

Identification of the human mitochondrial protein import receptor, huMas20p. Complementation of $\Delta mas20$ in yeast

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Abstract The human homolog of the *S. cerevisiae*/N. *crassa* mitochondrial protein import receptor, Mas20p/MOM19, has been identified and characterized. Sequence similarities between these three proteins is most pronounced within the NH₂-terminal third of the molecules. However, the mammalian protein exhibits only weak homology to the tetratricopeptide repeat B domain that is found in Mas20p/MOM19. huMas20p is targeted and inserted into the outer membrane of isolated rat heart mitochondria, in the N_{in}-C_{cyto} orientation. Antibodies directed against the soluble portion of huMas20p inhibited in vitro mitochondrial import of a diverse set of precursor proteins (including inner membrane uncoupling protein), but failed to block import of a fusion protein bearing the signal-anchor sequence of Mas20p itself. Finally, expression of huMAS20 complemented the respiratory defect of $\Delta mas20$ yeast cells. Together, these results demonstrate that huMAS20p is a component of the mammalian import apparatus.

Key words: Mitochondrion; Import receptor; Protein targeting

1. Introduction

The protein import machinery of the mitochondrial outer membrane is comprised of a dynamic complex of proteins that mediates translocation of cytosolic precursor proteins into or across the membrane [1,2]. Extensive genetic and biochemical studies in *S. cerevisiae* and *N. crassa* have so far identified several proteins within this complex that contribute directly to the initial recognition and/or binding of the precursor protein at the surface of the organelle. These include Mas20p/MOM19 [3,4], Mas70p/MOM72 [5,6], Mas22p/MOM22 [7,8] and Mas37p [9] in yeast/*Neurospora*, respectively. Together, these elements appear to cooperate in delivering the bound precursor into the translocation pore [10,11]. The requirement for multiple receptor subunits to sustain protein import into the mitochondrion presumably reflects the enormous diversity of cytosolic precursor proteins that must be recognized. Part of this diversity is reflected by the existence of a number of distinct mitochondrial targeting signals, each of which has quite different properties. These include positively-charged matrix-targeting signals and hydrophobic outer membrane signal-anchor sequences, both of which are usually located at the NH₂-termini of precursor proteins, as well as a number of less well defined internal signals within polytopic outer and inner membrane proteins. Additionally, certain precursor proteins may interact with the receptor complex via a cytosolic effector molecule [12]. Not surprisingly, therefore, different precursor proteins can

exhibit differential dependencies on individual receptor subunits [10,11].

To date, little has been learned about the import machinery of the mammalian outer mitochondrial membrane, other than the fact that like yeast and *Neurospora* mitochondria [13,14], import is dependent on surface-exposed, protease-sensitive components [15]. Characterization of the components of the mammalian import machinery, however, is important for several reasons. In particular, comparisons of evolutionarily distant relatives may help to identify conserved, and therefore functionally relevant, domains within the molecule. Additionally, mammalian mitochondria elaborate numerous metabolic and signal-transduction pathways that do not exist in lower organisms. Whether or not this added complexity in mitochondrial composition has influenced the evolution of the mammalian import machinery remains to be determined. Finally, the import machinery of yeast and *Neurospora* can manifest compensatory properties which may be critical for survival of the organism under certain adverse conditions. For example, enforced protein import can take place via a receptor 'by-pass' mechanism [16]. In the case of mammalian cells, it is not known whether or not such flexibility in the import machinery exists.

Here, we report the identification of a human homolog of Mas20p/MOM19 and demonstrate both a structural and functional similarity with the yeast and *Neurospora* counterparts. As well, however, differences between huMas20p and Mas20p/MOM19 were noted, which presumably reflect evolutionary differences in the import machinery between fungi and mammals.

2. Materials and methods

2.1. Isolation of huMAS20

The coding region of huMAS20 was cloned by RT-PCR of human fibroblast RNA using primers based on the sequence of HUMRSC145 (DBJ accession number D13641). In brief, 20 pmol of the antisense primer 5' CCGGAATTCTCATTCCACATCATCTTCAGCC were incubated with 1 µg of human fibroblast RNA in the presence of 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM each dNTP, 20 U RNasin and 20 U AMV reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min. Twenty pmol of the sense primer 5' CCAGAATT-CATGGTGGGTCGGAACAGCGCC and 2.5 U Taq polymerase were then added and incubated under standard PCR conditions [17]. The resultant PCR product was then cloned into the *EcoRI* site of the plasmid pBluescript KS.

