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Mitochondrial Targeting of Yeast Apoiso-1-cytochrome *c* Is Mediated through Functionally Independent Structural Domains

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An iso-1-cytochrome *c*–chloramphenicol acetyltransferase fusion protein (iso-1/CAT) was expressed in *Saccharomyces cerevisiae* and used to delineate two stages in the cytochrome *c* import pathway in vivo (S. H. Nye and R. C. Scarpulla, Mol. Cell. Biol. 10:5753–5762, 1990 [this issue]). Fusion proteins with the CAT reporter domain in its native conformation were arrested at the initial stage of mitochondrial membrane recognition and insertion. In contrast, those with a deletional disruption of the CAT moiety were relieved of this block and allowed to translocate to the intermembrane space, where they functioned in respiratory electron transfer. In the present study, iso-1/CAT was used to map structural determinants in apoiso-1-cytochrome *c* involved in the initial step of targeting to the mitochondrial membrane. Carboxy-terminal deletions revealed that one of these determinants consisted of the amino-terminal 68 residues. Deletion mutations either within or at the ends of this determinant destroyed mitochondrial targeting activity, suggesting that functionally important information spans the length of this fragment. Disruption of an α-helix near the amino terminus by a helix-breaking proline substitution for leucine 14 also eliminated the targeting activity of the 1 to 68 determinant, suggesting a contribution from this structure. A second, functionally independent targeting determinant was found in the carboxy half of the apoprotein between residues 68 and 85. This determinant coincided with a stretch of 11 residues that are invariant in nearly 100 eucaryotic cytochromes *c*. Therefore, in lieu of an amino-terminal presequence, apocytochrome *c* has redundant structural information located in both the amino and carboxy halves of the molecule that can function independently to specify mitochondrial targeting and membrane insertion in vivo.

The pathway of cytochrome *c* mitochondrial import, as defined in vitro, is unique among nucleus-encoded mitochondrial proteins (see reference 26 for references). Apocytochrome *c* is synthesized without an amino-terminal signal presequence (22, 25) and localized to the intermembrane space (IMS) in the absence of a membrane potential as an energy source (29). Moreover, in contrast to other IMS proteins which first enter the matrix and are subsequently exported to the IMS (9), cytochrome *c* is transported directly across the outer mitochondrial membrane (26). The import pathway is thought to involve the partial insertion of the apoprotein into the outer membrane, its specific interaction with the cytochrome *c* heme lyase, and its subsequent translocation to the IMS coupled to covalent heme attachment (26). The apocytochrome *c* can be arrested at an intermediate step in the import pathway by inhibiting heme modification (11). This intermediate is thought to be bound to the mitochondria in a membrane-spanning configuration accessible to the cytochrome *c* heme lyase (26). The specific determinants on the apoprotein required for mitochondrial membrane recognition and insertion have not been identified.

The fusion protein expression system described in the accompanying paper (19) was used to establish that the in vivo pathway for cytochrome *c* import closely resembles the pathway defined in vitro. This system consisted of chloramphenicol acetyltransferase (CAT) or copper metallothionein (CuMT) reporter proteins fused to the carboxy terminus of apoiso-1-cytochrome *c* (iso-1/CAT and iso-1/CuMT, respectively) and expressed in *Saccharomyces cerevisiae*. By altering the folded conformation of the reporter protein domain, it was possible to distinguish molecules arrested at the intermediate stage of mitochondrial membrane recognition and insertion from those translocated to the IMS and functional in respiratory electron transfer. Iso-1/CAT was targeted to the mitochondria and accessible to heme modification but remained membrane associated and therefore unable to support respiratory growth. In contrast, iso-1/CuMT was efficiently heme modified and remarkably effective in supporting respiratory growth in a strain deleted of the endogenous iso-1-cytochrome *c* gene. However, inclusion of copper in the growth medium, which converted the loosely structured apo-CuMT to the tightly folded holo-CuMT, specifically inhibited both heme attachment and respiratory function. Conversely, a deletion within the CAT domain of iso-1/CAT, which disrupted CAT activity, relieved the block in translocation and resulted in the ability of the fusion protein to accumulate in the IMS and function in respiratory electron transfer. Therefore, fusion proteins with a tightly folded reporter protein moiety were arrested as intermediates in the cytochrome *c* import pathway in that they were targeted to the mitochondria in a membrane-spanning configuration and accessible to the heme lyase. Unfolding of the reporter protein allowed their translocation to the site of cytochrome *c* function in the IMS and their catalytic interaction with the respiratory apparatus.

In the present study, a systematic mutational analysis of the iso-1-cytochrome *c* domain in iso-1/CAT was undertaken in order to map structural determinants involved in mitochondrial membrane recognition and insertion in vivo. Iso-1/CAT offers several advantages for mapping import determinants. First, the fusion protein is stably expressed in contrast to apoiso-1-cytochrome *c*, which turns over rapidly

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TABLE 1. Fusion proteins derived from M13 templates and complementary oligonucleotides

