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Removal of a Hydrophobic Domain within the Mature Portion of a Mitochondrial Inner Membrane Protein Causes Its Mislocalization to the Matrix

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We have examined the import and intramitochondrial localization of the precursor to yeast cytochrome *c* oxidase subunit Va, a protein of the mitochondrial inner membrane. The results of studies on the import of subunit Va derivatives carrying altered presequences suggest that the uptake of this protein is highly efficient. We found that a presequence of only 5 amino acids (Met-Leu-Ser-Leu-Arg) could direct the import and localization of subunit Va with wild-type efficiency, as judged by several different assays. We also found that subunit Va could be effectively targeted to the mitochondrial inner membrane with a heterologous presequence that failed to direct import of its cognate protein. The results presented here confirmed those of an earlier study and showed clearly that the information required to "sort" subunit Va to the inner membrane resides in the mature protein sequence, not within the presequence per se. We present additional evidence that the aforementioned sorting information is contained, at least in part, in a hydrophobic stretch of 22 amino acids residing within the C-terminal third of the protein. Removal of this domain caused subunit Va to be mislocalized to the mitochondrial matrix.

Most of the proteins that constitute a functional mitochondrion are encoded on nuclear DNA and synthesized on cytoplasmic ribosomes. They must then be directed to the organelle, imported, and sorted to one of four intramitochondrial compartments: the matrix, inner membrane, intermembrane space, or outer membrane. In recent years, much has been learned about protein import into mitochondria (for recent reviews see references 1, 19, 42, and 51). Proteins destined for this organelle usually carry an N-terminal extension (called a presequence or leader peptide) that is both necessary and sufficient for import (15, 24–26, 28). Import is also dependent upon surface components or receptors (34, 35, 37, 38, 41, 42, 53), two forms of energy (the hydrolysis of ATP and the electrical potential across the inner membrane [$\Delta\psi$]) (6, 14, 39, 40, 47), and soluble proteins located both inside (7, 36) and outside (13, 32) the mitochondrion. These polypeptides, which have been shown to be the products of a family of stress-induced genes, are thought to be "molecular chaperones" that maintain precursors in import or assembly-competent conformations (19, 51). Finally, precursor proteins are proteolytically processed by a metallopeptidase residing in the mitochondrial matrix (3, 4, 22, 30).

One aspect of mitochondrial import that is currently of wide interest concerns the mechanisms by which precursors are localized, or "sorted," within the organelle and where within the precursor the information necessary for sorting resides. It has been shown that several proteins located in the intermembrane space (or on the intermembrane face of the inner membrane) carry bipartite presequences (18, 20, 50) and are routed to their final destination via the matrix. Specifically, these proteins are first translocated to the matrix, where a portion of the presequence is cleaved. This initial cleavage is thought to expose a second targeting signal, which then redirects the protein back across the inner membrane (18, 20). The remainder of the presequence is then removed by an as yet uncharacterized protease(s) located in the intermembrane space (18, 20, 50).

While some progress has also been made towards understanding how proteins are sorted to the mitochondrial outer membrane (19, 21, 33) and matrix (19, 25, 26, 50), the delivery of proteins to the mitochondrial inner membrane is not well understood. For example, it is not clear whether inner membrane proteins carrying N-terminal presequences are routed via the matrix, in a manner similar to proteins of the intermembrane space (above), or whether they are translocated to the inner membrane directly (as is the case for the ATP/ADP carrier [41]). Moreover, many inner membrane proteins are subunits of hetero-oligomeric protein complexes. Therefore, it is possible that other subunits of the complex, prosthetic groups, and the actual assembly of the oligomer facilitate inner membrane localization.

Workers in our laboratory have been studying the import of a well-characterized mitochondrial inner membrane protein, subunit Va of yeast cytochrome *c* oxidase (9–11). In an earlier study, we demonstrated that heterologous presequences (or portions thereof) derived from proteins located in other mitochondrial compartments could efficiently deliver subunit Va to the inner membrane (17). The results of that study also uncovered preliminary evidence that the information which directed subunit Va to the inner membrane was not part of the presequence (17).

In this article, we report several new findings on the import of cytochrome *c* oxidase subunit Va. Results of in vivo experiments show that this protein can be efficiently delivered to the inner membrane with minimal targeting sequences, including one of only five amino acids. We also show that a mutant presequence which fails to direct import and localization of its cognate protein (human ornithine transcarbamylase [OTC]) can target subunit Va with nearly wild-type efficiency. Together, our combined in vivo results support our earlier speculation and conclusively demonstrate that the information necessary to sort subunit Va to

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the inner membrane is contained within the mature protein. Using an in vitro approach, we demonstrate that this sorting information resides, at least in part, within a hydrophobic stretch of amino acids at the C-terminal third of the protein.

