We have identified a novel mitochondrial targeting signal in the precursor of the DNA helicase Hmi1p of Saccharomyces cerevisiae that is located at the C terminus of the protein. Similar to classical N-terminal presequences, this C-terminal targeting signal consists of a stretch of positively charged amino acids that has the potential to form an amphipathic α-helix. Deletion of the C-terminal 36 amino acids of helicase resulted in loss of import into mitochondria, while deletion of the N-terminal 40 amino acids had no effect. When C-terminal regions of the helicase were placed at the C terminus of a passenger protein, dihydrofolate reductase, the resulting fusion proteins were directed into the mitochondrial matrix, and the C-terminal region of helicase became proteolytically processed. Import of helicase occurs in a C- to N-terminal direction; it requires a membrane potential and the TIM17–23 translocase together with mitochondrial Hsp70. Helicase is the only mitochondrial matrix protein identified thus far with a cleavable targeting signal at its C terminus.

The majority of mitochondrial proteins are encoded by the nucleus, synthesized in the cytoplasm, and imported into mitochondria through translocases in both the outer (TOM machinery) and inner (TIM machinery) membranes (1–3). Most proteins destined for the mitochondrial matrix bear targeting information at their N termini. These matrix targeting sequences (MTSs) are commonly 20–60 amino acids in length and are cleaved upon import into mitochondria by the mitochondrial processing peptidase (MPP) (4, 5). N-terminal presequences contain several positively charged amino acids, are notably devoid of negative charges, and contain periodic hydroxylated amino acid residues. Presequences are also periodically devoid of negative charges, and contain periodic positively charged amino acids, which are thought to be important for the interaction with the import machinery.

While proteins destined for the mitochondrial matrix generally possess N-terminal targeting signals, proteins of the outer and inner membranes often contain internal targeting information. Frequently these internal targeting signals consist in part, of hydrophobic membrane anchor segments. The transmembrane domain of the human Bcl-2, for example, has been shown to function as a mitochondrial signal anchor sequence that targets and inserts the protein into the outer membrane (7). In addition, a segment of positively charged amino acids located directly C-terminal to a single transmembrane domain functions together as an internal targeting signal in the inner membrane proteins Bap1p and cytochrome c_1 (8, 9). The majority of internal targeting signals from outer and inner membrane proteins, as well as from intermembrane space proteins, however, have not yet been characterized.

At least two different import translocases exist in the mitochondrial inner membrane which differ in their substrate specificity. Import of presequence targeted proteins into the mitochondrial matrix generally requires a membrane potential (Δψ) across the mitochondrial inner membrane and the TIM17–23 translocase machinery (10–14). The TIM17–23 translocase, along with the membrane-bound TIM44 and the matrix chaperone mt-Hsp70 (15–21), mediate the translocation across the inner membrane into the matrix. Alternatively, import of several inner membrane proteins, such as the ADP/ATP carrier and components of the TIM machinery itself, is mediated by the TIM22–54 translocase (22–24).

N-terminally targeted proteins traverse the import machinery in a linear fashion in an N- to C-terminal (N→C) direction. A recent analysis using artificial chimeras, however, has indicated that proteins can also be directed into mitochondria in the reverse direction, i.e. in a C- to N-terminal (C→N) direction when a targeting signal is placed at the C terminus of the protein (25). Are there any proteins that actually use backward import in vivo? Here we describe the characterization of the first protein, a DNA helicase, Hmi1p from Saccharomyces cerevisiae, which uses such a reverse import pathway. Hmi1p is required for mitochondrial DNA inheritance and is localized in the matrix. As demonstrated here, the helicase is targeted to mitochondria via a cleavable C-terminal signal. This targeting signal resembles classical N-terminal signals in terms of its net positive charge and amphipathicity. Deletion of the signal results in loss of import into isolated mitochondria, while its addition to the C terminus (and N terminus) of the dihydrofolate reductase (DHFR) passenger protein ensues import into the mitochondrial matrix. The identification of this novel C-terminal targeting signal indicates that the helicase may be a representative of a new class of mitochondrial proteins, which bear C-terminal targeting signals and which are translocated across the membranes in a C- to N-terminal direction.
**Experimental Procedures**

Recombinant DNA Techniques and Plasmid Constructions—The open reading frame (YOL095c) encoding the Hmi1p DNA helicase (706 amino acids) and truncated derivatives were amplified by PCR using yeast genomic DNA as a template. The Hel-ΔN construct lacked the first 40 amino acids of the helicase, while the Hel-ΔC lacked the last 36 amino acids. In the Hel-ΔN/ΔC construct, both the first 40 and last 36 amino acids of the helicase were deleted. The N-terminal primers (containing a BamHI restriction site for subsequent cloning) were as follows: 5'-CCCCGGATCCAGAGATGCAAGCCTA-3' for the full-length helicase and 5'-CCCCCCCTTATGCTGAAAGCCG-3' for the full-length and 5'-CCCCACGAGATGCAAGCCTA-3' for the Hel-ΔC and Hel-ΔN/ΔC constructs. All PCR products were then cloned using BamHI/HindIII into the in vitro transcription vector pGEM4 under the control of the SP6 polymerase.