Fusion protein	Template	Oligonucleotide
Δ69-108[Pro-14]	Δ69-108	5′-GTGCTACACCATTTAAAACTAGATGT-3′
Δ34-43/Δ69-108	Δ69-108	5′-CCACATAAGGTTCACTCTGGTCAA-3′
Δ69-108[Pro-38]	Δ69-108	5′-GTCCAAACTTGCCAGGTATCTTTG-3′
Δ52-68	Iso-1/CAT	5′-GCTGAAGGGTATATGTCAGAGTAC-3′
Pro-14	Iso-1/CAT	5′-GTGCTACACCATTTAAAACTAGATGT-3′
Δ52-68/Δ96-108	Δ52-68	5′-GACAGAAACGACTCCATCGATGGA-3′
Δ52-68/Δ86-108	Δ52-68	5′-GGTACCAAGATGTCCATCGATGGA-3′
Δ52-68/Δ75-108	Δ52-68	5′-GAGTACTTGACTTCCATCGATGGA-3′
Δ1-67	Iso-1/CAT	5′-CCGAATTTAAAATGAACATGTCAGAGTA-3′
Δ1-74	Iso-1/CAT	5′-CCGAATTTAAAATGAACCCAAAGAAATAT-3′
Δ1-84	Iso-1/CAT	5′-CCGAATTTAAAATGGCCTTTGGTGGGTT-3′
Δ1-67/Δ86-108	Δ1-67	5′-GGTACCAAGATGTCCATCGATGGA-3′

and does not accumulate in the cytosol (6). Second, the CAT moiety serves as an easily detected reporter molecule for assaying the targeting activity of even very small segments of the apoprotein. Third, the folded conformation of the CAT domain blocks translocation to the IMS, allowing the elucidation of structural features responsible for mitochondrial membrane recognition and insertion independent from those required for translocation and accumulation. Having established the compatibility of fusion protein structure with both the mitochondrial localization and electron transfer functions of cytochrome *c* in vivo (19), we now use this system to define regions of the apoprotein involved in mitochondrial targeting.

MATERIALS AND METHODS

Strains. *S. cerevisiae* NA1 (*MATa leu2-3 leu2-112 his4-34 ura3-52 URA3 HIS4-lacZ*) (19) was used as the host for the expression and mitochondrial targeting of the fusion proteins. Strain LSR1 (*MATa leu2-3 leu2-112 his4-34 ura3-52 hem1*) (19) is deficient in δ-aminolevulinic acid (δ-ALA) synthase and was used to measure heme modification of the fusion proteins. Strain N1B (*MATa leu2-3 leu2-112 his4-34 ura3-52 cyc1-1*) (23) is deficient in iso-1-cytochrome *c* and was used to determine the biological activity of the fusion proteins. All strains were constructed and transformed as described previously (19, 23).

DNA methods and construction of plasmids. Construction of YEp*CYC1/cat*, for expression of iso-1/CAT (iso-1-cytochrome *c*–chloramphenicol acetyltransferase fusion protein), was described previously (19). Carboxy-terminal deletions within the *CYC1* coding region were constructed by several strategies. First, deletions were introduced by *Bal*31 digestion of the *Eco*RV restriction fragment from the *CYC1* gene (25), followed by *Cla*I linker addition, *Eco*RI plus *Cla*I cleavage, and cloning of the resulting *Eco*RI-*Cla*I coding region fragments. This yielded deletions for the expression of mutant proteins lacking amino acids 103 to 108 (Δ103-108), Δ96-108, Δ84-108, Δ76-108, Δ69-108, Δ44-108, and Δ21-108. Second, the *Eco*RV *CYC1* fragment was digested with restriction enzymes at sites within the coding region. Following *Cla*I linker addition to the Klenow-treated fragments, *Eco*RI-*Cla*I fragments were cloned into the vector for expression of Δ53-108 and Δ36-108 fusion proteins. The *Eco*RI-*Cla*I *CYC1* fragment for expressing Δ10-108 was constructed by using complementary synthetic DNA oligonucleotides:

5′-AATTCAAGGCCGGTTCTGCTAAGAACAT -3′
3′- GTTCCGGCCAAGACGATTCTTGTAGC-5′

These form a 5′ *Eco*RI and 3′ *Cla*I overhang surrounding the initial *CYC1* coding region sequences. As a result of *Cla*I linker addition, the first residue of the 18-amino-acid bridge region separating the iso-1-cytochrome *c* and CAT domains (19) differs among the carboxy-terminal deletions from the S in iso-1/CAT as follows: P, Δ103-108; N, Δ84-108; P, Δ76-108; I, Δ69-108; H, Δ44-108; P, Δ36-108; H, Δ21-108; and N, Δ10-108.

Genes for the expression of fusion proteins with single amino acid substitutions or deletions within the *CYC1* coding region were constructed by site-directed oligonucleotide mutagenesis (13). The M13 templates and complementary oligonucleotides for the construction of each fusion protein are shown in Table 1. The fusion gene for the expression of Δ4-17 was obtained by isolating an *Mae*I fragment that encodes cytochrome *c* minus codons for the first 17 residues and one base of codon 18. Following S1 nuclease digestion, an *Eco*RI linker was added to recreate codons for amino acid residues 2, 3, and 18, and the *Eco*RI-*Cla*I fragment was cloned into the expression cassette. The genes for Δ4-17, Δ69-108, and Δ69-108[$Ser^{19,22}$] (serine substitutions at positions 19 and 22) were constructed by combining the *Dra*III-*Sph*I fragment that encompasses Δ69-108 with either the *Bam*HI-*Dra*III fragment containing the 4 to 17 deletion or the serine substitutions at residues 19 and 22 (19).

YEp*CYC1*, YEp*CYC1/CUP1*, and YEp*CYC1/cat*Δ for the expression of iso-1-cytochrome *c*, iso-1-cytochrome *c*–copper metallothionein (iso-1/CuMT), and iso-1/CATΔ, respectively, were constructed as described previously (19). All *CYC1-cat* junctions and site-directed modifications were verified by dideoxy sequence analysis (21).