2.2. In vitro import

Previous articles [18–21] describe the routine procedures used in this study. Briefly, mitochondria were purified from either rat heart [18] or *S. cerevisiae* strain D273–10B [22]. Import reactions were carried out at 30°C for 1 h in the presence of ³⁵S-labeled transcription-translation product in reticulocyte lysate (contributing 10% to final volume), and either rat heart mitochondria (30 µg protein) in 125 mM sucrose, 5 mM HEPES (7.5), 0.5 mM ATP, 2.5 mM NaSuccinate, 40 µM ADP, 1 mM

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K₂HPO₄, 1 mM dithiothreitol, 40 mM KCl, 1 mM MgAc₂, or yeast mitochondria (30 µg protein) in 0.6 M sorbitol, 50 mM HEPES (7.4), 50 mM KCl, 10 mM MgCl₂, 2.5 mM ethylenediamine tetraacetic acid, 2 mM KH₂PO₄, 2 mM ATP, 80 µM ADP, 5 mM NaSuccinate, 1 mM dithiothreitol. Rat and yeast mitochondria were then pelleted through a cushion of either 125 mM sucrose, 5 mM HEPES (7.5) or 0.6 M sorbitol, 50 mM HEPES (7.5), respectively. Integral membrane proteins were recovered following extraction with 0.1 M Na₂CO₃ (Fig. 2) [20], or 7 M urea (Fig. 3) [23,24].

2.3. Expression of huMAS20 in *E. coli* and generation of antibodies

Recombinant PCR technology was utilized to generate a bacterial expression construct, whereby an amino-terminal stretch of 6 histidine residues followed by the amino acid cysteine, was fused to the soluble portion of the huMAS20 cDNA (encoding amino acids 25–145). This hybrid molecule, designated huMas20p-ΔTM-6 × HIS, was placed under the control of the *ptac* promoter by cloning into the *Bam*HI-*Eco*RI site of the vector pGEX-2T (Pharmacia), in which the Glutathione S-transferase coding region has been removed [21]. Bacteria containing the huMAS20 expression vector were grown overnight and over-expression of huMAS20 induced with 1 mM isopropyl-β-thiogalactopyranoside for 3 h. The over-expressed protein was purified from inclusion bodies by denaturing in 7 M urea/100 mM KP_i pH 7.5 (U/P_i buffer) and binding to a Ni-NTA agarose column (Qiagen). The column was washed extensively in U/P_i buffer pH 5.8 containing first 1% Triton X-100, then 20% ethanol, and finally 1 M NaCl, and the pure protein was eluted with U/P_i + 1 M NaCl pH 4.0. Each fraction was neutralized with 1 M Tris pH 7.5. One litre of induced culture yielded approximately 2–3 mg of protein which was >95% pure. Chickens were immunized according to the method of Gassmann et al. [25]. Briefly, 500 µg of purified huMas20p-ΔTM-6 × HIS was mixed with an equal volume of Freund's complete adjuvant (Calbiochem) and the emulsion injected at two sites in the pectoral muscles. Two boosts were given 10 and 20 days following the initial immunization with 500 µg protein in Freund's incomplete adjuvant. Pre-immune eggs and day 30 post-immunization eggs were used to prepare yolk immunoglobulin. Protein from egg yolk was purified by isopropanol and acetone extraction according to the method of Bade and Stegemann [26]. Ig was purified from the total yolk protein using the gammaYolk kit (Pharmacia).

2.4. Expression of huMAS20 in yeast

The full-length coding region of the human MAS20 cDNA was placed under the control of the *ADHI* promoter by cloning into the yeast expression plasmid pSL1687 bearing the *URA3* gene as a marker. The strain YTB72 (*ura3*, *leu2*, *his3*, *trp1*, *mas20::LEU2*) was transformed directly from colony [27] with the recombinant plasmid, or vector alone. Colonies were streaked onto YPD plates (1% yeast extract, 2% peptone, 2% dextrose) and YPEG plates (1% yeast extract, 2% peptone, 3% glycerol and 3% ethanol). Yeast cells cured of the expression plasmid were obtained by counterselection on media containing 5-FOA. These *ura*[−] cells were streaked onto YPD and YPEG plates.

