A Novel, Bipartite Transit Peptide Targets OEP75 to the Outer Membrane of the Chloroplastic Envelope

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OEP75 is an outer envelope membrane component of the chloroplastic protein import apparatus and is synthesized in the cytoplasm as a higher molecular weight precursor (prOEP75). During its own import, prOEP75 is processed first to an intermediate (iOEP75) and subsequently to the mature form (mOEP75). Experiments conducted with stromal extracts indicated that iOEP75 was generated from prOEP75 by the activity of the stromal processing peptidase. The specific processing site was determined and used to divide the prOEP75 transit peptide into N- and C-terminal domains. To determine the targeting functions of the two domains of the transit peptide and of the mature region of prOEP75, we created a deletion mutant construct from prOEP75 and chimeric constructs between domains of prOEP75 and the precursor to a small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Analysis of these constructs by in vitro chloroplastic protein import assays revealed that the transit peptide of prOEP75 is bipartite in that the N- and C-terminal portions contain chloroplastic and intraorganellar targeting information, respectively.

INTRODUCTION

Many chloroplastic proteins are encoded by nuclear genes and synthesized as higher molecular weight precursors. An N-terminal stretch of amino acids, termed a transit peptide, targets these precursors to chloroplasts (reviewed in Archer and Keegstra, 1990; de Boer and Weisbeek, 1991; Theg and Scott, 1993). The transit peptides of precursors to stromal proteins are removed by a stromal processing peptidase when the precursors are translocated across the chloroplastic envelope. Proteins destined for the thylakoid lumen contain a bipartite transit peptide consisting of N- and C-terminal domains (reviewed in Robinson and Klösgen, 1994). The N-terminal domain acts as a stromal targeting signal and is removed in the stroma. The C-terminal domain subsequently directs the protein to the lumen. Most proteins destined for the chloroplastic inner envelope membrane (Li et al., 1992; Knight and Gray, 1995) and the thylakoid membrane (Hand et al., 1989; Cai et al., 1993) have transit peptides similar to those of precursors targeted to the stroma. Intraorganellar targeting information for these inner envelope membrane and thylakoid membrane precursor proteins apparently resides within the mature proteins. Among the known proteins of the chloroplastic outer envelope membrane, most are not synthesized as higher molecular weight precursors and therefore do not have a transit peptide (Salomon et al., 1990; Li et al., 1991; Fischer et al., 1994). The import pathway of these outer envelope membrane proteins is distinct from that of other known chloroplastic proteins in that they do not travel through the general import apparatus of the chloroplastic envelope.

Several components of this general import apparatus have been identified (reviewed in Gray and Row, 1995; Schnell, 1995). At least five outer envelope membrane proteins (OEPs) have been implicated in the import process. OEP34 (IAP34) has GTPase activity; however, its specific function is not known (Kessler et al., 1994; Seedorf et al., 1995). OEP44 (Com44) was identified by chemical cross-linking, and its function is also not known (Wu et al., 1994). OEP70 (SCE70) is a member of the heat shock cognate of proteins and may act as a molecular chaperone to maintain precursor proteins in an import-competent state (Ko et al., 1992). OEP75 may form at least part of the channel through which precursors pass (Perry and Keegstra, 1994). OEP86 (IAP86) is thought to be the receptor for precursors and, like OEP34, has GTPase activity (Hirsch et al., 1994; Kessler et al., 1994). OEP34, OEP40, and OEP70 are like most chloroplastic OEPs in that they are not synthesized as higher molecular weight precursors (Ko et al., 1992, 1995; Kessler et al., 1994; Seedorf et al., 1995). Surprisingly, however, both OEP75 and OEP86 are synthesized as higher molecular weight precursors containing N-terminal extensions (Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Tranel et al., 1995).

Although the precursor to OEP86 (prOEP86) has an N-terminal extension, it is unlikely that prOEP86 uses the general chloroplastic protein import apparatus. Whereas most chloroplastic transit peptides are characterized by a net positive charge, the transit peptide of prOEP86 has a net negative charge. Furthermore, import of prOEP86 was not inhibited by...
the presence of excess precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (prSS), a stromally targeted precursor protein (Hirsch et al., 1994).