Mitochondrial isolation. Mitochondria were isolated from transformant strains grown at 30°C in DL-lactate-containing medium (23) by glusulase treatment of cells, Dounce homogenization, and differential centrifugation as described previously (19). Specific targeting of the fusion proteins was assayed with trypsin (100 μg/ml, 30°C) treatment of mitochondrial fractions (2.0 mg/ml). Reactions were terminated with 1 mM phenylmethylsulfonyl fluoride plus loading dye, boiled for 3 min, and electrophoresed on 15% polyacrylamide–sodium dodecyl sulfate (SDS) gels, and the proteins were detected by immunoblotting with a mixture of antibodies to yeast cytochrome *c* and bacterial CAT as described previously (19, 23).

[^{14}C]δ-ALA labeling and immunoprecipitation. Total SDS-dissociated and boiled extracts were prepared from 5.0-ml LSR1 or N1B transformant cultures grown at 30°C in DL-lactate-containing medium supplemented with 40 μM [^{14}C]δ-ALA (Amersham) as described previously (19). Approxi-

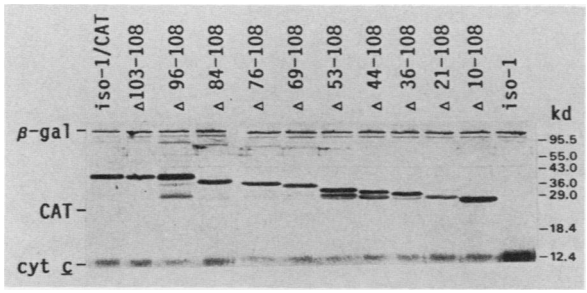

FIG. 1. Immunoblot detection of iso-1/CAT fusion proteins in total protein extracts. SDS-dissociated and boiled extract (100 μg) prepared from NA1 transformant strains grown in DL-lactate medium were separated on 15% polyacrylamide–SDS gels, transferred to nitrocellulose, and immunoblotted with a mixture of antibodies raised to yeast cytochrome *c*, bacterial CAT, and β-galactosidase as described in Materials and Methods. The fusion protein expressed in each transformant, specified as either iso-1/CAT or the indicated deletion (Δ) of amino acid residues within the iso-1 domain of iso-1/CAT, is shown above each lane. The last lane contains extract from a transformant overexpressing iso-1-cytochrome *c*. Yeast cytochrome *c* (cyt *c*) CAT, and β-galactosidase (β-gal) mark the positions of these proteins at the left, while molecular mass markers are listed on the right (in kilodaltons [kd]).

mately 500 μg of each extract was immunoprecipitated at 4°C overnight with antibodies to yeast cytochrome *c* and bacterial CAT, and the immune complexes were collected with 150 μl of protein A-agarose (Boehringer Mannheim Biochemicals). Washed precipitates were boiled in loading dye and electrophoresed on 15% polyacrylamide–SDS gels, and the labeled proteins were visualized by fluorography with 14% salicylic acid.

Miscellaneous. Expression of each fusion protein was screened for growth on medium containing chloramphenicol (1,500 μg/ml) (3) to confirm that the hybrid genes were in frame and by immunoblot analysis of total protein extracts as described previously (19). Protein determinations were done by the method of Lowry et al. (15), and densitometric scanning was accomplished with a Zeinah soft laser densitometer combined with a Hewlett-Packard 3390 integrator.

RESULTS

Expression and mitochondrial targeting of a series of iso-1/CAT fusion proteins with carboxy-terminal deletions in the apoiso-1 moiety. To demonstrate the utility of iso-1/CAT for mapping determinants involved in mitochondrial targeting, a series of fusion proteins having carboxy-terminal deletions within the iso-1 moiety were expressed in yeast cells. The expression of molecules ranging from the full-length iso-1/CAT to a fusion protein having only nine amino-terminal residues of apocytochrome *c* (Δ10-108) was monitored by immunoblotting of SDS-dissociated total protein extracts from transformant strains (Fig. 1). Fusion proteins were compared with the endogenous mitochondrial cytochrome *c* and with cytosolic β-galactosidase expressed from an integrated *HIS4-lacZ* fusion gene in the host strain (19). The results demonstrated that fusion proteins of the expected sizes ranging from 39.6 to 28.2 kDa were stably expressed (Fig. 1).

Iso-1/CAT was previously found to be associated with the mitochondrial fraction and resistant to external trypsin treatment (19). To determine which of the fusion proteins with carboxy-terminal deletions in the iso-1 domain were targeted to the mitochondria, crude mitochondrial fractions were