3. Results and discussion

A BLAST sequence homology search of current protein databases revealed the presence of a novel sequence, HUMRSC145 (DDBJ accession number D13641), that shared significant homology to *S. cerevisiae* Mas20p and *N. crassa* MOM19 (regions of homology of $p = 8 \times 10^{-6}$ and $p = 4 \times 10^{-4}$, respectively). As described in section 2, PCR primers were designed based on the sequence of the longest open reading frame of HUMRSC145, and the cDNA was cloned from human fibroblast mRNA. It yielded the same sequence as HUMRSC145 which, as suggested by the experiments described here, constitutes a structural and functional homolog of yeast Mas20p. Based on this homology, we have named the protein huMas20p (hu = human).

Fig. 1 shows an optimized amino acid alignment of MOM19, Mas20p, and huMas20p. The sequence of the predicted rat

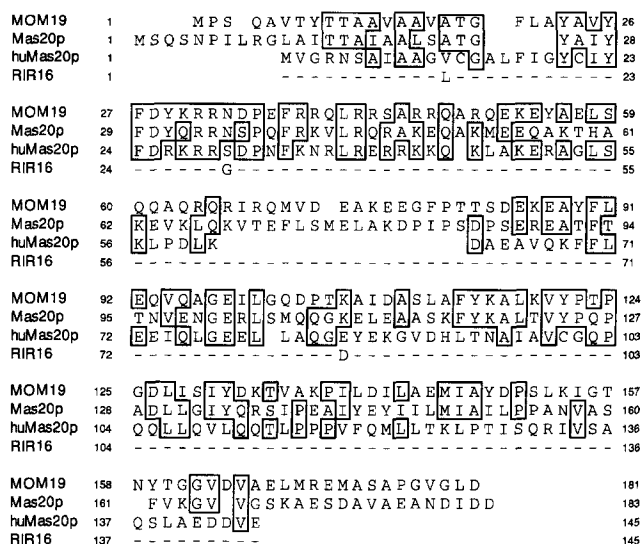


Fig. 1. Amino acid alignment of *S. cerevisiae* Mas20p, *N. crassa* MOM19, human Mas20p and rat RIR16. The sequence alignment was analyzed using the MAP algorithm [38] at the Baylor College of Medicine search launcher. Spaces were introduced for maximal alignment. Dashes indicate identity between human huMas20p and rat RIR16 sequences. Identical residues are boxed.

homolog of huMas20p has recently been deposited in the database (RIR16, accession number U21871) and shows 97% identity to the human sequence (Fig. 1). huMas20p contains 145 amino acids and has a predicted molecular weight of 16.3 kDa. Like MOM19 and Mas20p, it contains a short hydrophilic NH₂-terminus (amino acids 1–6) containing one arginine and no negatively-charged residues, followed by the predicted transmembrane domain (amino acids 7–24). huMas20p has an overall sequence similarity of 50% with Mas20p and 61% with MOM19 (Program Manual for the Wisconsin Package, Version 8, September 1994. Genetics Computer Group, 575 Science Drive, Madison, Wisconsin USA 53711). The strongest regions of similarity reside toward the NH₂-terminus, immediately downstream of the transmembrane segment (Fig. 1). Of note, huMas20p lacks homology to the A domain of typical tetratricopeptide repeats (TPRs) [28], and shows only weak homology to the core motif of TPR B domains (TPR consensus -W-LG-Y-A-F-A-P-). The B domain has been identified in both Mas20p and MOM19 [29]. Like the A domain, the B domain has a high probability of forming an amphipathic α -helix, which is postulated to mediate protein-protein interactions [28]. In contrast to Mas20p and MOM19, however, helical wheel projections show little, if any, potential for such a structure in huMas20p (not shown). Nevertheless, functional studies will be required to determine if the weak B domain of huMas20p (aa 89–101) contributes to protein-protein interactions.

