The Human Mitochondrial Import Receptor, hTom20p, Prevents a Cryptic Matrix Targeting Sequence from Gaining Access to the Protein Translocation Machinery

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Abstract. Yeast Mas70p and NADH cytochrome b_5 reductase are bitopic integral proteins of the mitochondrial outer membrane and are inserted into the lipid-bilayer in an N_{in}-C_{cyto} orientation via an NH_2-terminal signal–anchor sequence. The signal anchor of both proteins is comprised of a short, positively charged domain followed by the predicted transmembrane segment. The positively charged domain is capable of functioning independently as a matrix-targeting signal in yeast mitochondria in vitro but does not support import into mammalian mitochondria (rat or human). Rather, this domain represents a cryptic signal that can direct import into mammalian mitochondria only if proximal components of the outer membrane import machinery are removed. This can be accomplished either by treating the surface of the intact mitochondria with trypsin or by generating mitoplasts. The import receptor Tom20p (Mas20p/MOM19) is responsible for excluding the cryptic matrix-targeting signal from mammalian mitochondria since replacement of yeast Tom20p with the human receptor confers this property to the yeast organelle while at the same time maintaining import of other proteins. In addition to contributing to positive recognition of precursor proteins, therefore, the results suggest that hTom20p may also have the ability to screen potential matrix-targeting sequences and exclude certain proteins that would otherwise be recognized and imported by distal components of the outer and inner membrane protein-translocation machinery. These findings also indicate, however, that cryptic signals, if they exist within otherwise native precursor proteins, may remain topogenically silent until the precursor successfully clears hTom20p, at which time the activity of the cryptic signal is manifested and can contribute to subsequent translocation and sorting of the polypeptide.

BIOCHEMICAL and genetic studies in yeast and Neurospora have identified a number of proteins within the mitochondrial outer membrane that are responsible for recognizing and translocating cytosolic precursor proteins into the organelle. Together, they form an import machine comprised of a dynamic complex of receptor molecules that feed the incoming precursor protein to an associated translocation pore (Ryan and Jensen, 1995; Kubrich et al., 1995; Lithgow et al., 1995; Lill and Neupert, 1996). Subunits of the receptor complex that have been identified to date include Tom20p (Ramage et al., 1993; Moczko et al., 1994), Tom22p (Kiebler et al., 1993; Lithgow et al., 1994), Tom37p (Gratzer et al., 1995), and Tom70p (Hines et al., 1990; Söllner et al., 1990), whereas Tom40p (Vestweber et al., 1989; Baker et al., 1990; Kiebler et al., 1990) and associated proteins (Tom7p and Tom8p, Söllner et al., 1992; Tom6p, Kassenbrock et al., 1993) likely cooperate to form the translocation pore (for current nomenclature, see Pfanner et al., 1996). The existence of multiple receptor subunits may reflect an exceptionally diverse array of cytosolic precursor proteins whose delivery to the organelle is mediated by different types of signal sequences with quite different properties. These include positively charged matrix-targeting signals (Attardi and Schatz, 1988; Hartl and Neupert, 1990) and hydrophobic outer membrane signal–anchor sequences (McBride et al., 1992; Schlossmann and Neupert, 1995; Shore et al., 1995), both of which are usually located at the NH_2 termini of precursor proteins, as well as a number of internal signals within polytopic proteins of the mitochondrial outer and inner membranes whose character remains to be determined (e.g., uncoupling protein, Liu et al., 1988; porin, Smith et al., 1995). Additionally, the cytosolic signal recognition factor, mitochondrial-import stimulating factor (Hachiya et al., 1993), contributes to the recognition of at least certain precursor proteins by the mitochondrion, in part via direct interactions of mitochondrial-import stimulating factor with components of the receptor complex (Hachiya et al., 1995). Not surprisingly, therefore, different precursor proteins may exhibit differential dependen-

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cies on individual subunits of the receptor complex for recognition by the import machinery.