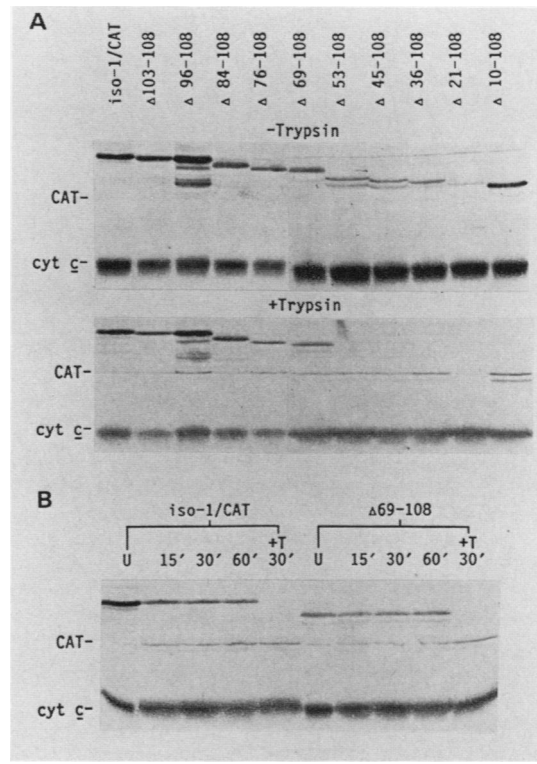

FIG. 2. Mitochondrial targeting of a series of iso-1/CAT fusion proteins with carboxy-terminal deletions in the apoiso-1 moiety. (A) Crude mitochondria (150 μg) isolated from NA1 transformant strains expressing iso-1/CAT or the indicated carboxy-terminal deletion within the iso-1 domain were either untreated (−Trypsin) or digested with trypsin (100 μg/ml) (+Trypsin) for 15 min at 30°C. Proteins were detected by immunoblotting as described in the legend to Fig. 1 with a mixture of antibodies to yeasts cytochrome *c* and bacterial CAT. (B) Crude mitochondrial fractions isolated from transformant strains expressing iso-1/CAT or the Δ69-108 deletion were untreated (U) or treated for the indicated times (in minutes) with trypsin (100 μg/ml) and analyzed by immunoblotting as above. +T, Mitochondria were treated with Triton X-100 prior to trypsin digestion. For each panel, the positions of CAT and yeast cytochrome *c* (cyt *c*) are shown at the left.

prepared from each transformant and treated with trypsin. In the absence of trypsin, each fusion protein was detected at the expected size in mitochondrial fractions (Fig. 2A). However, upon trypsin treatment of the same mitochondrial fractions for 15 min, only fusion proteins having at least 68 amino-terminal amino acid residues (Δ69-108) remained protease resistant, while those with 52 (Δ53-108) or fewer were degraded to a CAT-sized protein (Fig. 2A).

To determine the relative trypsin sensitivity of these proteins, mitochondria from a transformant expressing iso-1/CAT were compared with those expressing the Δ69-108 mutation over a time course of trypsin treatment (Fig. 2B). Iso-1/CAT and Δ69-108 were identical in their stability to trypsin digestion for at least 60 min. Pretreatment of mitochondria with Triton X-100 exposed both iso-1/CAT and Δ69-108 to proteolysis, demonstrating that an intact membrane structure was required for their resistance to trypsin (Fig. 2B, +T). Therefore, a fragment containing the amino-terminal 68 residues appears to be identical to the intact apoiso-1-cytochrome *c* in its ability to target the fusion proteins to the mitochondrial membrane in vivo.

To determine whether molecules associated with the mito-

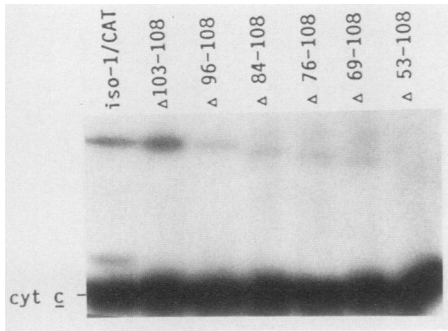

FIG. 3. Heme labeling of iso-1/CAT and carboxy-terminal deletions. Extracts prepared from LSR1 transformants expressing iso-1/CAT or the indicated deletion mutations (Δ103-108 to Δ53-108) were labeled with [^{14}C]δ-ALA and immunoprecipitated with a mixture of antibodies to yeast cytochrome *c* and bacterial CAT. The precipitates were collected with 150 μl of protein A-agarose and electrophoresed on a 15% polyacrylamide–SDS gel, and the labeled proteins were detected by fluorography. The autoradiograph was overexposed for 3 months at −80°C in order to detect the weakly labeled proteins. The position of the endogenous cytochrome *c* (cyt *c*) is shown at the left.

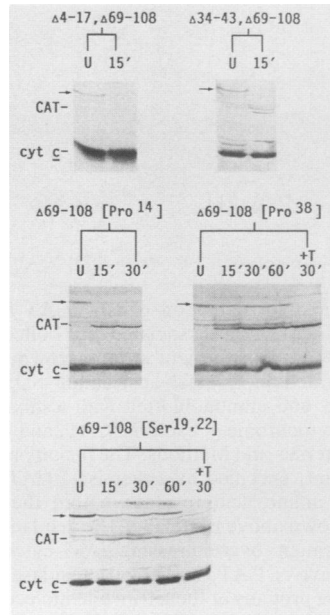

FIG. 4. Mitochondrial targeting of Δ69-108 fusion proteins containing mutations within the 1 to 68 targeting determinant. Mitochondria from NA1 transformant strains expressing fusion proteins with the indicated deletions (Δ4-17/Δ69-108 and Δ34-43/Δ69-108) or single amino acid changes (Δ69-108[Pro-14], Δ69-108[Pro-38], and Δ69-108[Ser^{19,22}]) were untreated (U) or treated with trypsin for the indicated times (in minutes), electrophoresed, and immunoblotted as described in the legend to Fig. 2. The positions of the fusion proteins (arrows), CAT, and yeast cytochrome *c* (cyt *c*) are shown at the left.

chondrial fraction were accessible to the cytochrome *c* heme lyase, the fusion proteins were expressed in a *hem1* strain deficient in δ-ALA synthase, the first enzyme in the heme biosynthetic pathway (27). This strain is unable to synthesize heme in the absence of exogenous δ-ALA (28). The transformants were grown in DL-lactate-containing medium supplemented with [^{14}C]δ-ALA, and the heme-modified proteins were visualized by autoradiography following immunoprecipitation and gel electrophoresis (Fig. 3). Fusion proteins having 68 or more amino-terminal residues (iso-1/CAT to Δ69-108) were radiolabeled, whereas one having 52 (Δ53-108) remained unlabeled. The shorter trypsin-resistant molecules were heme modified to a lesser extent, perhaps reflecting the degree to which they span the membrane to interact with the heme lyase or their ability to form a heme-binding pocket. Nevertheless, the accessibility of the fusion proteins to heme modification correlated with their protease-resistant association with the mitochondria.

