Hydrophobic Core but Not Amino-terminal Charged Residues Are Required for Translocation of an Integral Thylakoid Membrane Protein in Vivo*

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The integral membrane protein cytochrome f contains an amino-terminal signal sequence that is required for translocation into the thylakoid membrane. The signal sequence contains a hydrophobic core neighboring by an amino-terminal charged residue. Mutations that introduce charged amino acids into the hydrophobic core are inhibitory to cytochrome f translocation, and thus render cells non-photosynthetic. We have isolated both nuclear and chloroplast suppressors of these mutations by selecting for restoration of photosynthetic growth of Chlamydomonas. Here we describe the characterization of two chloroplast, second site suppressor mutations. Both suppressors remove the positively charged amino acid that borders the amino terminus of the hydrophobic core, and replace this arginine with either a cysteine or a leucine. The existence of these suppressors suggests that the hydrophobic core can be shifted in position within the signal sequence, and analysis of triple mutants in the signal confirms this hypothesis. Thus this signal that mediates translocation into the thylakoid membrane is characterized by a hydrophobic region whose exact amino acid content is not critical, and that need not be flanked on its amino terminus by a charged residue.

Signal sequences are often found as amino-terminal extensions that mediate the translocation of a protein across membranes (1). While these signals appear not to be conserved in sequence from bacteria to metazoans (2), they typically are characterized by an α-helical hydrophobic sequence bounded on its amino-terminal side by at least one positively charged residue (1). At least three paths into or across the thylakoid membrane have been described (3–5) and these can sometimes be distinguished by the type of signal sequence (6). Cytoplasmically synthesized proteins destined for the thylakoid are imported across the chloroplast envelope and then the amino-terminal region of the signal is removed by a chloroplaststromal protease (7). The remaining portion of the signal then directs the protein into or across the thylakoid (8). For luminal proteins such as those of the oxygen evolving complex and plastocyanin this signal is removed from the amino terminus within the lumen (8). The integral thylakoid membrane light harvesting chlorophyll protein has an internal, complex signal that is distributed throughout its three hydrophobic α-helices (9). Chloroplast-encoded proteins, such as cytochrome f, also target to the thylakoid with an amino-terminal signal, although some of these are not cleaved after translocation (10).

Genetic analysis indicates it is likely that both chloroplast and cytoplasmically synthesized proteins can use common translocation mechanisms (5). Our studies also show that proteins passing completely through the thylakoid membrane utilize a different path from those that become integral proteins (5). Biochemical experiments that study competition between various precursors and chimeric signal sequences (3), and that describe the energetics of protein translocation (11), distinguish at least three paths (12–15). Distinct pathways are indicated by these studies, but the possibility of common components cannot be ruled out; there may be multiple pathways into one (or two) common membrane translocases.

Mutations in signal sequences can lead to a loss of translocation, and a number of successful genetic selections have identified suppressors of bacterial signal mutations (16, 17). These extragenic suppressor mutations usually lie in proteins that interact with the mutant signal sequence, and the characterization of these suppressors has been an efficient way of identifying components of the membrane and cytoplasmic translocation machineries. We have used a genetic approach to characterize the translocation of cytochrome f, a chloroplast-encoded protein that spans the thylakoid membrane once (5, 10). Cytochrome f is a member of the photosynthetic electron transport chain, and as such is required for photosynthesis. Mutations in the hydrophobic core of the Chlamydomonas cytochrome f presequence inhibit the ability of cells to grow photosynthetically and by selecting for the restoration of photosynthetic growth, nuclear and chloroplast suppressors were isolated. We describe here the characterization of the chloroplast suppressor mutations and show that they lie within the cytochrome f signal sequence. We also demonstrate the necessity for a hydrophobic core, but not amino-terminal charged residues in the signal sequence for cytochrome f translocation.