3.1. Import of huMas20p

The first 28 amino acids of yeast Mas20p contain the information that targets this protein to the mitochondria and anchors it in the outer membrane. To investigate the targeting and topology of huMas20p, mitochondria were isolated from rat heart, and import of huMas20p compared to that of a fusion protein, Mas20p(1–28)DHFR, which contains amino acids 1–28 of yeast Mas20p fused to amino acids 3–186 of dihydrofolate

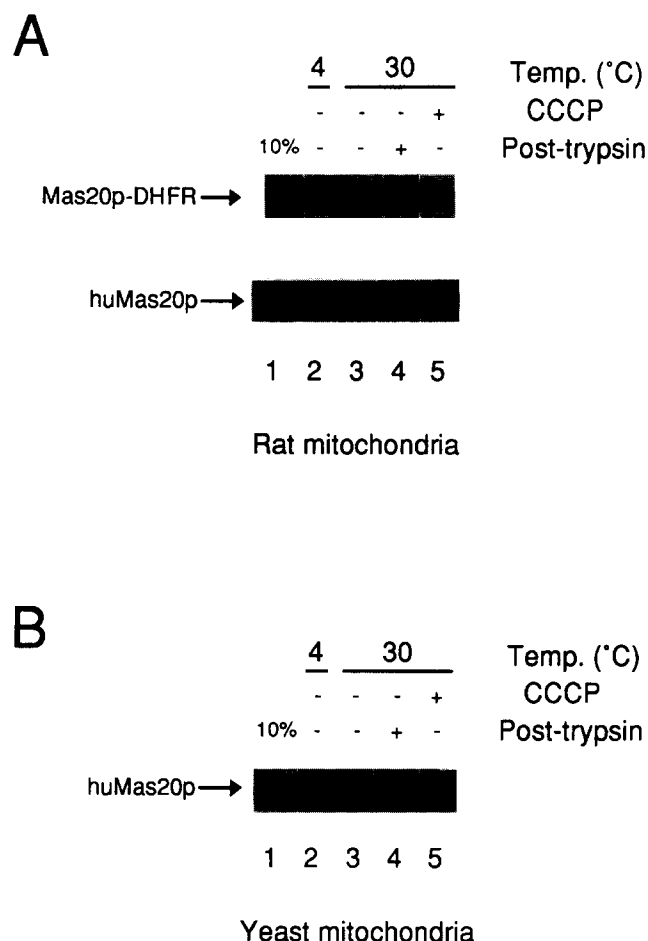


Fig. 2. Import of huMas20p into the outer membrane of mitochondria isolated from rat and yeast. (A) Import reactions included [35 S]Mas20p-DHFR (upper panel) or [35 S]huMas20p (lower panel) and were carried out in mitochondria isolated from rat heart at a concentration of 0.3 mg organellar protein/ml for 1 h at 4°C (lane 2) or 30°C (lanes 1, 3–5) in the presence (lane 5) or absence (lanes 1–4) of 1 μ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Membrane insertion was assayed by extraction with 0.1 M Na₂CO₃, pH 11.5 [20] (lanes 2, 3, 5). In certain cases, the import reaction mixtures were treated with 0.125 mg/ml trypsin for 20 min. on ice, followed by the addition of 1.25 mg/ml soybean trypsin inhibitor and a further incubation for 20 min on ice (lane 4). Lane 1, 10% of input radiolabelled protein. The radioactive signal for huMas20p was diffuse in lane 1 due to co-migration with globin in reticulocyte lysate. Arrows denote Mas20p-DHFR and huMAS20p. (B) Import reactions of [35 S]huMas20p carried out in mitochondria isolated from yeast at a concentration of 0.3 mg organellar protein/ml. Lane assignments are as in panel A. Arrow denotes huMAS20p.

reductase. As shown in Fig. 2A, the two proteins exhibited similar characteristics of import. Import and membrane insertion was assayed by the acquisition of resistance to extraction at pH 11.5 (Fig. 2A, lane 3). Resistance to alkaline extraction was dependent on the presence of mitochondria in the import reaction (not shown) and was sensitive to temperature (compare lanes 2 and 3). It was unaffected by the collapse of $\Delta\psi$ by CCCP (Fig. 2A, lane 5), suggesting that insertion into the outer membrane. This was consistent with the sensitivity of huMas20p and Mas20p(1–28)DHFR to exogenous trypsin following import (lane 4), and indicated that, as documented for yeast Mas20p [30] and *Neurospora* MOM19 [3], huMas20p was