Amino-terminal 68 residues of apoiso-1-cytochrome *c* constitute a distinct targeting determinant. The above deletion series demonstrated that the carboxy-terminal boundary of a larger determinant resided in the interval between residues 52 and 68 (Fig. 2A). To map the amino-terminal boundary of this determinant, residues 4 to 17 were deleted from the fusion protein containing the Δ69-108 mutation, and the resulting molecule (Δ4-17/Δ69-108) was tested for targeting activity. Activity was completely lost by removal of these amino-terminal residues in the Δ69-108 background (Fig. 4; Δ4-17/Δ69-108). These results indicated that the amino-terminal boundary of the 1 to 68 determinant may reside between amino acids 4 and 17. Removal of an internal region from residues 34 to 43 of the 1 to 68 determinant also destroyed mitochondrial targeting, suggesting that the targeting information spanned the entire fragment and that the ends alone were not sufficient for activity (Fig. 4; Δ34-43/Δ69-108).

One structural feature common to eucaryotic cytochromes *c* is an α-helical region near the amino terminus (14). Helical conformations are found in the signal prepeptides of other mitochondrial proteins (5). Although the carboxy-terminal deletion series demonstrated that the amino-terminal 52 residues of apocytochrome *c* were not sufficient for targeting of the fusion protein, residues 4 to 17 were clearly required for the activity of the 1 to 68 determinant. To evaluate the potential contribution of the amino-terminal α-helical region, the leucine at position 14 was converted to a helix-breaking proline by site-directed mutagenesis. A proline at this position has been reported to abolish cytochrome *c* function (8). This point mutation completely eliminated the targeting activity of the 1 to 68 determinant (Fig. 4; Δ69-108[Pro-14]). When histidine was replaced with proline at position 38, within a non-α-helical region, the 1 to 68 determinant retained full targeting activity (Fig. 4; Δ69-108[Pro-38]) even though, like Pro-14, Pro-38 is known to abolish cytochrome *c* function (8). In addition, as previously shown for the full-length iso-1/CAT (19), the heme-binding cysteines, which are essential for catalytic activity, were not required for targeting of the 1 to 68 determinant (Fig. 4; Δ69-108[Ser^{19,22}]). Therefore, the strong α-helical structure at the amino terminus is necessary but not sufficient for the targeting activity of the 1 to 68 determinant. Moreover, although this determinant is accessible to the heme lyase, heme attachment is not required for its specific targeting in vivo.

Second, functionally independent targeting determinant coincides with a sequence that is invariant in nearly 100 eucaryotic cytochromes *c*. The above experiments established that 68 amino-terminal residues of apoiso-1-cytochrome *c* are sufficient for mitochondrial targeting. Three deletions (Δ53-108, Δ4-17/Δ69-108, and Δ34-43/Δ69-108) and one point mutation (Δ69-108[Pro-14]) within this determinant completely destroyed its activity. To examine whether the 1 to 68 determinant was essential within the context of the intact apoprotein, the Δ52-68, Δ4-17, and Pro-14 mutations were individually introduced into the full-length iso-1 moiety of

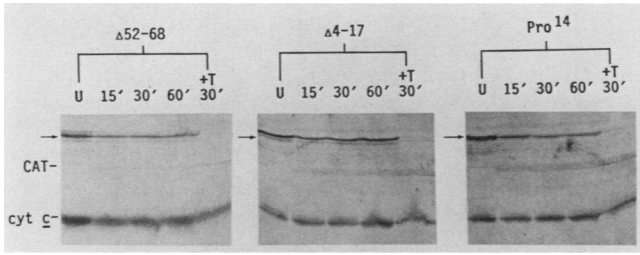

FIG. 5. Mitochondrial targeting of iso-1/CAT fusion proteins containing mutations that disrupt the 1 to 68 targeting determinant. Mitochondria isolated from NA1 transformant strains expressing iso-1/CAT and containing either the Δ52-68, Δ4-17, or Pro-14 mutation were untreated (U) or treated with trypsin for the indicated times (in minutes) and analyzed by immunoblotting as described in the legend to Fig. 2. The positions of the fusion proteins (arrows), CAT, and yeast cytochrome *c* (cyt *c*) are shown at the left.

iso-1/CAT, and the resulting fusion proteins were tested for mitochondrial targeting. Surprisingly, all of these mutated molecules were identical to iso-1/CAT in their trypsin-resistant association with mitochondria (Fig. 5). The inability of these mutations to disrupt targeting function within the context of the full-length fusion protein suggested that redundant targeting information may reside in the carboxy half of the apoprotein.

To define this targeting signal, a series of carboxy-terminal deletions were constructed from the fusion protein with the 52 to 68 internal deletion (Δ52-68) as the parent molecule. This molecule should be defective in the 1 to 68 determinant and thus allow unmasking of redundant signals in the carboxy half. A carboxy-terminal deletion to residue 99 (Δ52-68/Δ99-108) had no effect on targeting activity, while deletion to residue 86 (Δ52-68/Δ86-108) only partially diminished activity (Fig. 6). However, continued deletion to residue 76 (Δ52-68/Δ75-108) severely impaired mitochondrial targeting and rendered any bound material susceptible to trypsin digestion. These results are consistent with the presence of additional targeting information between residues 69 and 85.