Recent studies suggest that, following recognition by the receptor, subsequent vectorial movement of precursor proteins from the proximal to distal side of the outer membrane translocon may be mediated, in part, by a series of binding reactions involving the signal sequence (Bolliger et al., 1995; Hachiya et al., 1995; Mayer et al., 1995). Such findings raise important questions. For example, what is the specificity of these distal binding reactions relative to the interaction with the receptor complex? Also, with what degree of efficiency and specificity can an individual precursor protein bypass the receptor complex and still execute productive downstream interactions and, therefore, translocation into the organelle? Certainly, enforced protein import via a receptor bypass mechanism has been well documented in lower eukaryotes (Pfanner et al., 1988). Moreover, genetic studies have revealed that numerous genomic open-reading frames have the potential to encode sequences that can function as promiscuous matrix-targeting signals when located at the NH_2 terminus of a passenger protein (Allison and Schatz, 1986; Baker and Schatz, 1987; Hurt and Schatz, 1987; Roise et al., 1986). One mechanism to prevent unscheduled import of proteins into mitochondria, therefore, might be a receptor that on the one hand contributes to the positive recognition of a *bona fide* precursor protein but on the other excludes foreign proteins that bear a cryptic signal sequence from gaining access to downstream binding sites within the translocation machinery, an event that might allow the protein to be functionally imported.

Here we have gained insight into this problem as the result of finding that a domain located at the NH_2 termini of certain precursor proteins in yeast can function independently as a matrix-targeting signal in yeast mitochondria but not in mammalian mitochondria. Under normal conditions, mammalian Tom20p (Goping et al., 1995; Seki et al., 1995; Hanson et al., 1996) denies this domain access to the protein translocation machinery, but once access is achieved by physically removing the Tom20p barrier, this sequence is capable of mediating efficient import of attached proteins across both the outer and inner membranes. The physiological significance of these findings and the possible mechanisms whereby Tom20p prevents cryptic targeting sequences from gaining access to the import machinery are discussed.

Materials and Methods

General

Previous articles (Li and Shore, 1992*a*; McBride et al., 1992; McBride et al., 1995 and references therein) describe the routine procedures employed for recombinant DNA manipulations, transcription of pSP64 constructs, translation of the resulting mRNA in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, and import of radiolabeled precursor proteins into rat heart or liver mitochondria and mitoplasts. Purification and import into yeast mitochondria was performed according to Glick et al., (1992). Additional information is provided in the figure legends.

Recombinant Plasmids

pSP(pOMD29Δ16-29) (McBride et al., 1992) encodes amino acids 1–15 of Mas70p fused to amino acids 4–186 of dihydrofolate reductase (DHFR).[1] Following digestion with PstI and HindIII to remove the *MAS70* sequence, adapters were introduced that encode the following changes to wild-type Mas70p. (*a*) Lysine at position 9 was mutated to an isoleucine and isoleucine at position 12 was mutated to a lysine. The protein was designated Mas70p(1–15)K9:I12DHFR. The adapters were 5′-AGCTTATGAAGAGCTTCATTACAAGGAACATTACAGCCAAGTTGGCTGCA-3′ and 5′-GCCAACTTGGCTGTAATGTTCCTTGTAATGAAGCTCTTCATA-3′. (*b*) The positively charged amino acids at positions 2, 7, and 9 of wild-type Mas70p were replaced with glutamine, employing the adapters 5′ -AGCTTATGCAGAGCTTCATTACACAGAACCAGACAGCCATTTTGGCTGCA-3′ and 5′-GCCAAAATGGCTGTCTGGTTCTGTGTAATGAAGCTCTGCATA-3′. This new protein was designated Mas70p(1–15)KR2,7,9QDHFR. (*c*) Amino acids 1–15 of Mas70p were replaced with NADH cytochrome b_5 reductase (NCBR) amino acids 1–12 using the adaptors 5′-GCTTTTGAGTGAGATCTGGATAATCTGGAAAACATA-3′ and 5′-AGCTTATGTTTTCCAGATTATCCAGATCTCACTCAAAAGCTGCA-3′. This new protein was termed NCBR(1–12)DHFR. Finally, the DHFR sequence in pSP(pOMD29Δ16–29) was replaced with DNA-encoding amino acids 62–355 of rat preornithine carbamyl transferase (pOCT) by replacing a PstI-EcoRI fragment (Nguyen and Shore, 1987) with the corresponding restriction fragment generated from pSPO19 (Nguyen et al., 1986), yielding the new protein Mas70p(1–15)OCT. All changes were verified by nucleotide sequencing.

Results and Discussion

Mas70(1–15)DHFR and NCBR(1–12)DHFR Are Imported into Yeast but Not Rat Heart Mitochondria In Vitro

The NH_2-terminal signal–anchor sequences of yeast Mas70p and NCBR are responsible for targeting and inserting these proteins into the mitochondrial outer membrane in the N_{in}-C_{cyto} orientation, leaving the bulk of the protein facing the cytosol (Hase et al., 1984; McBride et al., 1992; Hahne et al., 1994). Interestingly, the signal anchor of NCBR is bifunctional and delivers a portion of the NCBR molecules to the intermembrane space in yeast mitochondria (Hahne et al., 1994). The signal anchor of both proteins is comprised of a short, amphiphilic, positively charged region at the extreme NH_2 terminus followed by the predicted transmembrane segment (Hase et al., 1984; Hahne et al., 1994). The NH_2 terminal domain of Mas70p can support matrix targeting of a fused passenger protein into mitochondria from yeast (Hurt et al., 1985) but not mammals (rat heart) (McBride et al., 1992). Fig. 1 documents this fact and shows that this disparity between mammalian and yeast mitochondria extends to NCBR as well. Import of porin, on the other hand, was observed for mitochondria isolated from either source.