MATERIALS AND METHODS

Strains and growth media. The *Saccharomyces cerevisiae* wild-type strains used in this study were D273-10B (ATCC 24657) and JM43 (*MATα leu2-3 leu2-112 his4-580 ura3-52 trp1-289*); both have been described previously (9, 10). The strain used for complementation studies was JM43-GD5ab (*MATα leu2-3 leu2-112 his4-580 ura3-52 trp1-289 cox5aΔ::URA3 cox5b::LEU2*), a derivative of JM43 in which the chromosomal copies of the *COX5a* and *COX5b* genes were disrupted with the *URA3* and *LEU2* genes, respectively (49). JM43-GD5ab has no detectable respiration and is incapable of growth on nonfermentable carbon sources (49). The growth and propagation of the yeast and *Escherichia coli* strains have also been described previously (10, 11, 17).

Vectors and plasmid constructions. The plasmids used in this study were derivatives of YCpLPS-5a (17) or pT7/T3-19 (Bethesda Research Laboratories [BRL]). The construction and use of the vector YCpLPS-5a as well as the plasmids YCpLPS-LL5a, YCpLPS-4(21)$^+$, and YCpLPS-4(11)$^+$ were described previously (17). The remainder of the YCpLPS plasmids used (Fig. 1B) were also constructed by procedures described in our earlier work (17). In-frame fusions between sequences encoding the human OTC, gly23, and Δ8-22 leader peptide regions and *COX5a* were generated as follows. YCpLPS-5a plasmid DNA was digested with *Bam*HI and *Sph*I and treated with T4 DNA polymerase in the presence of 50 μM dNTPs. This procedure generated a blunt-ended molecule by filling in the 5′ overhang of the *Bam*HI site while removing the 3′ overhang of the *Sph*I site. Gel-purified *Hin*fI-*Eco*RI fragments containing sequences from the leader peptide region of each OTC derivative (23) (provided by A. Horwich, Yale University) were treated with mung bean nuclease to flush the ends, ligated into the YCpLPS-5a DNA from above, and used to transform *E. coli* DH1 to ampicillin resistance. Correct orientation and reading frame of the various OTC-*COX5a* fusions were indicated by reconstitution of the upstream *Bam*HI site and generation of a new downstream *Sph*I site. Each YCpLPS construct (including those involving *COX4*) was also sequenced to further confirm that no other alterations in the DNA sequence had occurred. It should be noted that because of the construction, the N-terminus of the mature subunit was changed slightly (assuming that processing occurs at the native OTC site). These changes do not alter the function of the protein (see Results).

To generate mRNA in vitro, the *COX5a* gene was cloned behind the bacteriophage T7 promotor, yielding plasmid pT7/Va. Construction was accomplished by cloning a gel-purified *Bam*HI-*Pvu*II fragment from YCpLPS-5a into the RNA expression vector pT7/T3-19 (BRL) which had been cut with *Bam*HI and *Sma*I.

Construction of C-terminal subunit Va deletions. The C-terminal truncations in subunit Va were generated by taking advantage of endogenous *Pst*I and *Bal*I restriction sites (11). pT7/Va was linearized with either *Pst*I [pT7/Va(1-100)] or *Bal*I [pT7/Va(1-118)], followed by treatment with T4 DNA polymerase to blunt the ends. An *Hpa*I SMURFT linker (Pharmacia), containing an ocher stop codon in all three translational reading frames, was then inserted in each case. The plasmid pT7/VaΔ(101-118), which contains a subunit Va gene with an internal deletion, was created by digesting the parental vector with both *Pst*I and *Bal*I, treating with T4 DNA polymerase, and reclosing with T4 DNA ligase.

In vitro transcription and translation. Transcription reactions were performed in vitro according to the instructions provided for each lot of T7 RNA polymerase, which was purchased from New England BioLabs or Promega. Transcription reactions were extracted once with buffer-saturated phenol-chloroform-isoamyl alcohol (25:24:1), which terminated the reaction, and once with chloroform-isoamyl alcohol (24:1). The RNA was ethanol precipitated, pelleted, washed once with cold 70% ethanol, dried, and solubilized in an appropriate volume of diethylpyrocarbonate (DEPC)-treated sterile water. mRNA preparations were stored frozen at −70°C. They were routinely analyzed on agarose gels to assess their purity and integrity prior to translation in vitro.

Translation of mRNA in vitro was accomplished in nuclease-treated, messenger-dependent rabbit reticulocyte lysates purchased from Amersham or Promega. Reactions were performed in the presence of [^{35}S]methionine according to the manufacturer's instructions. In these lysates we found that capping was not required to achieve efficient translation of our mRNA preparations. Translation products were used immediately or divided into portions and stored frozen at −70°C.

In vitro import reactions. Mitochondria were prepared from *Saccharomyces cerevisiae* D273-10B (ATCC 24657) which had been grown to mid-log phase (100 to 120 Klett units with a green filter) in YPGE medium (48). The procedure was essentially that of Daum et al. (12), with minor modifications. These included the use of SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], adjusted to pH 7.2 with KOH) (20). Mitochondria were resuspended in SEM at a concentration of 2 to 5 mg/ml and either used immediately or stored frozen at −70°C in MFB (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS-KOH [pH 7.2], 33% glycerol). We could find no difference in the properties of mitochondria that were used immediately after isolation or after storage in MFB at −70°C (S. M. Glaser and B. M. Miller, unpublished results).