Having mapped this second targeting determinant, it was of interest to see whether this region of the apoprotein could function independently. Thus, an amino-terminal deletion of the first 67 residues (Δ1-67) was constructed and expressed in yeast cells. Despite the absence of residues 1 to 67, including the heme-binding cysteines, this molecule had full

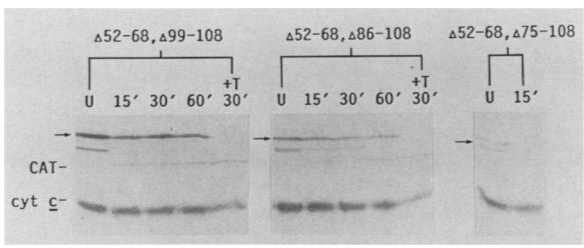

FIG. 6. Identification of a second targeting determinant by using a series of fusion proteins with carboxy-terminal deletions constructed within the Δ52-68 background. Mitochondria were isolated from NA1 transformant strains expressing the indicated CAT fusion proteins containing carboxy-terminal deletions combined with the internal Δ52-68 deletion (Δ52-68/Δ99-108, Δ52-68/Δ86-108, or Δ52-68/Δ75-108). These fractions were untreated (U) or digested with trypsin for the indicated times (in minutes) and analyzed by immunoblotting as described in the legend to Fig. 2. The positions of the fusion proteins (arrows), bacterial CAT, and yeast cytochrome *c* (cyt *c*) are shown at the left.

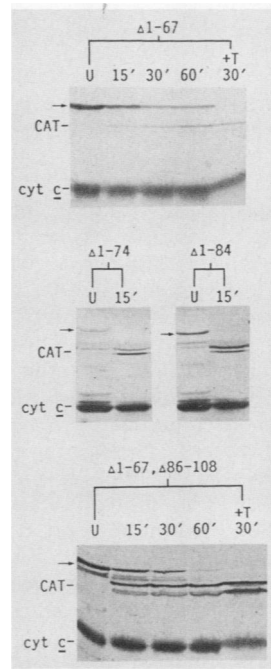

FIG. 7. Independent targeting function mediated by residues 68 to 85. Mitochondria from NA1 transformant strains expressing fusion proteins with the indicated deletions within the iso-1 domain (Δ1-67, Δ1-74, Δ1-84, and Δ1-67/Δ86-108) were untreated (U) or digested with trypsin for the indicated times (in minutes) and analyzed by immunoblotting as described in the legend to Fig. 2. Each immunoblot was treated with antibodies to yeast cytochrome *c* and CAT as described in the legend to Fig. 2. The positions of the fusion proteins (arrows), bacterial CAT, and yeast cytochrome *c* (cyt *c*) are shown at the left of each panel.

targeting activity (Fig. 7, Δ1-67). However, further deletion to residue 74 (Δ1-74) or 84 (Δ1-84) completely abolished this activity, indicating that the amino-terminal boundary of this second determinant resided between residues 68 and 75. To further test the independent targeting function of this region, a deletion to residue 86 was constructed in the Δ1-67 background (Δ1-67/Δ86-108). This molecule was sensitive to trypsin only after 60 min of digestion, indicating that it retained partial targeting activity. Therefore, the 18 residues between 68 and 85 can independently localize the CAT fusion protein to the mitochondrial membrane in vivo. Interestingly, this region contains the 11-amino-acid sequence between residues 75 and 85 (mammalian residues 70 to 80) that is invariant among the sequences of nearly 100 eucaryotic cytochromes *c* (8).

DISCUSSION

Mitochondrial biogenesis depends to a large extent upon targeting the protein products of nuclear genes to the correct submitochondrial compartment. For most of these proteins, the structural information responsible for the specificity of this process resides in an amino-terminal presequence that is proteolytically removed upon import (5). Cytochrome *c* lacks such a cleavable signal peptide and must therefore carry its targeting information within the structure of the apoprotein. In the present work, an in vivo fusion protein expression system was used to identify the mitochondrial targeting determinants of apoiso-1-cytochrome *c*. One important feature of this system is that, by using a reporter

protein with a folded conformation, the import of fusion proteins was arrested at the intermediate stage of membrane recognition and insertion (19). This feature allowed the identification of targeting determinants independent from those structures required for translocation to the IMS. A second unique feature of this system is that stable fusion proteins must participate in intracellular trafficking in vivo to arrive at their mitochondrial location. Thus, any auxiliary components such as cofactors, chaperonin activities, or receptors would be available for correct targeting.

Studies with in vitro systems have been inconclusive in defining regions of the apoprotein involved in mitochondrial uptake. A mixture of horse apocytochrome *c* fragments containing residues 1 to 65 (6 to 70 in the yeast numbering system) and 1 to 80 (6 to 85 in the yeast system) was unable to inhibit the uptake of the intact apoprotein into mitochondria in vitro (17). Under the same conditions, a fragment consisting of residues 66 to 104 (71 to 103 in the yeast system) was inhibitory to apoprotein uptake, but those with residues 81 to 104 (86 to 103 in the yeast system) or 66 to 80 (71 to 85 in the yeast system) were not. Similarly, carboxy-terminal segments of the apoprotein were protected from proteolysis upon insertion into artificial membrane vesicles (7). However, in contrast to these results, amino-terminal fragments 1 to 38, 1 to 59, and 1 to 65 (6 to 43, 6 to 64, and 6 to 70 in the yeast system, respectively) of apocytochrome *c* were found to bind more strongly to lipid vesicles than carboxy-terminal fragments, which generally exhibited weaker nonsaturable binding (12). Moreover, the same amino-terminal fragments were able to penetrate a lipid bilayer under conditions in which carboxy-terminal fragments could not. The differences in these various in vitro systems make it difficult to evaluate which results are biologically relevant to cytochrome *c* import.