Following import incubations with yeast mitochondria, Mas70(1–15)DHFR and NCBR(1–12)DHFR were recovered with the organelle (Fig. 1 *B*, lane *7*) and a significant fraction was resistant to subsequent treatment with trypsin (lane *8*). Acquisition of protease resistance was dependent on the presence of mitochondria (lane *2*) on $\Delta\Psi$ (i.e., it was abolished by carbonyl cyanide *m*-chlorophenylhydrazone [CCCP], lane *9*), physiological temperature (lane *10*), and the protein extractable at alkaline pH (not shown), all of which are consistent with import to the matrix compartment. In marked contrast, negligible protease-resistant precursor was recovered with rat heart mitochondria (Fig.

1. *Abbreviations used in this paper*: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DHFR, dihydrofolate reductase; NCBR, NADH cytochrome reductase; pOCT, preornithine carbamyl transferase.

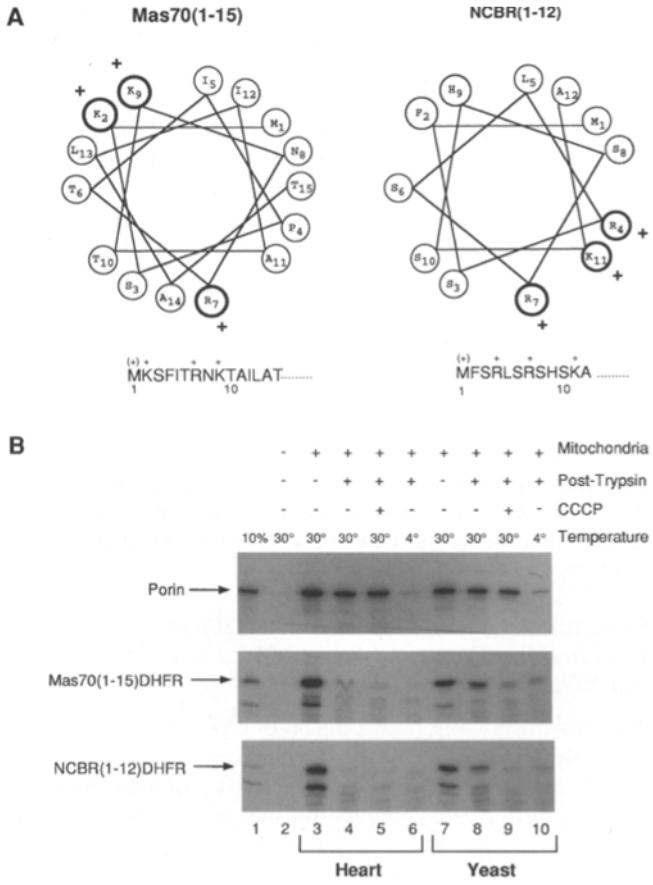

Figure 1. Mas70(1–15) and NCBR(1–12) direct protein import into yeast, but not rat heart, mitochondria. (*A*) Residues 1–15 of Mas70p (*left*) and 1–12 of NCBR (*right*) are shown as predicted α-helices, with amino acids indicated in the single letter code. Positively charged amino acids are circled in bold and indicated by a plus sign. (*B*) [^{35}S]porin (*top*), [^{35}S]Mas70(1–15)DHFR (*middle*) and [^{35}S]NCBR(1–12)DHFR (*bottom*) were generated by transcription–translation of recombinant plasmids and the resulting reticulocyte lysate incubated with mitochondria (0.5 mg/ml protein) purified either from rat heart (lanes *3–6*) (Li and Shore, 1992*b*) or from yeast strain D273-10B (lanes *7–10*) (Glick et al., 1992), in the presence (lanes *5* and *9*) or absence (lanes *2–4*, *6–8*, and *10*) of 1.0 μM CCCP, and incubated under standard import conditions for 30 min either at 30°C (lanes *2–5* and *7–9*) or 4°C (lanes *6* and *10*). Organelles were recovered from reaction mixtures by centrifugation either directly (lanes *3* and *7*) or following treatment with 0.125 mg/ml trypsin for 20 min at 4°C followed by a 10 min incubation with 1.25 mg/ml soybean trypsin inhibitor (lanes *4–6* and *8–10*) (McBride et al., 1992). They were subjected to SDS-PAGE and fluorography. Lane *1*, 10% input translation product; lane *2*, no mitochondria added to import reactions.