MATERIALS AND METHODS

PolymeraseChain Reaction Amplification and Sequencing—Chlamydomonas DNA was extracted using the rapid whole cell DNA isolation procedure (18). Thirty rounds of amplification were performed with 1 to 5 μl of DNA preparation, 0.2 mM dNTP, 1X reaction buffer, 1 μM primer petA2 (nucleotides –88 to –67), 5’-caatacgaccattagcctcac-3’, 1 μM primer peta221 (nucleotides 221 to 241) 5’-caatacgaccattagcctac-3’, 2.5 units of Taq (Boehringer) in 40 μl final volume. The polymerase chain reaction product was purified by precipitation with 50% isopropanol alcohol and 1 μM ammonium acetate. The amplified sequence was determined using the dSNA Cycle Sequencing System (Life Technologies, Inc.) using peta2 as the end labeled primer.

Mutagenesis of Cytochrome f Signal Sequence—The double mutant R10L/V16D was made by introducing the R10L mutation into the V16D mutant plasmid (5) using the digoxigenine-directed in vitro mutagenesis kit from Amersham Corp. The R10L mutation was made by creating the following nucleotide changes, 27–30 GCTG, where nucleotide 1
Suppressor Mutations of Signal Peptides

**Fig. 1. Sequence of wild type and mutant cytochrome f signal sequences.** Shown are the amino acid residues (numbered 1–30 below sequence) for the wild type (+) and mutant (italics on left) signal. A15E and V16D were engineered in vitro and introduced into Chlamydomonas. A15E-sup and V16D-sup, signals containing the selected suppressors. The autocorrelation of growth strains containing the indicated signal is shown on the right. The dark rectangular box above the sequence predicts the location of the hydrophobic core. Bold letters indicate mutant amino acids.

is the site of cytochrome f translation initiation (19). The triple mutant R10L/A12E/V16D was made by introducing the A12E substitution into the double mutant R10L/V16D psA (cytochrome f gene), using oligonucleotide mutagenesis. The following nucleotide changes were made: R10L, nucleotides 27–30 GCTG; A12E, nucleotides 33–36 TTAG; V16D, 45–47 AGA. Each of the mutant genes was identified in Escherichia coli using colony hybridization to mutant oligonucleotides (20) and plasmids were sequenced using Sequenase (U. S. Biochemical Corp.) to verify the existence of the mutation.

Transformation of Chlamydomonas reinhardtii—cc-125 was as described (5, 21, 22).

Identification of Mutant Transformants by Southern Analysis—The R10L/V16D and R10L/A12E/V16D Chlamydomonas mutants were identified by Southern analysis as described (5) with the following modifications. The R10L/V16D mutant was identified by hybridization with a R10L/V16D oligonucleotide. The R10L/A12E/V16D mutant was screened by hybridization with a R10L/A12E/V16D oligonucleotide. The temperature of washes at which the mutant oligonucleotide remains hybridized to the mutant DNA but not to wild type was 78°C for R10L/V16D and 74°C for R10L/A12E/V16D.

Spot Tests Analysis—Growth of strains was determined on acetate containing TAP medium and on minimal HS medium (21). Strains were grown in liquid TAP and then equal numbers of cells were spotted onto plates and grown under low light (15 μmol of photons/m² s) or high light (200 μmol of photons/m² s) for 5 days.

Northern analysis (5, 23) was quantified from 4 replicates using non-saturated signals and a Molecular Dynamics PhosphorImager and software.

RESULTS AND DISCUSSION

The signal sequence of cytochrome f contains a charged residue arginine (R) followed by 8 hydrophobic amino acids in a postulated α-helix followed by a glycine (G), α-helix breaking residue (Fig. 1). In a previous work (5), it has been demonstrated that mutant strains containing substitutions A15E or V16D (first letter indicates the wild-type amino acid at the numbered position, and the second letter, the mutant residue) showed reduced or no photosynthetic growth, respectively, as assayed by their inability to grow in the light in the absence of added carbon (no acetate, HS media). Western and pulse-chase analysis revealed that these mutations blocked the translocation of cytochrome f into the thylakoid membrane.