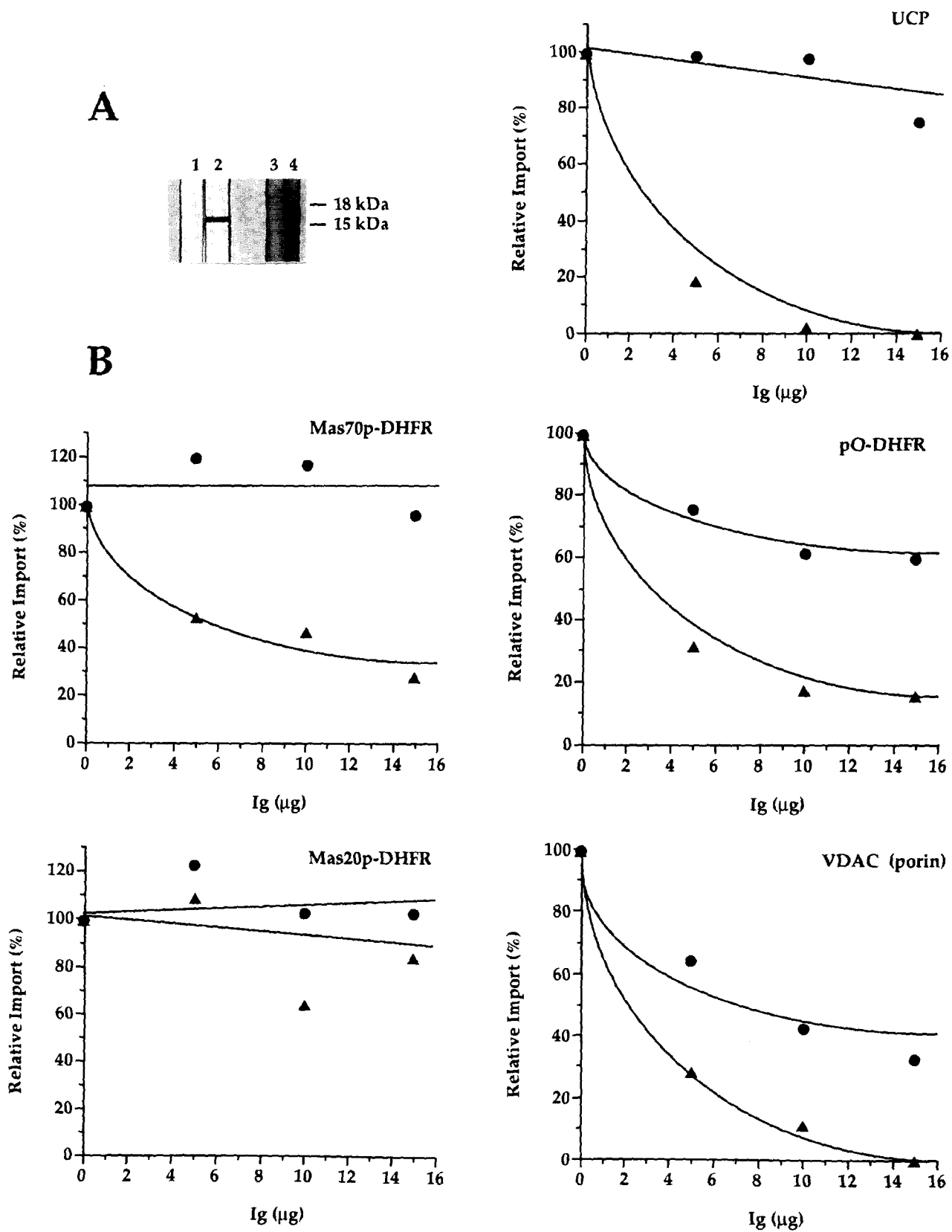
inserted into the outer membrane in the N_{in}–C_{cyto} orientation, leaving the bulk of the protein facing the cytosol. In addition, huMas20p was imported into mitochondria isolated from yeast (Fig. 2B). Similar to the import characteristics in rat heart mitochondria, membrane insertion into yeast mitochondria was demonstrated by the acquisition of resistance to extraction at pH 11.5 (Fig. 2B, lane 3), was temperature dependent (Fig. 2B, compare lanes 2 and 3), was unaffected by collapse of $\Delta\psi$ by CCCP (Fig. 2B, lane 5), and yielded an import product that was sensitive to exogenous trypsin (Fig. 2B, lane 4). Ten percent of input protein is shown in Fig. 2, lane 1. In this lane, the radioactive signal for huMas20p was diffuse due to co-migration of the protein with globin in reticulocyte lysate.