In vitro import reaction mixes (100 μl) contained 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS-KOH (pH 7.2), 3% bovine serum albumin, 1 mM ATP, and 10 mM K^+-malate–7.5 mM K^+-succinate (together called targeting buffer [TRB]), 2 to 10,000 cpm of radiolabeled precursor protein (generally contained in 0.1 to 2 μl) obtained from in vitro translations (above), and 50 to 100 μg of mitochondria. Reactions were routinely allowed to proceed for 10 to 30 min at 30°C and terminated by the addition of valinomycin to 10 μg/ml. When protease sensitivity was to be determined, an import reaction mix was divided in half, and proteinase K was added to one tube to 200 μg/ml. Digestion was allowed to proceed for 30 min at 0°C and then stopped by the addition of phenylmethylsulfonyl fluoride to 1 mM. Mitochondria were then collected by centrifugation and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Subfractionation of mitochondria was performed by the standard method (12), using the modifications described before (S. M. Glaser and M. G. Cumsky, J. Biol. Chem., in press).

Miscellaneous methods. Procedures for *E. coli* and yeast transformation, recombinant DNA work, DNA sequencing of double-stranded molecules, preparation of nucleic acids

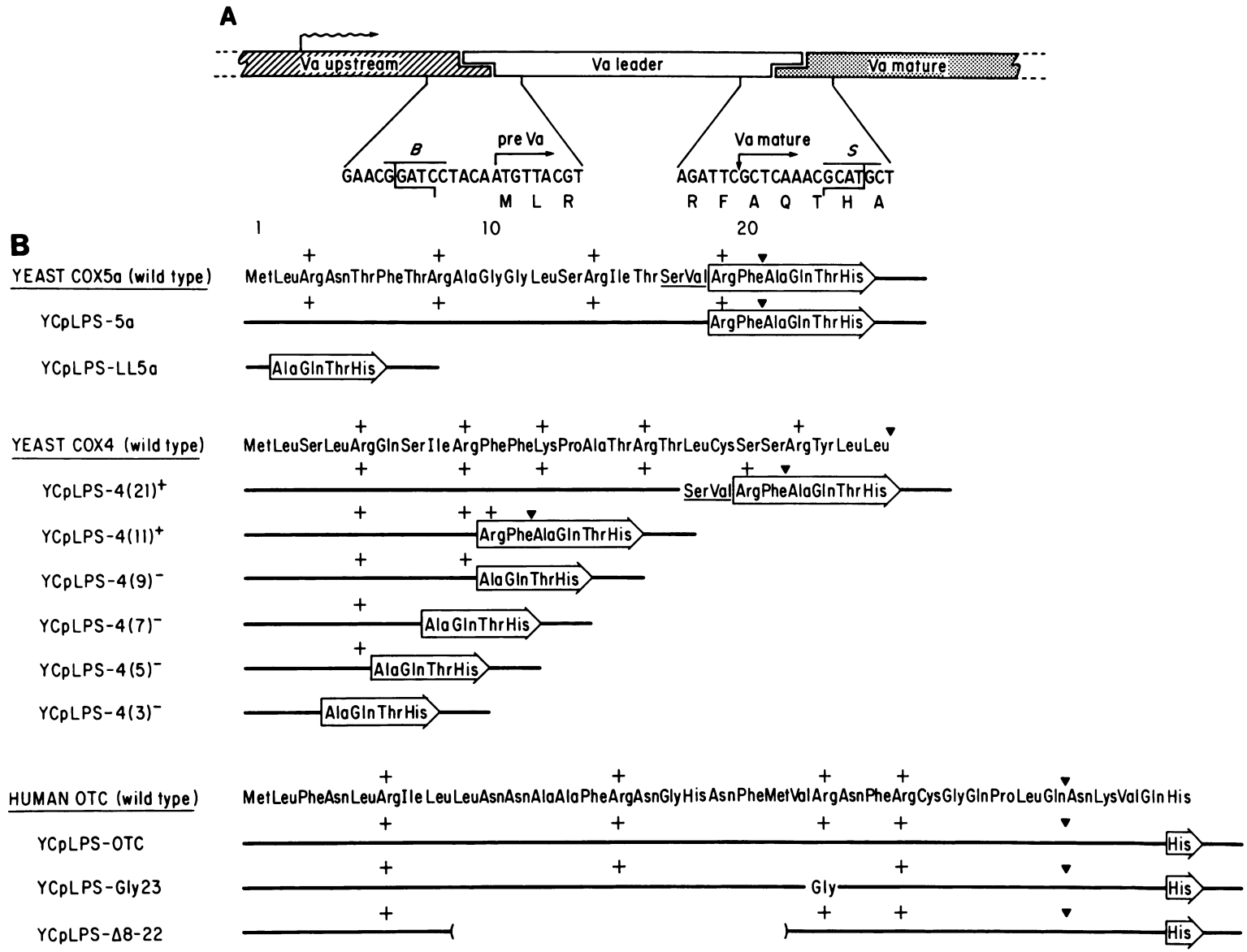

FIG. 1. *COX4-COX5a* constructions. (A) DNA and polypeptide sequence in the leader peptide region of *COX5a* in YCpLPS-5a. The diagram illustrates the positions of the *Bam*HI (B) and *Sph*I (S) cleavage sites. (B) Amino acid sequence of the leader peptide from each fusion protein used in this study. The sequences of the wild-type subunit IV, Va, and OTC leader peptides and the N-terminus of mature subunit Va are shown. The use of the vector and methods for generating precise fusions by using oligonucleotides and the construction of several of the derivatives have been described (17). Construction of the remainder of the fusion proteins diagrammed here is described in Materials and Methods. The residues inside the arrows [and underlined in the case of YCpLPS-4(21)$^+$] are derived from the subunit Va mature protein. Basic residues and the proteolytic processing site of the wild-type precursor proteins are also indicated (+ and arrowhead, respectively). Positioning of the arrowheads on the fusion protein sequences depicts only the position of the processing site on native presubunit Va or pre-OTC and not, for those fusions that undergo cleavage, a determined processing site.

and mitochondria, SDS-PAGE in the presence of glycerol and urea, and immunoblotting have been described (10, 11, 17; Glaser and Cumsky, in press). Except where noted in the figure legend, SDS-PAGE was performed with the buffer system of Laemmli (29). Fluorography was accomplished with En^{3}Hance (New England Nuclear Corp.).

RESULTS