The helicase fusion constructs were cloned into a pGEM4 vector containing either DHFR or DHFRmut in which the stop codon at the end of DHFR or DHFRmut had been removed by PCR amplification and recloning. DHFRmut is a derivative of the wild-type DHFR protein, which contains three point mutations Cys-7→Ser, Ser-42→Cys, and Asn-49→Cys (26). DHFRmut-Hel(388→706) was cloned as an EcoRI/HindIII fragment by taking advantage of the EcoRI restriction site at residue 388 of helicase. Similarly, DHFRmut-Hel(557→706) was cloned as an XbaI/HindIII fragment utilizing the XbaI site at amino acid 557. To obtain the DHFRmut-Hel(602→706) and Hel(602→706) constructs, the following primers (containing a HindIII restriction site) were used: 5'-CCCCCATCTAAGGAGAAGGGGAGCATGTCCAATTTCCC-3' for the full-length and 5'-CCCCAATCTAAGGAGAAGGGGAGCATGTCCAATTTCCC-3' for the N-terminal primers containing a HindIII restriction site. The resulting helicase XbaI/HindIII fragment was then cloned into the pGEM4 vector containing DHFRmut in the case of DHFRmut-Hel(602→706), or into the vector alone for the Hel(602→706) construct. The fragment encoding the last 37 and 47 amino acid residues of the helicase were amplified using the primers (containing XbaI restriction site) containing an XbaI site at amino acid 706 and 388, respectively. The C-terminal primer for the full-length and 5'-GAGATCTAGAGAAAGGGGAGCATGTCCAATTTCCC-3', containing a C-terminal helicase primer from above, was used. The helicase PCR products were then cloned as XbaI/HindIII fragments at the C terminus of DHFR in the pGEM4 expression vector. To clone the fragment encoding the last 37 amino acid residues of the helicase at the N terminus of DHFR, the N-terminal primer 5'-GAGAGAATTCGATGAAAGGCTAGATGCTGATG-3', containing the EcoRI restriction site, and C-terminal primer 5'-GAGAGAATTCGATGAAAGGCTAGATGCTG-3', containing a BamHI restriction site, were used. The helicase PCR product was then cloned as an EcoRI/BamHI fragment at the N terminus of DHFR in the pGEM4 expression vector.

Isolation of Mitochondria and Protein Import—All import experiments were performed in mitochondria isolated from the S. cerevisiae wild-type strain (D273-10B) unless otherwise indicated. D273-10B was sensitive to the D. discoideum drophobic protein lacking predicted transmembrane domain(s).

**RESULTS**

The DNA helicase Hmi1p is imported into the mitochondrial matrix in vitro. Radiolabeled helicase was synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine and incubated with isolated yeast mitochondria. Helicase became imported into mitochondria and was located in the mitochondrial matrix (Fig. 1A). This import was dependent on a membrane potential, Δψ. Import of helicase was proteolytically processed and could be extracted at alkaline pH, demonstrating it to be a soluble protein (Fig. 1B). This is also suggested by the tritiated acid sequence of the helicase, which predicts a hydrophilic protein lacking predicted transmembrane domain(s). During translation of the helicase mRNA in reticulocyte lysate, a series of smaller helicase by-products were also synthesized (Fig. 1A). These truncated helicase derivatives are generated by initiation of translation at ATG codons downstream from the start ATG codon; the translation initiation event is not repeatable in the presence of [35S]methionine and incubated with isolated yeast mitochondria.

**Miscellaneous**—The following procedures were performed according to published methods: protein determination (31), SDS-PAGE (32), and import of preproteins in the presence of methotrexate (8). The detection of proteins after blotting onto nitrocellulose was performed using the ECL detection system (Amersham Pharmacia Biotech).
structed to the start codon under these translation conditions. These by-products have varying N termini, but a common C terminus. Each of these N-terminally truncated derivatives present in the helicase lysate were imported efficiently into the mitochondrial matrix in a Δψ-dependent fashion (Fig. 1A).