3.2. Effect of anti-huMas20p antibodies on protein import

Previous studies have shown that antibodies against Mas20p [4, 30] and MOM19 [3] interfere with protein import in vitro. Here, we have been successful in raising antibodies in chickens to the highly conserved mammalian protein, and demonstrate that anti-huMas20p cross-reacts with an approximately 16 kDa protein present in rat heart mitochondria (Fig. 3A). As shown in Fig. 3B, pre-treatment of mitochondria isolated from rat heart with anti-huMas20p interfered with subsequent import of a diverse set of precursor proteins. These included: (1) a fusion protein, pO-DHFR [31], containing the matrix-targeting sequence of pre-ornithine carbamyl transferase fused to DHFR; (2) a fusion protein, Mas70p(1–29)DHFR [19], containing the signal-anchor sequence of Mas70p fused to DHFR; (3) VDAC (porin) [32], an integral β -barrel protein of the mitochondrial outer membrane; and (4) uncoupling protein (UCP) [33], a polytopic integral protein of the mitochondrial inner membrane which is a member of the ADP/ATP and P_i carrier family. Of particular note, however, the antibodies failed to block import of a fusion protein, Mas20p(1–28)DHFR (Fig. 2), bearing the signal-anchor sequence of Mas20p itself (Fig. 3B). This result supports the previous suggestion that MOM19 can be targeted to mitochondria independent of surface receptors [34]. The fact that Mas20p does not depend on itself for import and assembly into the import complex provides a mechanistic explanation for the fact that the wild-type gene can complement *Amas20* null strains.

3.3. Complementation of *Amas20* deficient yeast

The results of in vitro import suggested that huMas20p can correctly target mitochondria from a heterologous yeast source (Fig. 2B) and, therefore, raised the possibility of functional complementation between the two proteins in vivo. To test this, we examined the ability of *huMAS20* to complement the respiratory defect of *mas20* deficient yeast cells [4,29]. Equivalent amounts of *Amas20* yeast cells, transformed with either the non-recombinant pSL1687, or with pSL1687 driving the expression of *huMAS20* (Fig. 4, panel A), were streaked onto YPEG plates (Fig. 4, panel B). After 5 days of incubation at 30°C, only the cells expressing *huMAS20* were able to grow on the non-fermentable carbon source. Selection of *ura3*[–] cells that had lost the plasmid was accomplished by plating the cells on media containing 5-FOA. These cells were no longer able to grow on YPEG plates (Fig. 4, panel C). This result confirmed that the complementation of *Amas20 res*[–] cells was dependent on the expression of huMAS20 and was not due to the overexpression of an extragenic suppressor [35].

A**B**

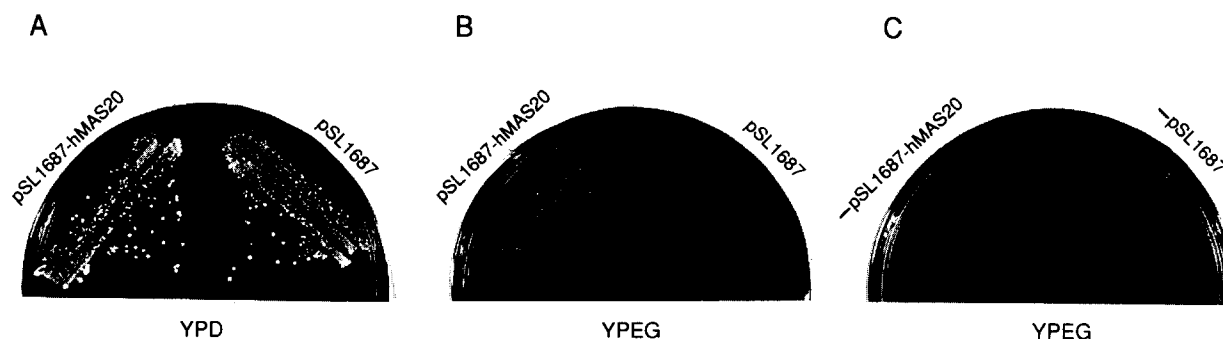


Fig. 4. Expression of human *MAS20* complements the respiration defect of a *MAS20*-deficient strain. The respiratory deficient *Δmas20* strain YTJB72 (*ura3, leu2, his3, trp1, mas20::LEU2*) was transformed with the *URA3* containing vector pSL1687, or with the recombinant construct hMAS20-pSL1687 wherein *huMAS20* expression is under the control of the *ADHI* promoter. The cells were streaked onto rich solid medium containing either (A) glucose (YPD) or (B) ethanol and glycerol (YPEG). (C) Subsequent 5-FOA resistant cells that had lost the *URA3*-based plasmid were streaked onto YPEG. All plates were incubated at 30°C for 5 days.