Construction and import of subunit Va fusion proteins. In a previous study, we analyzed the import of two hybrid proteins in which truncated forms of the cytochrome *c* oxidase subunit IV presequence were attached to the mature portion of subunit Va (17). In both cases tested, the shortened presequences targeted subunit Va to mitochondria, and to the inner membrane, with wild-type efficiency in vivo. One of these presequences (11 amino acids in length) was slightly shorter than that reported as the minimal sequence required to direct a heterologous protein, mouse dihydrofolate reductase (DHFR), to yeast mitochondria (26). Therefore, it seemed possible that import and localization of subunit Va could be accomplished with a subunit IV presequence which was shorter than that required for import of DHFR.

To test this possibility, we made several additional constructs using the vector YCpLPS-5a. This plasmid, which we call a leader peptide substitution (LPS) vector, facilitates the generation of precise fusions between essentially any presequence and mature subunit Va (Fig. 1A) (17). The plasmid also contains a yeast centromere to maintain copy number at approximately one per cell and, due to the upstream *COX5a* promotor, serves as an efficient expression vector in yeast cells.

The amino acid sequences of the various fusion proteins constructed are shown diagrammatically in Fig. 1B. In addition to wild-type and leaderless derivatives (encoded by plasmids YCpLPS-5a and YCpLPS-LL5a, respectively), the constructs encoded proteins containing successively shorter forms of the subunit IV presequence. Constructs designated with a superscript minus lack a site for proteolytic processing, since they result from the direct fusion of sequences encoding a truncated subunit IV presequence to the N-terminus of mature subunit Va. The two constructs containing the subunit Va processing site, which were constructed earlier and were included here as controls and for completeness, are designated with a superscript plus (17) (see legend to Fig. 1).

Each YCpLPS plasmid was transformed into the yeast strain JM43-GD5ab (referred to hereafter as GD5ab). This strain contains chromosomal disruptions of both endogenous subunit V genes, *COX5a* and *COX5b* (49). Because it lacks either form of subunit V, it has no cytochrome oxidase activity, cannot respire, and will not grow on nonfermentable substrates. All GD5ab-YCpLPS transformants were therefore analyzed for growth on the nonfermentable substrates glycerol-ethanol (YPGE medium) and lactate (YPL medium). Restoration of respiratory ability, seen as growth on either substrate, indicates that a given presequence has targeted subunit Va to the inner mitochondrial membrane. In addition to this complementation assay, YCpLPS transformants were subjected to two additional functional tests. We estimated cytochrome oxidase activity by the degree of staining with the redox dye *N,N,N',N'*-tetramethylphenylenediamine (31) and determined the growth rate of each transformant in liquid YPGE medium. While these functional assays are clearly not, by themselves, quantitative, accumulated studies in our laboratory have shown that when taken together they provide a reliable estimate of targeting efficiency. Evidence in support of this comes from the strong correlation between the results of the functional studies and those of two different in vivo tests, specifically the steady-state level of the various subunit Va derivatives in mitochondria (below) and their rate of import as determined by pulse-chase analysis (not shown).