Our previous work established that expression of rat cytochrome *c* in yeast cells could efficiently complement a deletion of the *CYC1* gene encoding apoiso-1-cytochrome *c* (23). This result suggested that highly conserved structures and/or sequences were involved not only in catalytic function but also in the subcellular localization of cytochrome *c*. Here, by systematically mutating the iso-1 moiety of iso-1/CAT, segments of the apoprotein containing targeting information were identified in vivo by their ability to specify the trypsin-resistant association of the fusion protein with the mitochondria. As shown previously (19), the stable association of the fusion protein with mitochondria in this assay depended upon the protection of the iso-1 moiety by the mitochondrial membrane.

Figure 8 shows a schematic comparison of the structures of fusion proteins used in this study with respect to both highly conserved and α-helical regions of the intact apoprotein and summarizes their targeting activity. These data led to the identification of two functionally independent targeting determinants, one between residues 1 and 68 and a second between residues 68 and 85. The ability to define the latter determinant to within 18 amino acid residues indicates that the system has sufficient resolution to detect very short stretches of targeting information. In addition, although there was some variation in the steady-state levels of the various fusion proteins, those that were not specifically targeted were expressed at levels similar to targeted molecules and were easily detected in untreated control lanes. Therefore, the inability of a given molecule to achieve a trypsin-resistant association with mitochondria can be largely attributed to the removal or disruption of targeting information.

The 1 to 68 determinant was first elucidated by a series of deletions from the carboxy terminus. Further deletions at either end or the middle resulted in a complete loss of targeting function, suggesting that the active region spanned the entire segment. This contrasts with in vitro results with lipid vesicles, suggesting that a horse apoprotein segment containing residues 1 to 38 (6 to 43 in the yeast system) was active in both membrane binding and penetration of a lipid bilayer (12). A more recent in vitro study points to a requirement for the first 58 residues of *Drosophila melanogaster* apocytochrome *c* for uptake into mouse liver mitochondria and confirms that the heme-binding cysteines are unnecessary for mitochondrial association (25a). The central region of the 1 to 68 determinant is predicted to have a nonhelical conformation and encompasses three phylogenetically conserved blocks (I, II, and III) of amino acid sequence (Fig. 8). Although all deletions that removed one or more of these blocks (Δ53-108, Δ34-43/Δ69-108, Δ52-68/Δ75-108, and Δ1-67) destroyed the targeting activity of this determinant, these conserved regions alone do not constitute the targeting signal. This central portion of the 1 to 68 determinant is flanked by amino- and carboxy-terminal α-helices. The latter appears to be unimportant because several molecules that had most of the carboxy α-helix removed (Fig. 8; Δ69-108, Δ69-108[Pro-38], and Δ69-108[$Ser^{19,22}$]) remained active. In addition, the heme-binding cysteines were not required because their conversion to serines did not abolish targeting. The amino-terminal α-helix is highly conserved in structure but not in sequence. A deletion within this structure (Fig. 8, Δ4-17/Δ69-108) and a helix-breaking Leu to Pro substitution at residue 14 (Δ69-108[Pro-14]) were equally effective in destroying targeting of the 1 to 68 determinant. This illustrates that single amino acid changes will be useful for defining specific structural motifs within a targeting determinant. Therefore, the amino-terminal helix appears to be necessary but not sufficient for the targeting function of this determinant. It is of interest in this context that a deletion of nine amino-terminal residues in iso-1-cytochrome *c* resulted in a reduced amount of holoprotein (24).

The amino-terminal 68 residues were not alone in specifying the targeting activity of the intact apoprotein. The fact that mutations that eliminated the activity of this determinant had no effect when introduced into the full-length iso-1/CAT fusion protein (Fig. 8, Δ52-68, Δ4-17, and Pro-14) suggested the presence of redundant targeting information in the carboxy end of the apoprotein. Furthermore, the efficient targeting of a fusion protein with an amino-terminal deletion to residue 67 (Δ1-67) demonstrated that this information could function independently. This region corresponds to that previously observed to compete for apoprotein uptake in vitro (17). Unlike the 1 to 68 determinant, the activity of the carboxy region could be further defined by deletion mapping to a short stretch of 18 residues (Fig. 8, Δ52-68, Δ86-108, and Δ1-67/Δ86-108) that retained at least partial function. Within this region is an 11-amino-acid sequence (residues 75 to 85) (the function of which is still uncertain) that is invariant among nearly 100 eucaryotic cytochromes *c*. However, residues 75 to 85 appeared to be insufficient for activity because an amino-terminal deletion to residue 74 (Δ1-74) was completely inactive.

In keeping with these results, recently constructed Tyr-72 to Pro or Pro-76 to Leu mutations completely abolished targeting in the Δ1-67 background but not in the full-length fusion protein (Nye and Scarpulla, unpublished). Tyr-72 lies within an α-helical region spanning residues 65 to 75, while