3.4. Conclusions

We have identified huMas20p as a component of the mammalian import apparatus. Amino acid analyses show that huMas20p is homologous to the fungal Mas20p/MOM19. Furthermore, complementation of the mitochondrial defects of *Δmas20* yeast cells by expression of huMAS20 demonstrates that huMas20p can function in vivo and productively interact with components of the yeast import machinery. In addition, like Mas20p/MOM19 [3,4,30], antibodies raised against the human protein inhibit in vitro import of a diverse set of cytosolic precursor proteins.

Nevertheless, important distinctions have been noted. For example, huMas20p lacks the highly conserved motif present within the tetratricopeptide repeat B domain that is shared among Mas20p/MOM19, Mas70p/MOM72, and Mas37p, and is believed to mediate protein-protein interactions [36]. Also, we found that, among the various precursor proteins examined, import of UCP was strongly inhibited by antibodies against huMas20p. UCP is a close relative of the ADP/ATP carrier (AAC) protein [37] which, in yeast and *Neurospora*, shows a preferential dependence for import on Mas70p/MOM72, rather than Mas20p/MOM19 [3,5,6,38]. Among other reasons, this has been interpreted to mean that the Mas70p and Mas20p subunits (with their respective partners) of the import receptor complex may associate reversibly rather than form a stable entity [10]. For this reason, antibodies against one receptor may not sterically interfere with the other. Our findings for mammalian mitochondria, on the other hand, suggest either that UCP depends directly on huMas20p for import, or that other components of the receptor complex (e.g. Mas22p), if they

exist, are indirectly affected by anti-huMas20p as a result of steric interference of the receptor complex as a whole. Finally, it is noteworthy that sequence conservation between eukaryotic Mas20p receptors is particularly pronounced only in regions immediately downstream of the transmembrane segment. Not only does this finding target these regions for functional significance (e.g. precursor protein recognition or interactions with other components of the import machinery), it provides a rational guide for further analysis of receptor function by mutagenesis.

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Fig. 3. Antibodies against huMas20p react with a 16 kDa protein of rat heart mitochondria and inhibit import of selective precursor proteins. (A) Ten μ g of purified huMas20p- Δ TM-6 \times HIS (lanes 1 and 2), and approximately 500 μ g purified rat heart mitochondrial protein (lanes 3 and 4) were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with total egg yolk protein from pre-immune (lanes 1 and 3) or huMas20p immunized (lanes 2 and 4) chickens. The primary antibody was detected with rabbit anti-chicken IgG conjugated to alkaline phosphatase (ICN). Color was developed using NBT/BCIP (Boehringer/Mannheim) according to the manufacturer's instructions. Molecular weight markers are indicated. (B) Standard import reactions (25 μ g purified rat heart mitochondria in 50 μ l import buffer) were performed as in Fig. 2 except that mitochondria were preincubated for 30 min. on ice with increasing amounts of pre-immune Ig (●) or Ig reactive against huMas20p (▲) prior to the addition of radiolabeled precursor proteins. Import reactions were terminated after 15 min at 30°C. Import is expressed relative to control import with no Ig added (100%). UCP, uncoupling protein; VDAC, human voltage dependent anion channel isoform-1; pO-DHFR, matrix targeting signal of pre-ornithine carbamyl transferase (amino acids 1–32) fused to dihydrofolate reductase; Mas70p-DHFR, signal anchor sequence of Mas70p (amino acids 1–29) fused to DHFR; Mas20p-DHFR, signal anchor of Mas20p (amino acids 1–28) fused to DHFR. Outer membrane insertion of Mas70p-DHFR and Mas20p-DHFR was assayed by extraction with urea [23,24] and import of the other precursors was assayed by protease protection [19,30,32].

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