose matrix.

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transcription-translation product with immobilized GST-Δ30hTom20 was competed by chemically pure unlabeled pODHFR isolated from expressing bacteria (26) (Fig. 2). These findings, together with the lack of interaction of pODHFR with GST alone, strongly suggest that the in vitro binding assay reflects bona fide precursor-receptor interactions.

The MTS of pODHFR Is Both Necessary and Sufficient for Binding of the Precursor Protein to hTom20—The binding reactions described in Figs. 1 and 2 were conducted with pODHFR derived as a transcription-translation product in reticulocyte lysate. To determine if precursor-receptor interactions can be detected using purified components, binding reactions were conducted using pODHFR isolated from expressing bacteria (26). Following elution from GST-Δ30hTom20-Sepharose, the protein was detected by Western blotting with anti-DHFR antibodies. As shown in Fig. 3, binding of chemically pure pODHFR was recorded for GST-Δ30hTom20 but not for GST (lanes 2 and 3). This interaction depended on the presence of the signal sequence in pODHFR (compare lanes 3 and 8) and was unaffected by the addition of translation-competent reticulocyte lysate to the binding reaction (compare lanes 2 and 3 with lanes 4 and 5).

Effects of ATP, NEM, Salt, and Detergent—Import of the pODHFR transcription-translation product into mitochondria in vitro is dependent on ATP (27) and is sensitive to inhibition by NEM (26). As shown in Fig. 4, however, depletion of ATP (lanes 3 and 4) or alkylation of lysate proteins with NEM following translation of pODHFR mRNA had little or no effect on subsequent binding to GST-Δ30hTom20. These findings are consistent with the observation that cytosolic components are not required for pODHFR interaction with GST-Δ30hTom20 (Fig. 3) and suggest that the ATP dependence and NEM sensitivity of import in vitro are at steps other than direct interaction of the precursor protein with Tom20.

In contrast, however, interactions of pODHFR with GST-Δ30hTom20 exhibited a striking sensitivity to salt and detergent (Fig. 5, A and B). Standard binding reactions contained an ionic composition that was similar to that used for in vitro import (32 mM KCl and 0.8 mM magnesium acetate) (28). Elevating the salt concentration by including NaCl in the binding reaction resulted in a progressive loss of interaction of pODHFR with GST-Δ30hTom20, such that ~50% of binding was lost at 200 mM. At the highest assayed level of NaCl concentration, the amount of binding was reduced to the background binding of pODHFR to GST (Fig. 5A). The low level of background binding of pODHFR to GST, on the other hand, was unaffected by increasing the salt concentration to 1 M NaCl. Inclusion of the nonionic detergents Triton X-100 or Tween 20 stimulated binding of pODHFR to GST-Δ30hTom20 by about 3-fold, and this effect for both detergents was detected at concentrations of the detergent which were immediately above the critical micelle concentration (0.015% v/v for Triton X-100 and 0.007% v/v for Tween 20), with no further stimulation observed (Fig. 5B).

The effects of detergent and salt on hTom20-precursor interactions were also analyzed for yTom70(1–29)DHFR and human VDAC. The former is a hybrid protein bearing the NH2-terminal signal anchor sequence of yeast Tom70p (amino acids 1–29) fused to DHFR (23), and the latter is the mammalian equivalent of the β-barrel protein, porin (29). Both proteins are efficiently targeted and inserted into the outer membrane of mammalian mitochondria in vitro by a pathway that is inhibited by antibodies against Tom20 (18). As shown in Fig. 6, Triton X-100 stimulated binding of pODHFR to GST-Δ30hTom20, had no effect on binding of yTom70(1–29)DHFR, and inhibited the interaction of VDAC with the receptor (Fig. 6, compare lanes 4 and 5). Likewise, the effects of elevated concentrations of NaCl also were variable depending on the precursor examined.
Mutations in hTom20 Which Differentially Affect Its Ability to Recognize Precursor Proteins—Two regions of hTom20 were targeted for mutagenesis. First, a cluster of acidic residues at the extreme COOH terminus of the molecule, Glu-Asp-Asp-Val-Asp-COOH (residues 141–145), were deleted and the mutation was designated Q108A. Second, a predicted amphiphilic helix, spanning amino acids 104–114, which contains a uniform Gln face (Fig. 7A) was modified by introducing an Ala point substitution at amino acid 108, and the mutation was designated Q108A. The mutations were introduced into GST-A30hTom20, and their effect on the ability of hTom20 to recognize various precursor proteins was examined. As shown in Fig. 7B, neither mutation had a significant effect on binding of pODHFR and yTom70(1–29)DHFR. However, they both dramatically reduced binding of VDAC and UCP. The latter is a polytopic integral protein of the inner membrane (31) which, like VDAC, contains complex, multiple targeting regions within the molecule (31).

A Cryptic MTS Does Not Interact with hTom20—Previous studies have shown that the NH2-terminal 15 amino acids of yeast Tom70 can function as a cryptic signal and direct import of an attached reporter protein into the matrix compartment of mammalian mitochondria in vitro, but only if hTom20 is physically removed from the import machinery (22). To determine if this quality control property of hTom20 correlates with its ability to recognize and interact with the cryptic MTS, a fusion protein bearing the cryptic signal, yTom70(1–15)DHFR, was examined for binding to GST-A30hTom20. Compared with pODHFR, such binding was negligible (Fig. 8). It is likely, therefore, that the ability of Tom20 to refuse entry of the cryptic signal into the import machinery does not involve a direct physical interaction with the signal.