1 *B*, compare lane *4* with lanes *5* and *6*) or mitochondria from human cells (not shown), despite the fact that, like yeast mitochondria (Fig. 1, lanes *7–10*), the heart mitochondria supported robust import of porin (lanes *3–6*), as well as the entire Mas70p signal anchor (McBride et al., 1992) and many other proteins. Import and insertion of porin into the outer membrane of yeast and heart mitochondria was measured by assaying temperature-sensitive and $\Delta\Psi$-independent acquisition of resistance to trypsin (Mihara et al., 1982; Gasser and Schatz, 1983) (Fig. 1 *B*).

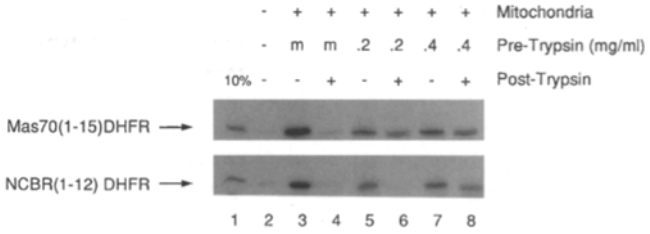

Figure 2. Activation of import of Mas70(1–15)DHFR and NCBR (1–12)DHFR by pretreatment of heart mitochondria with protease. Mitochondria purified from rat heart (0.5 mg/ml protein) were incubated with 0.2 mg/ml (lanes *5* and *6*) or 0.4 mg/ml (lanes *7* and *8*) trypsin for 45 min at 4°C at which time soybean trypsin inhibitor (SBTI) was added in 50-fold excess for a further 20 min incubation at 4°C. As a mock control (*m*; lanes *3* and *4*), incubation with 0.8 mg/ml trypsin was carried out in the presence of SBTI. The mitochondria were recovered by centrifugation and incubated with [^{35}S]Mas70(1–15)DHFR (*top*) or [^{35}S]NCBR(1–12)-DHFR (*bottom*) under standard import conditions for 30 min at 30°C (lanes *3–8*). Following the import reaction, the mitochondria were centrifuged through a 0.5 ml 250 mM sucrose/10 mM Hepes, pH 7.5 cushion, and either resuspended directly in SDS sample buffer (lanes *3*, *5*, and *7*) or resuspended in import medium to 0.5 mg/ml protein and incubated with 0.125 mg/ml trypsin for 20 min at 4°C, followed by 10 min in the presence of SBTI (lanes *4*, *6*, and *8*). Mitochondria were recovered, subjected to SDS-PAGE, and analyzed by fluorography. Lane *1*, 10% of input [^{35}S]precursor protein; lane *2*, import in the absence of organelles.

Activation of Import of Mas70(1–15)DHFR and NCBR(1–12)DHFR into Heart and Liver Mitochondria

Because the NH_2 termini of Mas70(1–15)DHFR and NCBR (1–12)DHFR have the potential to form positively charged, amphiphilic helices typical of matrix-targeting signals (Fig. 1 *A*), it was predicted that these domains might in fact constitute cryptic signals that are denied entrance to heart mitochondria due to a screening mechanism located on the surface of the organelle. Consistent with this possibility, Fig. 2 demonstrates that pretreatment of intact rat heart mitochondria with trypsin permitted subsequent uptake of Mas70(1–15)DHFR and NCBR(1–12)DHFR, resulting in the acquisition of protease protection for both proteins (compare lane *4* with lanes *6* and *8*). Interestingly, NCBR(1–12)DHFR required a higher concentration of trypsin in the pretreatment period than did Mas70(1–15)DHFR (Fig. 2). In both cases, however, trypsin pretreatment reduced the amount of total precursor that was subsequently recovered with the surface of the organelle (Fig. 2, compare lane *3* with lanes *5* and *7*) but had no effect on the level of a marker protein, sulfite oxidase (not shown, see Nguyen et al., 1993), in the intermembrane space, indicating that trypsin had not broached the outer membrane. When exposed, sulfite oxidase is otherwise highly sensitive to trypsin (Ono and Ito, 1984; Nguyen et al., 1993). It would appear, therefore, that one or more proteins on the surface of heart mitochondria promote binding of these precursor proteins to the surface of the organelle. However, a surface-exposed protein(s) also prevents these precursors from subsequently passing across the outer membrane. Under the conditions employed, trypsin presumably removed this barrier while leaving other components of