TABLE 1. Complementation and function assays

Strain and plasmid	Complementation[a]		TMPD staining[b]	Doubling time[c] (h)	% of wild-type growth rate[d]
	Glycerol	Lactate			
JM43 (no plasmid)	++	++	++++	2.8	100
GD5ab (no plasmid)	−	−	−	NA[e]	NA
GB5ab-YCpLPS-5a	++	++	++++	2.8	100
GB5ab-YCpLPS-LL5a	−	−	+	NA	NA
GD5ab-YCpLPS-4(21)$^+$	++	++	++++	2.8	100
GD5ab-YCpLPS-4(11)$^+$	++	++	++++	3.0	92.9
GD5ab-YCpLPS-4(9)$^-$	++	++	++++	2.7	103.6
GD5ab-YCpLPS-4(7)$^-$	+	−	+	NA	NA
GD5ab-YCpLPS-4(5)$^-$	++	++	++++	2.8	100
GD5ab-YCpLPS-4(3)$^-$	−	−	+	NA	NA
GD5ab-YCpLPS-OTC	++	++	++++	3.0	92.9
GD5ab-YCpLPS-gly23	++	++	++++	3.0	92.9
GD5ab-YCpLPS-Δ8-22	++	++	++++	3.3	82.2

[a] Complementation refers to growth on a solid nonfermentable carbon source. ++, Wild-type growth; +, weak growth; −, no growth.

[b] Degree of staining by the cytochrome oxidase dye *N,N,N,N'*-tetramethylphenylenediamine (TMPD). Symbols: ++++, wild type; ++, reduced; +, faint; −, no staining.

[c] Growth rates were determined in YPGE medium and are expressed as the doubling time in hours

[d] In reference to the growth rate of JM43, calculated by subtracting the percent difference in growth rate between a given strain and JM43 from 100%.

[e] NA, Not applicable (did not grow).

The results of the analysis are presented in Table 1. The N-terminal nine or more residues of the subunit IV presequence directed efficient import of subunit Va. Moreover, the N-terminal five residues of this presequence (Met-Leu-Ser-Leu-Arg) also targeted subunit Va to mitochondria with an efficiency indistinguishable from that of its native presequence. Presequences composed of the N-terminal seven and three amino acids of the subunit IV leader failed to direct import of subunit Va; in each case, the results of the three functional assays indicated that these fusion proteins behaved like the leaderless form of subunit Va. This was not surprising in the case of the three-residue peptide encoded on the 4(3)$^-$ fusion, since it was quite short and did not contain a basic amino acid (Fig. 1B). However, the failure of the seven-residue peptide to direct import of subunit Va was not expected, given the efficiency with which the 4(5)$^-$ fusion was imported. At present, a precise molecular explanation for the latter observation is lacking.

To corroborate the results of the functional tests biochemically, we prepared mitochondria from all the strains analyzed in Table 1 and determined the steady-state level of subunit Va in each by immunoblotting. The results of the experiment (Fig. 2) are in perfect agreement with the data in Table 1. Transformants that were phenotypically like wild-type or positive control strains in the functional assays contained wild-type or nearly wild-type levels of subunit Va in mitochondria. Likewise, little or no subunit Va could be detected in YCpLPS-4(7)$^-$ or YCpLPS-4(3)$^-$ mitochondria.

The results of our studies on the subunit IV-subunit Va fusions are significant in two respects. First, they suggest

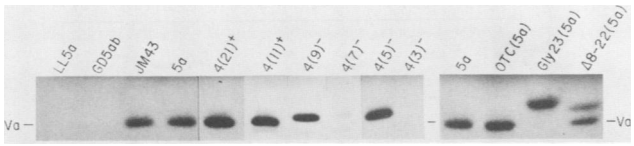

FIG. 2. Steady-state levels of subunit Va in mitochondria. Mitochondrial protein (25 μg) isolated from the untransformed yeast strains GD5ab and JM43 or from transformants harboring the indicated fusions (Fig. 1B) was run on 11.8% glycerol–urea gels (10, 17). The gels were analyzed by immunoblotting with anti-subunit Va antisera and ^{125}I-protein A. The position of mature subunit Va is indicated (Va).

that the import of subunit Va is normally very efficient, since it can be effectively targeted to mitochondria with a minimal (five-amino-acid) presequence. Second, and of greater importance, is that these studies provide additional evidence that the information required to localize subunit Va to the inner membrane resides in the mature protein, not in the presequence. Evidence in support of this comes from the fact that it is unlikely that a five-amino-acid peptide contains both targeting and sorting information. In addition, the N-terminal 12 residues of the subunit IV presequence directed DHFR to the matrix (26), while subunit Va was directed to the inner membrane by essentially the same (or shorter forms of the same) peptide.

The conclusions just described were derived from studies that relied exclusively on subunit IV-subunit Va fusion proteins. Thus, we could not rule out the possibility, however unlikely, that such fusions represented a unique or novel situation. We therefore constructed another set of fusions involving a completely different presequence. Here, sequences encoding the wild-type and two mutant forms of the human OTC presequence were fused to those of mature subunit Va by using the YCpLPS-5a vector (Fig. 1B). The function of the OTC presequence has been studied extensively both in vitro and in vivo (8, 23, 24, 27). It has been shown that when expressed in yeast cells, the wild-type human OTC precursor is efficiently transported into the mitochondrial matrix, where it is processed and assembled into an enzymatically active form that can substitute for the corresponding yeast enzyme (the product of the *ARG3* gene [8]). The two altered OTC presequences, gly23 and Δ8-22, are both totally deficient in directing OTC into rat liver mitochondria in vitro (23). The Δ8-22 presequence also fails to deliver OTC to the mitochondrial matrix (its normal destination) in vivo. When this precursor was expressed in HeLa cells, only 21% was associated with the mitochondrial fraction (27). While the gly23 presequence directed OTC to the matrix of HeLa cells, the imported precursor was not processed or assembled into an active trimer (27). Finally, when expressed in yeast cells, both mutant precursors failed to yield an active OTC, and neither protein was found in the matrix (8). Their precise intramitochondrial location was unclear, however (8).