Previously (Tranel et al., 1995), we presented evidence that the import pathway of prOEP75 involved the general chloroplastic protein import apparatus. The amino acid composition of the prOEP75 transit peptide is similar to that of other chloroplastic transit peptides, and import of prOEP75 is inhibited by the presence of excess prSS.

In this study, we provide additional evidence that prOEP75 uses the general chloroplastic protein import apparatus en route to its final location in the outer envelope membrane. Our findings indicate that (1) the N terminus of the prOEP75 transit peptide initiates the precursor on the generalized chloroplastic import pathway and (2) the C-terminal portion of the prOEP75 transit peptide causes divergence from this generalized import pathway and prevents translocation of OEP75 into the stroma.

RESULTS

prOEP75 Is Processed to Intermediate-Sized OEP75 by the Stromal Processing Peptidase

When incubated with isolated chloroplasts, prOEP75 is processed stepwise to the mature form (Tranel et al., 1995). An intermediate-sized product (iOEP75) and the mature product (mOEP75) obtained from an import reaction of prOEP75 with intact chloroplasts are shown in Figure 1A. Previously (Tranel et al., 1995), we speculated that the N terminus of the prOEP75 transit peptide was a chloroplastic targeting domain and that during import it would be removed by the stromal processing peptidase (SPP) (Vander Vere et al., 1995), generating iOEP75. To test this hypothesis, we incubated radiolabeled prOEP75 with a stromal extract. As shown in Figure 1B, incubation of prOEP75 with the stromal extract resulted in the accumulation of iOEP75 but not mOEP75. We also performed the stromal processing assay with prSS (Figure 1C). prOEP75 and prSS were processed with similar efficiency. To determine whether prSS and prOEP75 are processed by the same protease, we performed a competition experiment in which excess, unlabeled prSS is added in an effort to block the processing of prOEP75. As can be seen in Figure 1B, the addition of prSS greatly reduced the accumulation of iOEP75, indicating that its appearance is dependent on the SPP. The mature domain of prSS (mSS) was not an efficient competitor of prOEP75 processing.

Based on molecular size determination from SDS-PAGE, 3 to 4 kD of the prOEP75 transit peptide was removed by the SPP. To determine the specific processing site, we used radiolabeled protein sequencing. prOEP75 was labeled by the incorporation of 3H-leucine and incubated with intact chloroplasts under standard import conditions, that is, 3 mM ATP for 30 min at room temperature. Under these conditions, typically 10 to 20% of the added prOEP75 is associated with chloroplasts—one-half as iOEP75 and the other half as mOEP75. The resultant iOEP75 was isolated by using SDS-PAGE, transferred onto a ProBlott membrane, and subjected to sequencing by Edmann degradation.

The amount of radioactivity released after each cycle is presented in Figure 2A. A strong peak of radioactivity was released after the second cycle, followed by at least 10 cycles during which no radioactive peaks were released. There are four potential processing sites that could give rise to this sequence pattern. That is, there are four leucine residues, underlined in Figure 2B, that do not have another leucine residue immediately upstream of it (toward the N terminus) or within 10 residues downstream from it (toward the C terminus). Among these four potential cleavage sites, however, the removal of 3 to 4 kD by the SPP is consistent only with the cleavage site being between residues 35 and 36. If this is the cleavage site, then an additional peak of radioactivity should have been released after the seventeenth cycle. A small broad peak was observed between cycles 17 through 20. Thus, all of the data are consistent with the conclusion that the SPP processing site is between residues 35 and 36.