the import machinery (e.g., Tom40p) at least partially functional. Damage to the import machinery might explain the doublet bands that are seen in autoradiograms of Mas70(1–15)DHFR and NCBR(1–12)DHFR following trypsin-activated import and subsequent treatment with protease (Fig. 2, lanes *4*, *6*, and *8*). This presumably reflects incomplete import, and consequent cleavage, of at least some polypeptide chains.

The inability of Mas70(1–15) (Fig. 3) and NCBR(1–12) (not shown) to support import of a passenger protein into intact mitochondria was also observed for rat liver mitochondria and was observed when the passenger protein, DHFR, was replaced by the mature portion of pOCT, a mitochondrial matrix protein which is efficiently imported in vitro (Fig. 3). The inability of these precursors to import into intact mammalian mitochondria, therefore, is a property of the cryptic targeting signal rather than due to the type of passenger protein employed.

In addition to trypsin pretreatment of mammalian mitochondria as a means of activating protein import directed by Mas70(1–15) or NCBR(1–12), Figs. 3 and 4 also show that mitoplasts, in place of intact mitochondria, permit efficient import mediated by these sequences. Whereas intact mitochondria and mitoplasts were equivalent in their ability to import pOCT (Fig. 3, *top*), only the mitoplasts sustained import under the direction of Mas70(1–15) (Fig. 3, *bottom*) or NCBR(1–12) (Fig. 4, *bottom*). Again, import into mitoplasts was assayed by the acquisition of protease protection that was dependent on the presence of the organelle (Fig. 4, compare lanes *2* and *4*), on an intact electrical chemical potential (Fig. 4, compare lanes *4* and *5*), and on physiological temperature (compare lanes *4* and *6*). As expected, import directed by Mas70(1–15) into mitoplasts was ablated when the positively charged amino acids at positions 2, 7, and 9 were converted to uncharged glutamine residues (Fig. 4, compare *first* and *third panels*), a finding consistent with the evidence that import across the inner membrane is initiated by an electrophoretic force that is imposed by $\Delta\Psi$ on a positively charged signal sequence (Martin et al., 1991; McBride et al., 1995). Import, however, was sustained following a rearrangement of the positively charged amino acids within Mas70(1–15) (Fig. 4, *second panel*). Finally, import of Mas70(1–15)DHFR into mitoplasts was competed by a sequence corresponding to the matrix-targeting sequence of pOCT, either in the form of a synthetic signal peptide (Gillespie et al., 1985) (Fig. 5, *left*) or in the form of a bacterial expression product, pODHFR (Sheffield et al., 1990), in which the pOCT signal sequence has been fused to DHFR (Fig. 5, *right*). The extent and pattern of competition for import of the transcription–translation products of Mas70(1–15)DHFR and pODHFR were very similar (Fig. 5), strongly suggesting that the two proteins were imported into mitoplasts by a common mechanism.

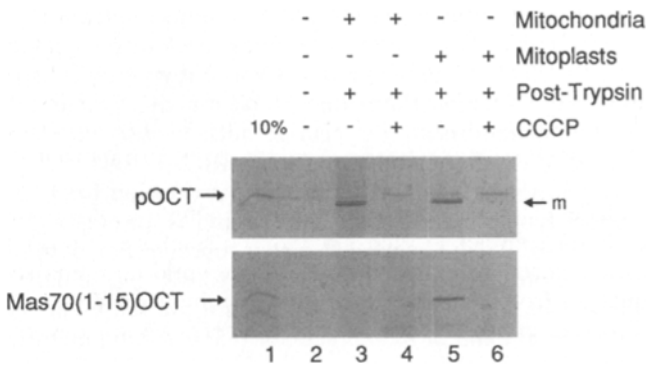

Figure 3. Amino acids 1–15 of Mas70p mediate import of a reporter protein into mitoplasts but not into intact mitochondria. [^{35}S]pOCT and [^{35}S]Mas70(1–15)OCT were incubated with intact mitochondria or mitoplasts purified from rat liver (McBride et al., 1995) (0.5 mg protein/ml) under standard import conditions for 30 min at 30°C (lanes *3–6*), in the presence (lanes *4* and *6*) or absence (lanes *3* and *5*) of 1.0 μM CCCP. Organelles were recovered from reaction mixtures by centrifugation either directly (*top*) or following treatment with 0.125 mg/ml trypsin for 20 min at 4°C, followed by a 10 min incubation with 1.25 mg/ml soybean trypsin inhibitor (*bottom*, lanes *3–6*). They were subjected to SDS-PAGE and fluorography. Lane *1*, 10% of input [^{35}S]precursor protein; lane *2*, import in the absence of organelles. *m*, processed pOCT.