The Presence of the C-terminal Region of Helicase Is Essential for Import—To further investigate the location of the targeting information, we generated truncated derivatives of helicase where either the N-terminal 40 amino acid residues were deleted (Hel-ΔN), or the C-terminal 36 residues (Hel-ΔC), or both N- and C-terminal (Hel-ΔN-ΔC) (Fig. 2A). Import of Hel-ΔN was dependent on a membrane potential and was accompanied by proteolytic processing to a form with an ~3 kDa lower apparent molecular mass. As was observed with the authentic helicase, a series of smaller (N-terminal truncated) translation by-products present in the Hel-ΔN lysate, were also translocated into the mitochondrial matrix. Neither Hel-ΔC nor Hel-ΔN-ΔC was imported into mitochondria. (One of the internal translation by-products of both Hel-ΔC and Hel-ΔN-ΔC proteins, this truncated product seems to be generated from initiation of translation at the internal methionine, Met-120. In this case, a stretch of positively charged amino acids directly after Met-120 may be acting as a cryptic N-terminal matrix-targeting signal.)

In conclusion, the very C-terminal region, but not the N-terminal region of the helicase protein is essential for targeting to mitochondria.

FIG. 2. The C-terminal 36 amino acid residues of helicase are essential for import. Radiolabeled Hel-ΔN, Hel-ΔC, and Hel-ΔN-ΔC proteins were imported into isolated mitochondria for 20 min at 25 °C, either in the presence (+NADH) or absence (+Valinomycin) of a Δψ. Mitochondria were then either mock-treated or subjected to PK (40 μg/ml) treatment under non-swelling or swelling conditions. All samples were analyzed as in Fig. 1. p, precursor; m, mature protein.

The Targeting Signal of the Hmi1p Helicase Is Located at Its C Terminus—To precisely localize the targeting signal in the helicase protein, a number of fusion proteins were created and their import was analyzed. In a first set of experiments, C-terminal portions of the helicase were placed at the C terminus of DHFR (Fig. 3A). A mutated form of the DHFR was used (DHFRmut), which does not fold to its native conformation, and hence is exquisitely sensitive to added protease if it remains outside the mitochondria in such an import assay (26). DHFRmut-Hel(388–706) contains approximately the C-terminal half of helicase (Fig. 3A). This protein was efficiently imported into the mitochondrial matrix in a Δψ-dependent fashion and became processed (Fig. 3B). Two other constructs containing smaller portions of helicase, DHFRmut-Hel(557–706) and DHFRmut-Hel(602–706), were also imported and processed. To exclude the possibility that the passenger protein, DHFRmut, contained any cryptic targeting information and was responsible for the import observed, a truncated helicase protein, Hel(602–706), comprising the last 104 amino acid resi-

FIG. 3. The C terminus of helicase can direct the DHFR passenger protein into the mitochondrial matrix. A, DHFR-helicase fusion constructs. Shaded areas represent various lengths of the C-terminal region of helicase placed at the C terminus of DHFR. B, the internal methionine, Met-120. In this case, a stretch of positively charged amino acids directly after Met-120 may be acting as a cryptic N-terminal matrix-targeting signal. A mutated form of the DHFR was used (DHFRmut), which does not fold to its native conformation, and hence is exquisitely sensitive to added protease if it remains outside the mitochondria in such an import assay (26). DHFRmut-Hel(388–706) contains approximately the C-terminal half of helicase (Fig. 3A). This protein was efficiently imported into the mitochondrial matrix in a Δψ-dependent fashion and became processed (Fig. 3B). Two other constructs containing smaller portions of helicase, DHFRmut-Hel(557–706) and DHFRmut-Hel(602–706), were also imported and processed. To exclude the possibility that the passenger protein, DHFRmut, contained any cryptic targeting information and was responsible for the import observed, a truncated helicase protein, Hel(602–706), comprising the last 104 amino acid resi-
Radiolabeled helicase, Hel(557–706), and Hel(602–706) were imported for 20 min either as described previously (+Mn²⁺/Mg²⁺) or in the following buffer (+EDTA/o-Phe): 3% (w/v) bovine serum albumin, 50 mM HEPEs (pH 7.2), 0.5 M sorbitol, 80 mM KCl, 2 mM potassium phosphate, 10 mM EDTA, and 2 mM o-phenanthroline (o-Phe). Import was performed at 12 °C, as the block of MPP activity by EDTA/o-Phe is much more effective than at 25 °C. Following import, mitochondria were then either mock-treated or subjected to PK (40 μg/ml) treatment under non-swelling or swelling conditions. All samples were analyzed by SDS-PAGE, blotting onto nitrocellulose, and autoradiography. p, precursor; m, mature protein.