The consensus cleavage site for the stromal processing peptidase is (VII)-X-(A/C)IA (where X is any amino acid; Gavel and von Heijne, 1990). In addition, arginine residues are common between positions -6 and -10 from the cleavage site. The determined SPP processing site for prOEP75 only weakly resembles the consensus cleavage site in that a cysteine residue is present at position -1 and an arginine residue is present at position -7. In all previously known cases in which a cysteine residue is present at position -1, an isoleucine residue

Figure 1. prOEP75 Is Processed to iOEP75 by the SPP

(A) The radiolabeled prOEP75 translation product (Tr) was incubated with chloroplasts during a 30-min import assay (see Methods). The products of the import assay (C, chloroplasts without fractionation) were analyzed by SDS-PAGE and fluorography. (B) and (C) Radiolabeled prOEP75 or prSS, respectively, was incubated with or without stromal extract for 90 min at room temperature. The stromal extract was prepared as described in Methods. Unlabeled mSS or prSS (1.8 μM final concentration) was added as a competitor to the reactions as indicated; (+) indicates the presence of the component, and (−) indicates the absence of the component. Products of the reactions were analyzed by SDS-PAGE and fluorography.
is present at position -3 (Gavel and von Heijne, 1990). The cleavage site of prOEP75 is an exception to this observation.

Having determined the site at which the SPP cleaved the transit peptide to generate iOEP75, we were able to subdivide prOEP75 into three regions, the N- and C-terminal regions of the transit peptide (n75 and c75, respectively) and the mature region (m75). A schematic diagram showing these divisions and the nomenclature that we use throughout this article is given in Figure 3A. After subdividing the transit peptide into two regions, we were able to begin addressing the function of each region. To do so, we created a deletion mutant construct from prOEP75 and chimeric constructs between domains of prOEP75 and the transit peptide (tSS) and mSS, respectively, as shown in Figure 3B. To name these chimeric constructs, we simply listed, in order and joined by hyphens, the regions of the parent proteins that are present in the construct. For example, the construct that has the C-terminal domain of the pr75 transit peptide inserted between the transit peptide and mature regions of prSS was named tSS-c75-mSS. By using in vitro assays to analyze the import characteristics of these constructs, we were able to make inferences regarding the function of each of the three regions of pr75 in its import pathway (described below).

**Figure 2.** The SPP Cleaves prOEP75 between Residues 35 and 36.

(A) ³H-leucine-labeled prOEP75 was incubated with chloroplasts, and import was allowed to occur. The resultant iOEP75 was isolated by SDS-PAGE, blotted onto a ProBlott membrane, and subjected to protein sequencing. Radioactivity released after each cycle of Edmann degradation was determined by liquid scintillation spectroscopy.

(B) Shown is the amino acid sequence of the prOEP75 transit peptide. All of the leucine residues are in boldface; leucine residues that do not have another leucine immediately upstream from them or within the 10 residues downstream from them are underlined. The vertical arrow indicates the deduced stromal processing site.

**Figure 3.** Schematic Diagram of prOEP75, prSS, and a C-Terminal Truncation of prOEP75, and the Nomenclature for Domains within the Precursors.

The lengths of the rectangles representing the proteins and the domains within the proteins are drawn to scale.

(A) prOEP75 (pr75) is divided into its transit peptide (t75) and mature domain (m75). The transit peptide is further divided at the stromal processing site into N-terminal (n75) and C-terminal (c75) domains. iOEP75 (i75) consists of the C-terminal domain of the transit peptide and the mature domain. The black rectangle within c75 shows the location of a hydrophobic stretch of amino acids.

(B) prSS is divided into its transit peptide (tSS) and mature domain (mSS).

(C) A C-terminal truncation of pr75 was obtained by creating a stop codon in the pr75 cDNA clone (see Methods).
protein targeted to the thylakoid lumen was recovered almost exclusively in the thylakoid fraction (Figure 4E).

Similar import and fractionation analyses were performed with the reciprocal construct n75-mSS (Figure 4C). When n75-mSS was incubated with chloroplasts under import conditions, the protein was processed to a product of the expected size for mSS. Although the efficiency of import was low, the product was resistant to external protease, verifying that import had occurred (data not shown). Most (approximately three-fourths) of the processed product (mSS) was recovered in the soluble fraction. The fractionation pattern of the mature product is very similar to that produced upon fractionation of mSS derived from imported prSS (Figure 4D).