Each OTC construct shown in Fig. 1B was transformed into GD5ab and analyzed in a manner identical to that used previously. As shown in Table 1, each form of the OTC presequence was functional when attached to subunit Va, although the Δ8-22 derivative was slightly less effective than either of the other two (it grew in liquid YPGE medium with a slightly slower doubling time). Immunoblots of mitochondria prepared from representative transformants were again consistent with the functional data (Fig. 2), as the amount of subunit Va detected within the organelles was at or near wild-type levels. The immunoblots also showed that the wild-type OTC presequence was cleaved from the subunit Va moiety. This result was consistent with earlier observations suggesting that the yeast processing machinery is able to recognize the human OTC processing site (8). Interestingly, the gly23-subunit Va precursor was not processed, while the Δ8-22 derivative was processed poorly (Fig. 1; previous results indicated that cleavage of a presequence is not required for import or activity of subunit Va [17]). In the case of Δ8-22-subunit Va, it is not yet clear whether both the processed and unprocessed forms of the subunit are active in the holoenzyme (Fig. 2, last lane).

The observation that subunit Va was efficiently targeted to mitochondria by a presequence that failed to deliver its cognate protein (the Δ8-22 presequence) is consistent with our previous results. It also strengthens the conclusion that import of this polypeptide does not require a strong targeting signal and is thus highly efficient. Also in line with the results presented earlier was our observation that subunit Va was

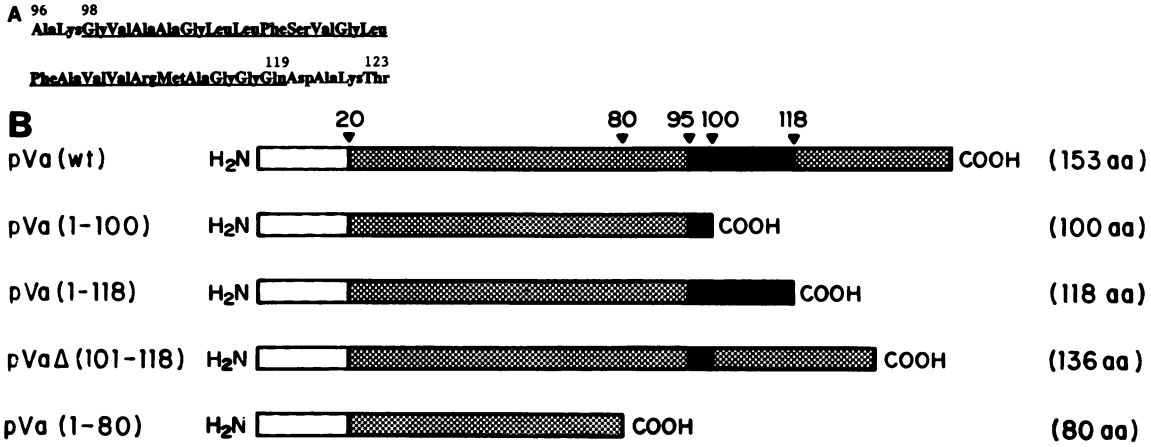

FIG. 3. (A) Amino acid (aa) sequence of subunit Va in the hydrophobic region. The proposed localization domain is underlined. Residues are numbered according to their position in the 153-amino-acid precursor (11). (B) C-terminal deletions of subunit Va. Schematic depiction of wild-type presubunit Va [pVa(wt)] and the various derivatives constructed (see Materials and Methods for details). The 20-residue presequence is shown unshaded, the hydrophobic domain (from hydropathy plots [11]) is shaded, and the remainder of the polypeptide is stippled. The length of the various precursors is shown in parentheses at the right.

efficiently localized to the inner membrane when attached to the wild-type OTC presequence. Since the same presequence directs OTC to the matrix space of yeast (or mammalian) mitochondria (8, 27), the result demonstrates once again that the information required to route subunit Va to the inner membrane resides within the mature portion of the protein.

Identification of region necessary for inner membrane localization of subunit Va. From previous work in other laboratories, it is known that subunit Va is an integral protein of the inner mitochondrial membrane, with at least part of the polypeptide embedded within the lipid bilayer (5, 44, 45). Nevertheless, the protein contains a large proportion of hydrophilic amino acids (44, 45), and an examination of structural and hydropathic plots of its sequence revealed only a single hydrophobic domain long enough to span a membrane (11). This domain (Fig. 3A) resided in the C-terminal third of the protein, between amino acids 98 and 119 (numbering from the start of the 153-residue precursor). It contained only a single charged amino acid (Arg), which was located near the C-terminal boundary of the domain at position 115. While the amino acid sequence of subunit Va and related subunits in *S. cerevisiae* (subunit Vb [11]), *Neurospora crassa* (subunit V [46]), and bovine heart (subunit IV [43, 46]) are highly divergent in this region, it is striking that the hydrophobic nature of the domain is remarkably conserved (46). Thus, we speculated that the region might be important for subunit Va function and, possibly, for inner membrane localization.