Substitution of yTom20p with hTom20p in Yeast Mitochondria Prevents Import of Mas70(1–15)DHFR and NCBR(1–12)DHFR

We previously cloned and partially characterized the human homolog of Tom20p and demonstrated that *hTOM20* complemented the respiratory defect of *Δtom20* in yeast (Goping et al., 1995), indicating that hTom20p can func-

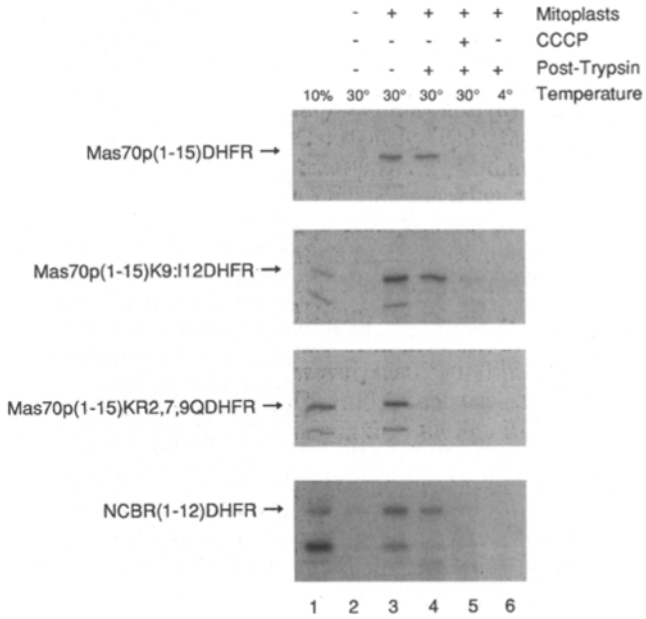

Figure 4. Import of Mas70(1–15)DHFR and NCBR(1–12)DHFR into mitoplasts. [^{35}S]Mas70p(1–15)DHFR (*top*), Mas70p(1–15)K9:I12DHFR (*second*), Mas70p(1–15)KR2,7,9QDHFR (*third*), and NCBR(1–12)DHFR (*bottom*) were obtained by transcription–translation and their import into rat liver mitoplasts assayed. Conditions and manipulations are described in Fig. 3. Lane *1*, 10% of input precursor protein; lane *2*, import in the absence of mitoplasts; lanes *3–6*, import in the presence of mitoplasts; lanes *4–6*, treatment with trypsin following the import reaction; lane *5*, import in the presence of 1.0 μM CCCP; lane *6*, import at 4°C.

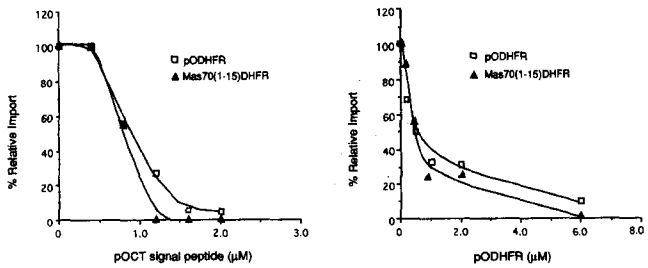

Figure 5. Synthetic matrix-targeting signal peptide and bacterial-expressed matrix precursor protein compete for import of Mas 70p(1–15)DHFR into mitoplasts. Solutions containing 7 M urea, 10 mM Hepes (pH 7.4), and various concentrations of either the peptide pO(1–27)cys (Gillespie et al., 1985) or purified pODHFR (amino acids 1–36 of pOCT fused to amino acids 4–186 of DHFR) obtained from expressing bacteria (Sheffield et al., 1990) were flash diluted 25-fold into a standard import reaction lacking translation product and incubated for 10 min at 4°C. Reactions contained a final concentration of 0.28 M urea and the indicated concentration of pO(1-27)cys (0–2 μM, *left*) or pODHFR (0–6 μM, *right*). Import into mitoplasts was initiated upon addition of reticulocyte lysate containing [^{35}S]pODHFR or [^{35}S]Mas70p(1–15) DHFR, and the mixture incubated at 30°C for 15 min. Trypsin (0.125 mg/ml) was added and the incubation continued for 20 min at 4°C, followed by a further incubation with 1.25 mg/ml soybean trypsin inhibitor for 10 min. Mitoplasts were recovered by centrifugation and analyzed by SDS-PAGE and fluorography. Bands corresponding to trypsin-protected Mas70p(1–15)DHFR and processed pODHFR were quantified using a BAS 2000 Bioimager (Fuji Photo Film Co., Tokyo, Japan), with the products obtained in the presence of CCCP (see Figs. 1 and 3) employed as baseline. Imported products obtained in the absence of competitor peptide or bacterial pODHFR were arbitrarily set at 100%.