Analysis of the import of n75-mSS and tSS-i75 led to two conclusions: (1) n75 is capable of targeting a passenger protein to the chloroplastic import apparatus, and (2) the main targeting function of n75 is to target t75 to the import apparatus.

Figure 4. n75 Is Functionally Interchangeable with tSS.

The radiolabeled precursor was incubated with chloroplasts under import conditions. Intact chloroplasts were then repurified through a Percoll cushion. Two-ninths of the reaction was analyzed directly (C, chloroplasts before fractionation). The remaining seven-ninths were lysed and separated over sucrose gradients into soluble (S), envelope (E), and thylakoid (T) fractions. All samples were analyzed by SDS-PAGE and fluorography. Tr indicates one-forty-fifth the volume of the translation product that was added to the initial reaction in (A), (B), (D), and (E) or one-nineth of the volume added to (C).

(A) Import and fractionation of tSS-i75.
(B) Import and fractionation of pr75.
(C) Import and fractionation of n75-mSS.
(D) Import and fractionation of prSS.
(E) Import and fractionation of prPC (precursor to plastocyanin).

Apparently, then, pr75 initially follows the general chloroplastic import pathway, which normally would lead to the translocation of the precursor across the envelope. Information that causes divergence of prOEP75 from this generalized import pathway must be contained within i75.

Intraorganellar Targeting Information Is within c75

To determine whether c75 is responsible for diverting prOEP75 from the generalized import pathway, we deleted this region from pr75 to obtain n75-m75. Also, we fused tSS directly to m75. Results from import and fractionation analyses of these precursors are shown in Figure 5. n75-m75 was processed to a product of the same size as m75 (Figure 5A). Fractionation analysis revealed that the product was present in both the soluble and the membrane fractions. Also, some precursor was recovered in the membrane fractions. This precursor is most likely associated with the exterior face of the outer envelope membrane because it was susceptible to external protease (described below). n75-m75 was not efficiently imported or processed, as was observed for n75-mSS. Thus, n75 was not an efficient chloroplastic targeting domain when taken out of its native context, that is, when c75 was not adjacent to it. Nonetheless, it was apparent that without c75, a significant portion (approximately one-fourth) of m75 was not correctly targeted to the envelopes but rather to a soluble fraction.

Figure 5. c75 Contains Intraorganellar Targeting Information.

(A) and (B) Import and fractionation of n75-m75 and tSS-m75, respectively, as described in the legend to Figure 4. Tr indicates one-nineth in (A) or one-forty-fifth in (B) the volume of translation product added to the reaction.

(C) Import of i75. Radiolabeled i75 was incubated with chloroplasts under import conditions. After 5 min (5'), chloroplasts from one-third of the reaction were repurified through a Percoll cushion. The remaining chloroplasts were repurified through a Percoll cushion after 20 min (20'). Half of these were then treated with thermolysin (0.2 mg per mL for 30 min on ice) and pelleted. All samples were analyzed by SDS-PAGE and fluorography. Tr, one-thirtieth the volume of translation product added to the reaction; Th, sample that was treated with thermolysin after import.
Comparison of results obtained from import and fractionation of tSS-m75 with those obtained with tSS-i75 also led to the conclusion that c75 contains intraorganelar targeting information. As shown in Figure 5B, tSS-m75 was efficiently imported, and the majority of the product, presumably m75, was in the soluble fraction. Some m75 was also recovered in the envelope and thylakoid fraction, although it is not visible in Figure 5B. Comparison of Figures 5A and 5B with Figures 4B and 4A, respectively, reveals that c75 has a profound effect on the targeting of m75. Thus, we concluded that c75 is at least partly responsible for diverting pr75 from the generalized import pathway.