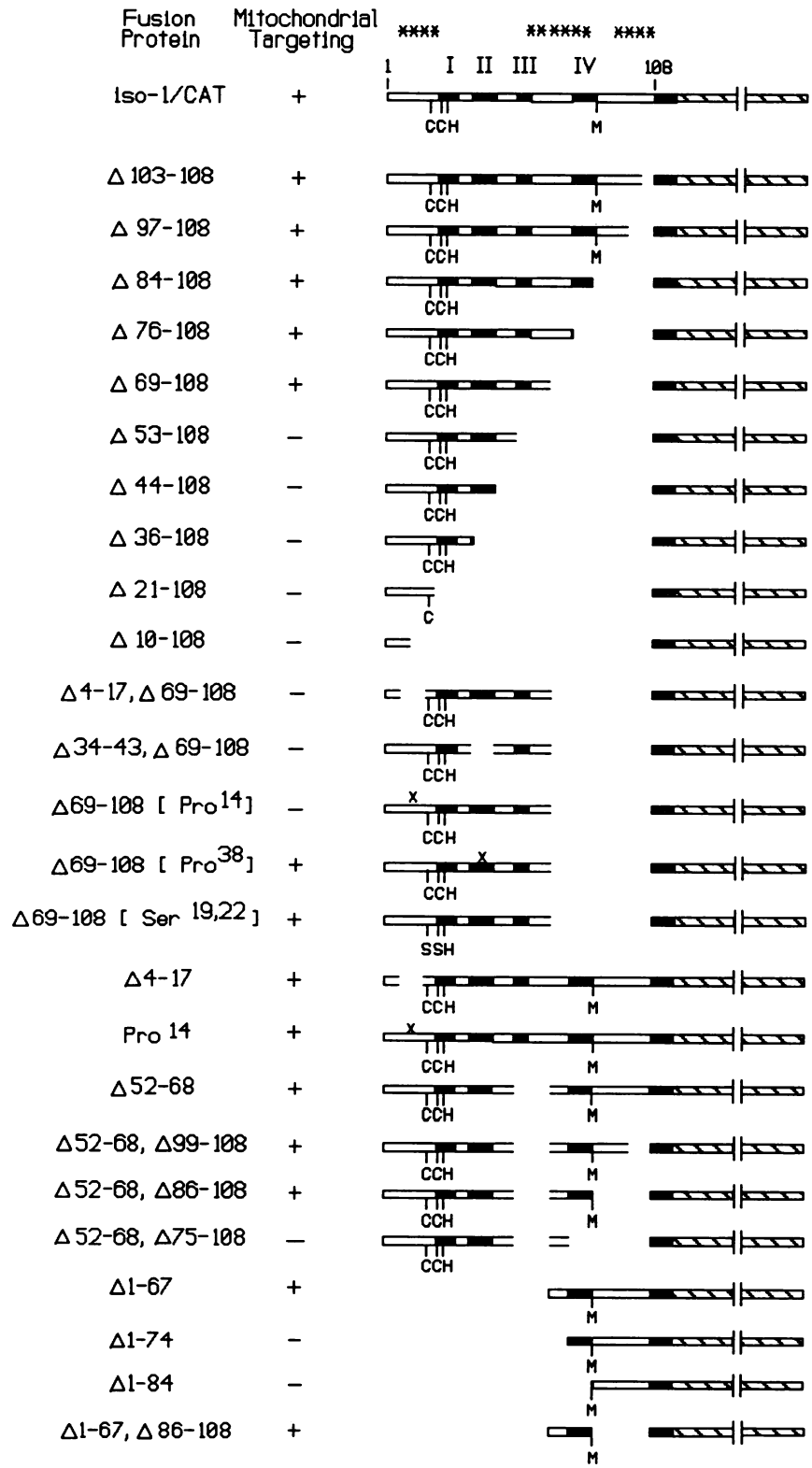

FIG. 8. Summary of the structures and mitochondrial targeting activities of mutationally altered iso-1/CAT fusion proteins. The full-length iso-1/CAT fusion protein depicted at the top shows iso-1-cytochrome *c* residues 1 to 108 fused to bacterial CAT (hatched boxes) through an 18-amino-acid bridge sequence (stippled boxes) (19). Also indicated are sequence blocks conserved in eucaryotic cytochromes *c* (solid boxes labeled with Roman numerals above), predicted α-helical regions (asterisks) (4, 12, 14), and the positions of heme-binding cysteines 19 and 22 (C), histidine 23 (H), and methionine 85 (M). Deletions and amino acid replacements (denoted by gaps and by X, respectively) in the iso-1 domain are diagrammed, with their name and mitochondrial targeting activity shown to the left.

Pro-76 is one of the 11 consecutive invariant residues (8). The overlap of targeting information with this highly invariant sequence suggests that the 68 to 85 determinant may confer specific recognition through a receptor or binding activity. For example, the conserved sequence may be recognized by the heme lyase through a site different from that used for heme attachment. Alternatively, other receptors and binding proteins have been postulated to participate in cytochrome *c* import (10, 18). If such proteins do exist, they may require a conserved sequence within the apoprotein for recognition specificity.

In conclusion, the mitochondrial localization of iso-1/CAT fusion proteins is mediated through information found in both the amino and carboxy halves of apoiso-1-cytochrome *c*. These function independently and thus represent redundant information for fusion protein targeting. Redundant import determinants have also been observed in other nucleus-encoded mitochondrial proteins. For the F_1-ATPase β-subunit, the precursor containing the amino-terminal presequence, but not the mature form, was competent for import in vitro (16). However, the presequence was found to be dispensable for import in vivo because of redundant information elsewhere in the amino terminus of the mature protein (1). Also, 111 amino-terminal residues of the yeast ADP/ATP carrier protein could direct dihydrofolate reductase to an intermediate import stage (2), while truncation of similar residues in the *Neurospora crassa* ADP/ATP carrier did not disrupt import (20). It is important to emphasize that while the signals identified here for apocytochrome *c* are indistinguishable in fusion protein targeting, they may play very different roles in apoprotein uptake. For example, the 1 to 68 determinant may insert into the lipid bilayer and make the cysteines accessible to the heme lyase, while the 68 to 85 determinant may be involved in the specific recognition of a receptor or binding protein. The utility of fusion proteins for studying these determinants independent from each other and from other steps in the import pathway should contribute to the understanding of the structural requirements for apocytochrome *c* import.

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