tionally substitute for the yeast receptor and sustain import of yeast precursor proteins in vivo. As shown in Fig. 6 (*top*), mitochondria isolated from the *hTOM20* strain of yeast supported import of porin into the mitochondrial outer membrane in vitro with characteristics very similar to import of porin into wild-type (wt) yeast mitochondria. In both cases, most of the porin that was recovered with the wt or hTom20p yeast mitochondria was resistant to protease treatment following import at 30° but not at 4°C. In contrast, temperature-sensitive import of Mas70(1–15)DHFR was observed for wt yeast mitochondria (Fig. 6, *bottom*, lanes *3–5*) but not for hTom20p yeast mitochondria (lanes *6–8*). Most of the Mas70(1–15)DHFR that was recovered with wt yeast mitochondria following import at 30°C was resistant to protease (compare lanes *3* and *4*), whereas this acquisition of protease resistance was significantly reduced following import at 4°C (lane *5*). Following import into hTom20p yeast mitochondria at 30°C, however, acquisition of protease resistance of Mas70(1–15) DHFR was low (compare lanes *6* and *7*) and, in fact, was near the level of the 4°C control (lane *8*). Similar results were also obtained with NCBR(1–12)DHFR (not shown). Thus, hTom20p yeast mitochondria exhibit similar characteristics to rat heart and liver mitochondria with respect to the lack of protein import under the direction of Mas70(1–15) and NCBR(1–12) signal sequences (see Figs. 1 and 3). Also, mitochondria from *Δtom20* yeast (Ramage et al., 1993) failed to import Mas70(1–15)DHFR and NCBR(1–12)DHFR (not shown). However, it is not known if this means that yTom20p directly contributes to positive recognition of these precursors or if deletion of yTom20p indirectly results in disruption of components that otherwise interact with yTom20p and are required for import.

Finally, while it cannot be rigorously excluded that replacement of yTom20p with hTom20p in yeast mitochondria nonspecifically impairs the import machinery utilized by Mas70(1–15)DHFR and NCBR(1–12)DHFR for translocation to the matrix, it is unlikely that this is the case. We have previously demonstrated that *hTOM20* complements the respiratory defect in *mas20*-null yeast (Goping et al., 1995), and therefore, the human receptor must support import of numerous proteins, including other matrix proteins, into yeast mitochondria. Also, the human receptor supports porin import (Fig. 6), and it is well documented that insertion of porin into the outer membrane extensively overlaps the early steps in protein import to the matrix (Pfaller et al., 1988; Kiebler et al., 1993). Lastly, Tom20p is part of a single heterooligomeric receptor complex in yeast (Hauke et al., 1996), a complex into which hTom20p can assemble (Seki et al., 1995). It is unlikely, therefore, that hTom20p would have nonspecific effects on the import machinery that mediates uptake of proteins bearing cryptic matrix-targeting signals without affecting import of other proteins. Rather, we conclude that hTom20p selectively excludes these cryptic signals from gaining access to the downstream translocation machinery.

Conclusions

We have shown that a sequence which can function as an active matrix-targeting signal in yeast mitochondria is a cryptic signal in mammalian mitochondria and is denied access to the translocation machinery of the mammalian organelle by Tom20p. Thus, Tom20p has two functions: it contributes to positive recognition of precursor proteins and also constitutes a barrier, or guardian, against sequences that otherwise can be recognized by distal components of the import machinery and can be functionally imported into the organelle. While the underlying mechanism of such screening by Tom20p is not known, there are two obvious explanations. Tom20p might be situated within the receptor complex in such a way that it shields the translocation pore and physically prevents cryptic targeting sequences from bypassing the receptor complex and gaining direct access to downstream components within the import machinery with which the cryptic signal can functionally interact. Alternatively, Tom20p, perhaps in cooperation with other components of the import machinery, may recognize both specific and related cryptic signals but may vectorially release into the translocation pore only those proteins bearing the correct sequence. This is consistent with the finding that binding of Mas70(1–15)DHFR and NCBR(1–12)DHFR to rat mitochondria was reduced following pretreatment of the organelle surface with protease (see Fig. 2). Such discrimination between different ligands is common during signal transduction events controlled by GTP-binding proteins, including the release of ER-targeted proteins from signal recognition particle to the translocon following binding of the signal recognition particle