The recovery of the products during fractionation of n75-m75 and tSS-m75 was inefficient. In Figures 5A and 5B, compare the amount of product in the stromal fraction, lanes S, with the amount in the whole chloroplast sample, lanes C. A similar comparison with prSS, in Figure 4D, shows a much better recovery of product. We suspected that if m75 were being targeted to the stroma, it would be degraded by endogenous proteases. However, incubation of intact chloroplasts at room temperature for 15 min after an import reaction with n75-m75 did not result in an appreciable loss of the product (data not shown). Another possibility is that m75 was lost from the soluble fraction during acetone precipitation of that fraction. In fact, only 70 to 85% of m75 in the soluble fraction was recovered when precipitated with acetone (data not shown). Thus, the results presented in Figures 5A and 5B underestimate how much of the product was actually in the soluble fraction.

Can c75 alone direct m75 to the chloroplastic envelope? The results presented in Figure 5C indicate that it cannot. When incubated with chloroplasts, only a small percentage of i75 bound to the chloroplasts, and no processing of i75 to m75 was observed. Furthermore, all of the bound i75 was susceptible to external protease, indicating that the protein was not incorporated into the outer envelope membrane.

The data presented thus far suggest separate functions for n75 and c75. n75 appears to act as a typical transit peptide in that it directs the precursor into the general chloroplastic import pathway. c75, in a subsequent step, causes the precursor to diverge from this general pathway. Thus, i75 is best described as a bipartite transit peptide and is analogous to the bipartite transit peptides of thylakoid lumen proteins. The N-terminal portion of the transit peptide targets the precursor to the chloroplast, whereas the C-terminal portion of the transit peptide contains intraorganelar targeting information.

To test more rigorously whether c75 contains information for diverting m75 from the generalized import pathway, we attempted to use this domain to divert prSS from the generalized pathway. The c75 domain was inserted into prSS to obtain tSS-c75-mSS. Results presented in Figure 6A reveal that when incubated with chloroplasts, tSS-c75-mSS was processed into two major products. These two products are similar in size and of the approximate size expected if prSS were removed. It is likely that a second processing site was created at the junction of tSS and c75. Minor changes in the primary structure in the vicinity of precursor processing sites can lead to aberrant processing (Wasmann et al., 1988; Archer and Keegstra, 1993). When fractionated, both products behaved the same and are collectively referred to as c75-mSS. Approximately half of c75-mSS was recovered in the envelope and thylakoid fractions. We interpret this to mean that c75 diverted some of the precursor from the generalized import pathway.

The failure to direct more of the product to the envelope may be attributed to the fact that n75 plays some role in envelope targeting. However, the results presented in Figure 6B indicate that this is not the case. When t75-mSS was incubated with chloroplasts, the major product, presumably c75-mSS, gave a similar fractionation pattern, as did c75-mSS when derived from tSS-c75-mSS. That is, about half of the product was in the soluble fraction and the other half was in the membrane fractions. Why is all of the product not targeted to the envelope fraction? Perhaps m75 contains information to direct itself to the envelope. Alternatively, m75 may not contain specific targeting information but is necessary to anchor the protein to the outer envelope membrane once it is targeted there.

A cDNA clone encoding a C-terminal truncation of pr75, designated pr75\(\Delta\)251 and schematically represented in Figure...
3C, was constructed (see Methods). This cDNA clone encodes the first 246 residues of pr75, followed by codons for cysteine, isoleucine, asparagine, valine, and a stop codon. Results from import and fractionation analyses of pr75Δ251 are given in Figure 6C. The major product derived from pr75Δ251 was no more efficiently targeted to the envelope than was the major product derived from t75-mSS. Thus, either additional targeting information is present within residues 247 to 809 of m75 or i75Δ251 is not able to anchor itself efficiently in the outer envelope membrane.