To test this hypothesis, we generated subunit Va derivatives that lacked the hydrophobic domain (Fig. 3). These included several C-terminal truncations, as well as an internal deletion of the region. Because such constructs would not, a priori, be expected to remain enzymatically active, further import and localization analysis was performed in vitro. Radiolabeled precursors corresponding to each of the constructs shown in Fig. 3 were generated by in vitro transcription and translation (Glaser and Cumsky, in press) (see Materials and Methods), and each was tested for its ability to be imported into isolated yeast mitochondria (16). Three of the precursors, pVa(1-100), pVa(1-118), and pVaΔ(101-118), were taken up and processed by mitochondria in a manner dependent upon a membrane potential (the ionophore valinomycin prevented import in each case) (Fig. 4). The pVa(1-80) precursor was the sole exception and represented a special case. Although it was clearly translated in the reticulocyte system, it was apparently unstable; after incubation with isolated mitochondria, none could be detected in either the mitochondrial or postmitochondrial fractions (not shown). We did not study it further.

The three derivatives which were taken up by mitochondria were analyzed for their intramitochondrial locations. In vitro import experiments were again performed with each precursor, and the mitochondria from each reaction were separated into total soluble and membrane fractions (12; Glaser and Cumsky, in press). Analysis of these fractions revealed that the two proteins which retained the hydrophobic domain, Va(1-118) and native subunit Va, were in the membrane fraction (Fig. 5). Although the membranes were not fractionated further, it is reasonable to assume, based on properties of subunit Va and the results presented above, that both proteins reside in the inner membrane. On the other hand, Va(1-100) and VaΔ(101-118), which both lacked the hydrophobic domain, were found in the soluble fraction (Fig. 5). Because this fraction contained components of both the matrix and intermembrane space, it was not immediately clear which compartment the proteins were in. However, the fact that each was proteolytically processed indicated that they had been at least transiently exposed to the matrix. Subsequent experiments, in which the contents of the intermembrane space were released selectively, demonstrated that both proteins were in the matrix (not shown). Thus, when taken together, the results in Fig. 5 demonstrate that the removal of amino acids 101 to 118 caused subunit Va to be mislocalized to the matrix. We conclude, therefore, that the domain between amino acids 101 and 118 is necessary for inner membrane localization of subunit Va.

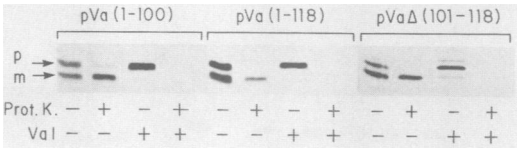

FIG. 4. In vitro import of subunit Va derivatives. Reaction mixes contained 5×10^3 cpm of the indicated ^{35}S-labeled precursors (usually in 1 μl or less of reticulocyte lysate) and 50 μg of mitochondria in TRB (Materials and Methods). Reaction mixes were made to 100 μl total volume with TRB and incubated at 30°C for 10 min. Where indicated (Val), the mitochondria were pretreated with 10 μg of valinomycin per ml before the reaction was performed. Proteinase K digestions (Prot. K) were performed as described in Materials and Methods. The mitochondria were then reisolated by centrifugation and prepared for SDS-PAGE. Gels (15%) were run with the buffer system of Laemmli (29), dried, and fluorographed. The positions of the precursor (p) and mature (m) forms of each derivative are indicated. For ease of viewing, the mature forms of each derivative are aligned. See Fig. 5 for actual mobilities and positions in gel.

DISCUSSION

The results presented in this study enhance our overall understanding of the import and localization of yeast cytochrome *c* oxidase presubunit Va. Our data show that subunit Va can be efficiently directed to mitochondria and to the inner membrane with extremely poor targeting sequences. One, consisting of the N-terminal five amino acids of the subunit IV presequence [4(5)$^-$], contains a single basic residue. It is, to date, the shortest presequence reported as being capable of targeting an otherwise nonimportable protein (LL5a) to mitochondria. Another presequence, OTC Δ8-22, failed to properly target its cognate protein (OTC) to

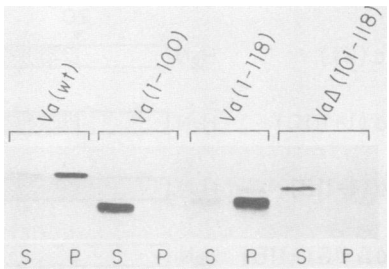

FIG. 5. Intramitochondrial localization of subunit Va derivatives. In vitro import reactions with the indicated ^{35}S-labeled precursors were performed as described in the legend to Fig. 4 and Materials and Methods. Following proteinase K digestion of each reaction mix, they were fractionated into total soluble (S) and membrane (P) fractions by standard methods (12; Glaser and Cumsky, in press). Samples were analyzed by SDS-PAGE and fluorography as described in the legend to Fig. 4.