A15E and V16D strains were subjected to methanesulfonic acid ethyl ester mutagenesis and suppressors were selected for the ability to restore the cells to photosynthetic growth by plating 10⁶ cells on HS (nonacetate containing media which selects for photosynthetic growth) in light greater than 200 μmol of photons/m² s (5). One hundred and twenty A15E suppressors, and two V16D suppressors were obtained. Multiple variations of growth conditions and alternative mutagens (such as UV) were tried but no additional suppressors were obtained (5). In the absence of methanesulfonic acid ethyl ester treatment no suppressors were detected in over 10⁶ cells. Crosses of both V16D suppressor strains, and two of the A15E suppressor strains (all mating type +) with a wild-type mating type – strain produced progeny that all grew photosynthetically. Because the chloroplast genome, and thus the V16D and A15E mutations, are predominately inherited from the mating type + strain (21), we concluded that these four strains carried chloroplast suppressor mutations. The remaining 118 A15E suppressors strains tested carry nuclear suppressors. These loci are referred to as tip (thylakoid insertion protein) mutants and will be described elsewhere.

To determine the nature of the chloroplast suppressors of A15E and V16D, the cytochrome f signal peptide coding sequence from each of the four strains was amplified by polymerase chain reaction and sequenced (data not shown). In both V16D suppressor strains the codon for Arg-10 (cgc) was changed to Leu (ctc), and in both A15E suppressing strains, Arg-10 was changed to Cys (tgc, Fig. 1). No other changes in the signal sequence were found. Reversions of the original mutation were not found, which was expected as the initial mutations involved three base alterations for A15E and two base alterations for V16D. To determine that the suppression of V16D was due only to the Arg-10 substitutions and not to some other additional mutation in the chloroplast genome, we synthesized in vitro the double mutant R10L/V16D and transformed this gene into a wild-type Chlamydomonas using particle gun bombardment and homologous recombination in the chloroplast genome (24). Initial transformation was scored by co-transformation with a spectinomycin/streptomycin resistance (sp² or st²) marker in the chloroplast rRNA gene (24). Through multiple subculturing (single cell cloning) and selection of sp² or st² in these transformed lines, all ~70 copies of psA (cytochrome f gene) can be replaced, and this occurs through random segregation and selection for sp² or st². Strains were propagated non-photosynthetically on TAP media. The presence of only mutant and no wild-type gene copies was detected using mutant or wild-type oligonucleotides and Southern blotting of total cell DNA. Fig. 2 shows that the labeled oligonucleotide R10L/V16D hybridizes only to DNA isolated from the mutant strains, and not the wild type. No wild type cytochrome f signal sequences are present in the DNA isolated from the R10L/V16D strain.

The strains carrying the double mutation R10L/V16D were then tested for growth in the absence of acetate as an indication of their ability to translocate cytochrome f. Fig. 3 shows that the V16D strain cannot grow photosynthetically on HS media, and A15E has 10% the amount of photosynthetic growth of wild type (5). Both of the strains carrying the selected chloroplast suppressors of A15E and V16D (V16Dsup and A15Esup, Fig. 3), and the in vitro engineered double mutant strain (R10L/V16D) grow on HS. Thus the R10L substitution alone is sufficient to suppress the V16D mutation. Fig. 3 demonstrates that
each of these strains can grow heterotrophically on TAP medium, which contains acetate, but different heterotrophic growth rates are seen, especially for V16D. The reasons for this reduced growth are not known, although effects on non-photosynthetic growth have been seen for a variety of other mutants that affect chloroplast function (21). Whole cell extracts were made from each strain, run in a denaturing acrylamide gel, and the Western blot was probed with anti-cytochrome f antiserum and also with antiserum to the chloroplast large subunit of ribulose-bisphosphate carboxylase/oxygenase (LSU) to verify equal loading (Fig. 3, panels W). In the wild type, the in vitro synthesized suppressor strain, and in the suppressor strain, cytochrome f accumulates to normal levels. Northern analysis also demonstrates that mRNA for each wild type and mutant strain accumulates to wild-type levels (Fig. 3, panel N), as compared to the levels of 16 S rRNA.