The C-terminal Targeting Signal Is Proteolytically Processed by MPP following Import—To test whether the processing of the helicase and derivatives thereof was catalyzed by the mitochondrial processing peptidase (MPP), import of helicase and various derivatives was performed in the presence or absence of divalent cations (Fig. 4). MPP activity is dependent on divalent cations and therefore is inhibited in the presence of chelators such as EDTA and o-phenanthroline. The processing of helicase, Hel-ΔN, DHFRmut-Hel(557–706), as well as Hel(602–706), was inhibited in the presence of the chelators. Furthermore, despite that each of these precursors imported contained a different N-terminal sequence; all were processed with similar efficiencies upon import. We conclude that MPP is recognizing and cleaving a C-terminal segment of helicase.

The Targeting Signal of Helicase Can Operate in Both N→C and C→N Import Directions—to demonstrate that the extreme C-terminal region of helicase contains the mitochondrial targeting signal, two further fusion proteins were constructed. The C-terminal 37 amino acid residues were placed either at the C terminus of wild-type DHFR, DHFR-HelC37, or like a traditional mitochondrial targeting signal at the N terminus of DHFR, HelC37-DHFR. Unlike DHFRmut, wild-type DHFR forms a tightly folded structure. Upon incubation with isolated mitochondria in the presence of a Δψ, these fusion proteins were imported into the matrix and both were proteolytically processed (Fig. 5A). Thus, the C terminus of helicase comprises a targeting signal that can operate in the “normal” forward reaction, N-terminal to C-terminal (N→C) import, as well as in a reverse direction (C→N), as is the authentic situation for the helicase. Import of the DHFR moiety demonstrated the ability of the helicase targeting signal to drive the import of a folded domain into the mitochondrial matrix in both directions. Import in a C→N direction also occurred with similar kinetics as import along the standard N→C route (Fig. 5B). Furthermore, using a slightly longer derivative, DHFR-HelC47, translocation into the matrix was inhibited when performed in the presence of methotrexate (Fig. 5C). Thus proteins imported in a reverse direction, can be arrested as translocation intermediates.

Where is the MPP cleavage site in the C-terminal targeting sequence of Hmi1p? Three potential cleavage sites may seem possible from the analysis of the amino acid sequence of this region of the helicase. Both Arg-679 and Arg-684 with their respective flanking sequences are unlikely candidates for representing typical Arg(-2) recognition sites (1, 2, 33); in contrast, Arg-691, which is followed by Ala-Tyr, conforms to a classical cleavage motif. The data in Fig. 5A indicate that the putative cleavage site is, indeed, located close to the C terminus. A larger segment of the precursor was cleaved off when the
C-terminal 37 amino acid residues of the helicase were at the N terminus of DHFR than when they were placed at the C terminus of the DHFR protein. It is therefore likely that cleavage occurs after Ala-692, resulting in the removal of the C-terminal 14 residues.

**Import of Helicase Is Facilitated by the TIM17–23 Machinery Together with mt-Hsp70**—Preproteins targeted to mitochondria by N-terminal cleavable presequences are imported across the inner membrane via the TIM17–23 machinery driven by ATP-hydrolysis in conjunction with mt-Hsp70. Does helicase use the same import machinery? Radiolabeled helicase and DHFR\(^\text{mut}\)–Hel(602–706) were imported into mitochondria harboring a modified form of Tim23, Tim23fs. Tim23fs mitochondria display a strongly reduced capacity to import N-terminally targeted proteins such as pSu9(1–69)–DHFR\(^\text{mut}\) (Fig. 6A and Ref. 22). Import of helicase and DHFR\(^\text{mut}\)–Hel(602–706) into Tim23fs was also significantly reduced when compared with the isogenic wild-type mitochondria.

In order to test the requirement for mt-Hsp70, import of helicase into mitochondria isolated from the ssc1–3 yeast mutant was performed. This yeast strain harbors a mutated form of mt-Hsp70 and has a temperature-sensitive growth phenotype. Mitochondria prepared from this strain grown at the permissive temperature are defective in importing preproteins, if they are exposed to a brief incubation at 37 °C, prior to import (28). Import of the control protein, pSu9(1–69)–DHFR\(^\text{mut}\) into the ssc1–3 mitochondria was strongly inhibited following this pretreatment (Fig. 6B). Import of helicase and DHFR\(^\text{mut}\)–Hel(602–706) into the ssc1–3 mitochondria was also reduced in comparison to wild-type mitochondria. The presence of slightly smaller fragments of these proteins in a protease protected location indicated, however, that in contrast to the N-terminally targeted proteins, they had become at least partially imported into the ssc1–3 mitochondria (Fig. 6B). Similar translocation intermediates were observed following the import of helicase and derivatives into matrix ATP-depleted wild-type mitochondria, conditions also where the activity of mt-Hsp70 is compromised (results not shown).