mitochondria in vivo or in vitro (23, 27). Not only did the Δ8-22 presequence direct efficient import of subunit Va in vivo, but recent results from our laboratory have shown that it efficiently targets subunit Va to isolated yeast mitochondria in vitro (B. R. Miller, unpublished results). Finally, the $4(9)^-$ presequence, which we have shown to be an effective targeting signal when attached to subunit Va in vivo and more recently in vitro (B. R. Miller, unpublished results), was unable to target mouse DHFR to yeast mitochondria in vitro (26).

Since subunit Va can be effectively imported with minimal or defective presequences as well as presequences that fail to direct import of other proteins, it is possible that presubunit Va is taken up by mitochondria more efficiently than other precursor proteins. Several recent results from our laboratory are consistent with this view and offer an explanation for it. Specifically, these results indicate that, in solution, presubunit Va is loosely folded. It is extremely sensitive to proteases, it can be imported into isolated mitochondria at temperatures as low as 0°C, it requires only low levels of ATP for import, and import is not facilitated by urea denaturation of the protein (B. R. Miller and M. G. Cumsky, submitted for publication). Thus, we suggest that the loose structure adopted by subunit Va in solution enhances its ability to be taken up by mitochondria.

Another factor that could contribute to the efficiency with which subunit Va is imported is the contribution of targeting information from the mature protein. It has been shown the β subunit of the yeast F_1-ATPase contains redundant targeting signals, one of which is in a region reported to be at the N-terminus of the mature protein (2). While the leaderless form of subunit Va was not imported into mitochondria (Table 1, Fig. 2), our results cannot conclusively rule out the possibility that some targeting information resides in the mature protein. Studies currently in progress are testing this possibility directly.

The results of this study have also provided further documentation that the information necessary for localizing subunit Va to the inner membrane resides within the mature protein. The results prompted us to look directly for a sequence that might be involved in localization or sorting. This sequence was found, at least in part, in the C-terminal third of the polypeptide.

We propose that the domain between amino acids 98 and 119 is an inner membrane localization signal. At present, we do understand, in molecular terms, the way in which the signal directs subunit Va sorting. Based on parallel studies with other translocated polypeptides, however, we suggest it is likely that the putative signal functions in one of three ways. These involve mechanisms whereby the signal acts as a "stop-transfer" or membrane-anchoring domain, a protein-interactive or assembly domain, or as a secondary targeting signal that acts after translocation of the Va precursor to the matrix.

The hydrophobic nature of this region (it contains only a single charged amino acid near the C-terminal boundary of the domain, which is not necessarily part of the signal; see below and Fig. 3A) is consistent with its acting as a stop-transfer signal or membrane anchor. Such signals are known to stall the translocation of proteins across the membrane of the endoplasmic reticulum (52) and the mitochondrial outer membrane (21, 33). It should be noted that the use of a stop-transfer sequence to localize subunit Va necessarily predicts that the Va precursor does not cross the inner membrane completely. While we have thus far failed to observe a matrix-localized translocation intermediate (B. R. Miller, unpublished results), the existence of such an intermediate cannot yet be rigorously ruled out. It is clear, however, that at least the N-terminal portion of the protein must be at least transiently exposed to the matrix, since the presequence is cleaved by the metalloprotease localized there.

A second way in which the localization signal may work also involves preventing translocation across the inner membrane. This model would predict, however, that the domain traps subunit Va in the membrane via a direct physical interaction with a polypeptide, perhaps another subunit of the holoenzyme. While no direct evidence supporting this view is currently available, it is appealing because it involves a mechanism whereby localization of the protein and its assembly into the holoenzyme are coupled.

Finally, presubunit Va may be targeted back to the inner membrane after transfer to the matrix. This model predicts that the localization signal functions in a manner analogous to that of the sorting information contained in bipartite presequences (18, 20). That is, after translocation into the matrix, and presumably while complexed with hsp60 and/or other components of a soluble translocation apparatus (36), the signal would then act to redirect the polypeptide to the inner membrane.

Although each model described above is presently speculative, studies that will enable us to distinguish between them are in progress. We are using the yeast in vitro import system to elucidate the precise pathway by which presubunit Va is routed to the inner membrane and to identify the mitochondrial components that decode the putative signal. Several additional questions pertaining to the putative signal are also of interest. For example, although the results presented in this study show that the hydrophobic domain is necessary for intramitochondrial localization, we have not yet shown whether the region is alone sufficient for it. We have also not determined whether alternative sequences of similar composition and character can function in its place or defined precisely the boundaries of the signal. Ongoing studies will hopefully provide answers to some of these important questions. The successful completion of these studies should enhance our overall understanding of mitochondrial import and of the way in which proteins are targeted to the inner mitochondrial membrane.

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