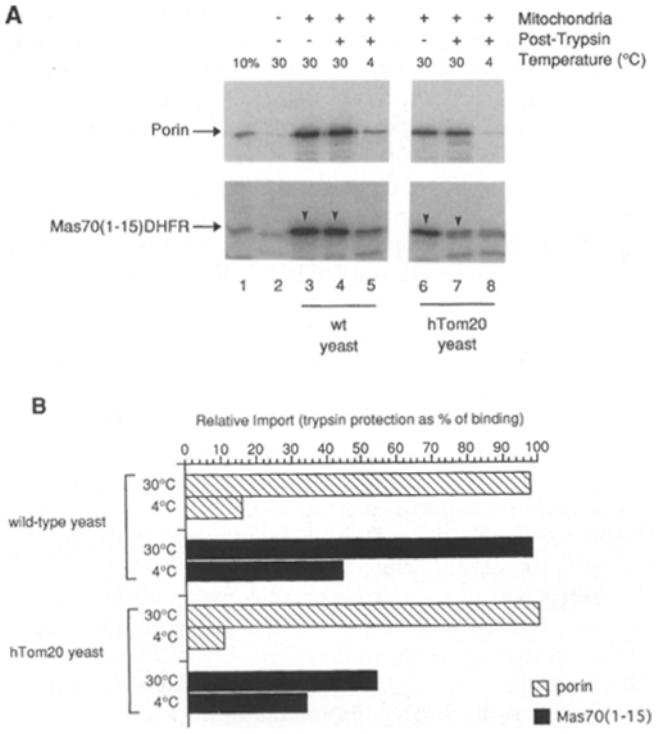

Figure 6. hTom20p prevents import of Mas70(1–15)DHFR into yeast mitochondria. (*A*) [^{35}S]porin (*top*) or [^{35}S]Mas70(1–15) DHFR (*bottom*) were incubated with mitochondria purified either from wild-type yeast (lanes *3–5*) or from *Δmas20* yeast complemented with the hTom20 receptor (Goping et al., 1995). Incubation was performed for 30 min at either 30°C (lanes *3*, *4*, *6*, and *7*) or 4°C (lanes *5* and *8*) under standard import conditions. Mitochondria were collected either directly (lanes *3* and *6*) or following trypsin treatment (lanes *4*, *5*, *7*, and *8*). Lane *1*, 10% of input [^{35}S]precursor protein; lane *2*, import in the absence of organelles. (*B*) Autoradiograms in (*A*) were quantified by laser densitometry and analyzed using image analysis software (Image v1.57, NIH). The radioactive bands in (*A*), lanes *3* and *6* were arbitrarily set at 100. Porin, *hatched bars*; Mas70(1–15)DHFR, *solid bars*.

signal sequence complex to signal recognition particle receptor (Miller and Walter, 1993). An analogous ATP-dependent transfer of precursor proteins between components of the mitochondrial receptor complex has been documented for precursors that are complexed with mitochondrial-import stimulation factor (Hachiya et al., 1995; Komiya et al., 1996; Mihara and Omura, 1996). Whatever the mechanism, however, screening of potentially cryptic targeting sequences by Tom20p may play an important role in preventing missorting of proteins to mitochondria in the eukaryotic cell.

Finally, our results may be relevant to postreceptor sorting of precursor proteins within the mitochondrion. For example, cryptic matrix-targeting sequences would be expected to remain topogenically silent until they pass beyond Tom20p and, therefore, would not interfere or compete with other topogenic sequences within the polypeptide that are recognized by this receptor. One class of proteins for which this may be relevant are polytopic integral proteins of the inner membrane, where it has been observed that positively charged domains within the polypeptide are preferentially located on the matrix side of the inner membrane (Gavel and von Heijne, 1992). Recognition of these domains as cryptic matrix-targeting signals at the inner membrane during import could represent an important determinant for proper transmembrane topology by driving paired transmembrane helices into the bilayer (Liu et al., 1988). Conversely, the lack of recognition of these sequences at the outer membrane may allow the polypeptide to adopt a conformation which is incompatible with integration into the lipid-bilayer of the outer membrane, thereby resulting in proper sorting to the inner membrane.

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