Both V16D and A15E introduce charges into the hydrophobic core of the signal sequence, and Arg-10 lies at the amino-terminal boundary of the hydrophobic core. Thus the R10L and R10C mutations may simply allow the hydrophobic core to shift toward the amino terminus thereby effectively eliminating the charged residues from the hydrophobic core (Fig. 4). Alternatively, both Ala-15 and Val-16 lie on the same face of the predicted $\alpha$-helix, and Arg-10 lies on the opposite face when this region is displayed as a helical wheel. Thus the elimination of the charge by R10L (or R10C) may permit that face, rather than the newly charged Ala-15/Val-16 face, to interact with the terminal end of the hydrophobic core to be inhibitory. If the $\alpha$-helix face hypothesis is correct, then A12E mutation should have no effect in the triple mutant.

The triple mutant R10L/A12E/V16D was constructed in vitro by oligonucleotide-directed mutagenesis, and introduced into a wild-type Chlamydomonas strain using sp’st’ for initial selection. Strains carrying the three mutations in cytochrome f were screened by single cell cloning and hybridization with the mutant oligonucleotide containing the R10L/A12E/V16D mutations (Fig. 2). Homoplasmic strains were obtained that carry the changes, and the presence of mutant but not wild-type copies of petA was verified by Southern blotting using mutant or wild-type oligonucleotide probes (Fig. 2). These new strains were tested for their ability to grow photosynthetically, and for steady state levels of cytochrome f mRNA and protein. The results are shown in Figs. 3 and 4. While all strains accumulate normal or near normal levels of cytochrome f mRNA, the triple mutant (R10L/A12E/V16D) does not grow photosynthetically in the absence of acetate (HS versus TAP, Fig. 3). Nucleic acid probes to the 16 S rRNA were used to standardize the RNA from four individual experiments, and the ratio between the cytochrome f and 16 S rRNA varied at most 10% between individual strains. Western analysis indicates that cytochrome f does not accumulate in the strain carrying the triple mutation, but does in the wild type, the suppressor (R10L/V16D), and the strain having only A12E. Western blotting the same samples with anti-ribulose-bisphosphate carboxylase/oxygenase LSU demonstrates that approximate equal amounts of protein were indeed analyzed. Thus the data are consistent with the shifting of the hydrophobic core toward the amino-terminal region, and not with the orientation of specific amino acids residues on the helical wheel.

The cytochrome f signal sequence is required for translocation across the thylakoid membrane in vivo (5). This signal sequence, similar to the bacterial signal sequences, requires a region that is predicted to be $\alpha$-helical and vacant of charged residues (1). The results presented here demonstrate that the exact sequence content of the hydrophobic core is not critical, although it cannot include charged residues. Moreover, the charged amino acid that flanks the amino-terminal side of the hydrophobic core is not essential for the accumulation of cytochrome f.

It has been shown previously that the A15E and V16D mutations cause an accumulation of small amounts of labile cytochrome f precursor which cannot be detected in wild type cells (5), and the data indicated that translocation was greatly slowed and even abolished in the case of the V16D mutant strain. One possible mechanism of elevating the reduced levels of mature cytochrome f in the A15E and V16D mutations thereby suppressing their effect may have been to increase the cytochrome f expression levels. However, the mRNA and protein levels in the chloroplast suppressor strains that restore photosynthetic growth are similar to wild type. Thus overexpression is not the mechanism of suppression in this example.
This is supported by the observation that the suppression of V16D and A15E is due solely to the R10L/C substitution, and these changes alone would be unlikely to drastically increase cytochrome f expression.

The cytochrome f signal sequence is similar to those sequences directing proteins to the bacterial inner membrane as this hydrophobic core is bounded by an amino-terminal charged residue which is not essential for translocation in vivo (1). However, in vitro studies with bacterial membranes indicate that mutations in this charged region result in a reduced rate of translocation, although this appears to be protein specific (1, 17, 25). Our results cannot rule out the possibility that the cytochrome f suppressor mutations that fully restore cytochrome f levels in vivo subtly change the kinetics of thylakoid translocation, and that our in vivo assay is not sufficiently sensitive to detect these changes.

The molecular and genetic analysis of the four nuclear suppressors tip (5) should help to clarify the role of the cytochrome f signal, to identify the proteins that mediate translocation, and to evaluate the extent to which the “multiple translocation pathways” of the thylakoid actually interact and overlap.

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