In summary, translocation of helicase across the inner membrane occurs at the TIM17–23 import site. Passage into the mitochondrial matrix requires the action of mt-Hsp70; however, this requirement does not appear to be as stringent as that of N-terminally targeted proteins.

**DISCUSSION**

The recently identified DNA helicase, Hmi1p, is located in the mitochondrial matrix\(^2\) (Fig. 1A). As demonstrated here, this helicase uses a unique targeting mechanism for its import into mitochondria. Hmi1p is directed to and imported into the matrix by virtue of a C-terminal cleavable targeting signal.

The C-terminal ~36 amino acid residues of the helicase contain a mitochondrial targeting signal. Following import of
helicase into the matrix, part of this targeting signal (most likely the last 14 C-terminal residues) is proteolytically removed, an event presumably catalyzed by MPP (Fig. 7A). Similar to classical N-terminal targeting signals, the C-terminal targeting signal of helicase comprises several positively charged amino acid residues and is devoid of negatively charged ones. The C-terminal 18 amino acid residues of the helicase precursor display the ability to form a positively charged amphipathic α-helix (Fig. 7B).

The helicase targeting signal can direct the passenger protein DHFR into the mitochondrial matrix when placed either in its authentic C-terminal position or when placed at the N terminus of DHFR. In both cases the targeting signal undergoes efficient proteolytic processing following import. The helicase import signal, therefore, can direct a protein into the matrix, part of this targeting signal (most likely the last 14 C-terminal residues) is proteolytically removed, an event presumably catalyzed by MPP (Fig. 7A). Simultaneous proteolytic cleavage of the targeting signal in both cases can drive the DHFR into the mitochondrial matrix when placed either in the N- or the C-terminal position. When placed at the N terminus of DHFR, in both cases the targeting signal underlies DHFR into the mitochondrial matrix when placed either in the N- or the C-terminal position.

Additionally, helicase targeting signal comprises a mitochondrial targeting signal (most likely the last 14 C-terminal residues) is proteolytically removed, an event presumably catalyzed by MPP (Fig. 7A). Simultaneous proteolytic cleavage of the targeting signal in both cases can drive the DHFR into the mitochondrial matrix when placed either in the N- or the C-terminal position. When placed at the N terminus of DHFR, in both cases the targeting signal underlies DHFR into the mitochondrial matrix when placed either in the N- or the C-terminal position.

Heliase employs the TIM17–23 translocase and mt-Hsp70. Inhibition of import in the ssc1–3 mutant defective in Hsp70 function, however, was not complete. The production of translocation intermediates, arrested during the process of import across the mitochondrial membranes, was not detected with representatives of the classical N→C import pathway. These data indicate that the C→N import of the helicase protein, although it appears to require mt-Hsp70 for efficient import of the mature protein, may have less stringent requirements for mt-Hsp70 than the standard N→C import process.

The DNA helicase Hmi1p (open reading frame YOL095c) has high degree of similarity to the Staphylococcus aureus PCRA helicase (34) and the Escherichia coli Rep helicase (35). Compared with these proteins, Hmi1p has an extended C terminus (~60 amino acids) of unrelated sequence. One other mitochondrial DNA helicase has been characterized in S. cerevisiae, Pif1p, which contains a predicted N-terminal presequence (36). Why might the two mitochondrial helicases in S. cerevisiae be targeted to mitochondria in different fashions? Both helicases contain conserved ATP-binding motifs. In the case of the Pif1p, which contains a predicted N-terminal presequence (36), the ATP-binding site is located internally in the protein. In contrast, with the Hmi1p it is present at the extreme N-terminal region of the protein. The presence of an N-terminal presequence in close proximity to the ATP-binding domain may somehow interfere with or modulate folding of this domain before or after translocation across the mitochondrial membranes. The pathway of C→N import implies a post-translational mechanism of translocation. Whether there might be a need for such a mechanism in the case of the Hmi1 helicase remains an open question.

Until now, the search for putative N-terminal targeting signals has been the predominant method of identifying possible mitochondrial proteins. Upon searching the S. cerevisiae protein data base, however, many unidentified open reading frames were found that contain positively charged amino acids at their C termini. Thus, additional mitochondrial proteins with C-terminal targeting signals may exist that have been overlooked so far.

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