DISCUSSION

Tom20 is but one component of a multimeric receptor complex in the mitochondrial outer membrane, which also includes Tom70/71–72, Tom37, and Tom22 (1, 2, 6, 32–34). Nevertheless, extensive genetic and biochemical analyses have revealed an important role for Tom20 in the overall function of the receptor complex in mediating import of most precursor proteins (10, 11, 13–22). To date, however, these studies have been conducted on Tom20 as a native component of an intact import machinery in mitochondria, and therefore contributions from other components have been difficult to exclude. Here, we have examined direct interactions between hTom20 and precursor proteins by expressing the hTom20 cytosolic domain as a GST fusion protein in bacteria and developing an assay that discriminates between specific and nonspecific interactions in the binding reaction. By these criteria we established that a fusion protein bearing the cryptic signal, yTom70(1–15)DHFR, was examined for binding to GST-A30hTom20. Compared with pODHFR, such binding was negligible (Fig. 8). It is likely, therefore, that the ability of Tom20 to refuse entry of the cryptic signal into the import machinery does not involve a direct physical interaction with the signal.

FIG. 5. Effects of NaCl and detergent on pODHFR binding to GST-A30hTom20. Panel A, line graph of the effects of increasing amounts of NaCl to pODHFR binding to GST-A30hTom20 (●) or GST (○). Panel B, line graph of the effects of increasing amounts of Tween 20 (●) or Triton X-100 (○) to pODHFR binding to GST-A30hTom20. Data points represent the average values of at least three experiments.
these various proteins revealed several important features about hTom20 function.

Consistent with observations made with intact mitochondria (21), we found that precursor-hTom20 interactions were sensitive to salt concentration (100–1,000 mM) (Fig. 5), implying that electrostatic interactions are involved in the binding reaction. Of note, however, pODHFR and yTom70(1–29)DHFR were more sensitive than VDAC to elevated concentrations of NaCl, suggesting that binding of VDAC to Tom20 may involve a different type of interaction. This conclusion was supported by the finding that mutations in Tom20 were identified which drastically curtailed the ability of the receptor to interact with VDAC and UCP but had negligible influences on binding of pODHFR and yTom70(1–29)DHFR. Not only may different precursor proteins interact with the receptor through different physical interactions, therefore, but these binding reactions may also involve different regions of the Tom20 molecule. In fact, the notion that Tom20 has more than one binding site for precursor proteins was first suggested to explain the correlation between a Tom20 proteolytic fragment (10) and the differing sensitivities of some precursor proteins to import into elastase pretreated mitochondria (35, 36).

**FIG. 7.** Helical wheel projection and histogram plots of binding reactions involving mutations of hTom20. Panel A, helical wheel projection. The α-helical structure was predicted for hTom20 amino acids 104–114 (shown) using the Predicted Protein PHD E-mail Server PHD based on the program PHDsec (41), the E-mail server Protein Sequence Analysis (PSA) (42, 43), the PSSP prediction programs SSP, NNSSP, and SSPAL (44–47) (all of them are freeware on the Internet), and also the DNA-Strider 1.0 α-helical prediction. Dark circles represent glutamine residues. Mutation of Gln108 to Ala108 is represented by an arrow. Panel B, binding reactions represented as histogram plots. In vitro translated [35S]pODHFR, [35S]yTom70(1–29)DHFR, [35S]VDAC, and [35S]UCP were incubated with glutathione-Sepharose that had been prebound with GST (lane 1), GST-Δ30hTom20 (Wt, lane 2), GST-Δ30hTom20 Q108A (lane 3), or GST-Δ30hTom20 Δ141–145 (lane 4). Binding is shown relative to the amount of radiolabeled protein bound by GST-Δ30hTom20 (Wt). The results shown are the average values of at least five experiments.
"Materials and Methods." 10% of the input lane 2 prior to receptor binding (40).

Of particular interest was the finding that binding of pODHFR to hTom20 was enhanced by detergent and that this effect became dramatically apparent at or above the critical micelle concentration of the detergent (Fig. 5). Binding of yTom70(1–29)DHFR, in which the MTS of pODHFR has been replaced by a signal anchor sequence, showed no sensitivity to detergent, whereas VDAC binding was actually inhibited by detergent (Fig. 6). Although the enhancing effects of detergent on pODHFR interactions with hTom20 may involve several parameters, these findings are consistent with the observation that the pODHFR MTS is a lipophilic sequence that adopts an amphipathic helix upon binding to detergent micelles (37). In column chromatography, pODHFR, but not yTom70(1–29)DHFR, migrated with a higher hydrodynamic radius in the presence of detergent than in its absence,2 suggesting that detergent was bound to the amphipathic matrix targeting sequence (38, 39). Therefore, it may be that in vitro, such a detergent-induced helix is a preferred structure for recognition by the receptor. During import into intact mitochondria, such a preferred structure could be manifested as a result of interactions of the MTS with the surface of the membrane lipid bilayer prior to receptor binding (40).

Finally, we analyzed a cryptic MTS derived from the hydrophilic, positively charged NH₂-terminal domain of the yeast Tom70 signal anchor sequence (amino acids 1–10). This sequence can direct attached passenger proteins into the matrix compartment of mammalian mitochondria only if Tom20 is removed from the receptor complex, and conversely, replacing yTom20 with hTom20 prevents import of the same protein into yeast mitochondria (22). These results suggest that hTom20 performs a quality control function that restricts bypass import into mammalian mitochondria (22). Two models were proposed to explain this observation. First, hTom20 might interact with both cryptic and active MTS sequences but reject the cryptic MTS and disallow it from proceeding to distal binding sites within the import machinery. Alternatively, hTom20 might physically block the translocation pore and occlude the cryptic

2 Schleiff, unpublished observations.