Import of t75-mSS and pr75Δ251 resulted in the accumulation of lower molecular weight products in addition to the major products described above (Figures 6B and 6C). The identity of these products is not known. They may have resulted from subsequent processing of c75, or they may be degradation products.

c75 Prevents Translocation across the Inner Envelope Membrane

Why were the major import products from tSS-c75-mSS, t75-mSS, and pr75Δ251 recovered in both soluble and membrane fractions? One explanation is that c75 merely slows translocation, allowing the passenger protein to anchor itself in the outer envelope membrane. In the absence of such an anchor (e.g., with mSS as the passenger protein), the protein might be fully imported into the stroma. Alternatively, c75 may completely block translocation into the stroma, in which case the import products recovered in the soluble fraction would not be in the stroma but rather in the intermembrane space. In either case, the products recovered in the membrane fractions might be due to the specific association of c75 with the envelopes.

To determine whether the soluble import products derived from tSS-c75-mSS, t75-mSS, and pr75Δ251 were in the stroma or intermembrane space, we determined their susceptibility to trypsin. Under controlled conditions, trypsin gains access to the intermembrane space but not to the stroma (Cline et al., 1981; Lübeck et al., 1996; Scott and Theg, 1996). After incubation with precursor proteins, chloroplasts were repurified through a Percoll cushion and divided into three aliquots. Each aliquot was then incubated with or without trypsin or with thermolysin, as described in Methods. Thermolysin treatment was included so that we could distinguish between proteins that had crossed the outer membrane and those that were peripherally associated on its exterior. After the protease reactions, protease inhibitors were added, and intact chloroplasts were repurified, washed, and analyzed by SDS-PAGE and fluorography. Figure 7 shows the results of the protease analysis.

mSS derived from prSS was resistant to both thermolysin and trypsin (Figure 7A), verifying that neither of the proteases were gaining access to the stroma. m75 and i75 derived from pr75 were very susceptible to trypsin and partly susceptible to thermolysin (Figure 7B), verifying that both proteases were active under our experimental conditions. The major import products derived from tSS-c75-mSS, t75-mSS, and pr75Δ251 behaved identically to each other in terms of their susceptibility to thermolysin and trypsin (Figures 7C to 7E). In each case, the products were resistant to thermolysin but susceptible to trypsin. This is similar to what was observed for m75 derived from pr75. m75 was recovered in the membrane fractions; however, only about half of the products derived from tSS-c75-mSS, t75-mSS, and pr75Δ251 were recovered in the soluble fractions (described above). Thus, we concluded that the major soluble import products derived from tSS-c75-mSS, t75-mSS, and pr75Δ251 are in the intermembrane space.
m75 derived from imported n75-m75 or tSS-m75 was resistant to both thermolysin and trypsin (Figures 7F and 7G), indicating that m75 had been translocated into the stroma. This finding is consistent with the idea that the targeting function of n75 is to initiate pr75 on the generalized import pathway and that c75, not m75, is primarily responsible for causing divergence from the generalized import pathway.

Investigation of the Targeting Information within c75

Earlier, we speculated that a hydrophobic region within c75 (denoted schematically in Figure 3A) may act as a stop-transfer domain and thereby direct m75 to the outer envelope membrane (Tranel et al., 1995). Having shown that c75 contains intraorganellar targeting information, we wanted to determine whether in fact the hydrophobic region was important to this function. Site-directed mutagenesis was performed to replace some of the hydrophobic residues within this region with hydrophilic residues. Specifically, the alanine residues at positions 61 and 66 and the leucine residue at position 72 were changed to arginine residues. (During mutagenesis, an off-site mutation also occurred, changing tyrosine at position 76 to proline [see Methods].) The hydrophobicity profiles of the transit peptides of wild-type pr75 and the mutant (designated pr75mut4) are presented in Figure 8A. As shown in Figure 8B, despite the decreased hydrophobicity of pr75mut4, its fractionation pattern after an import reaction was indistinguishable from that of wild-type pr75. A mutation of just one hydrophobic residue (pr75A66R) also did not affect targeting (Figure 8C). Thus, it appears that the overall hydrophobicity of c75 is not important to its targeting function.

The hydrophobic region within c75 was predicted to form an α-helical structure (data not shown). To determine whether this putative structure is important to the function of c75, we used site-directed mutagenesis to change the alanine at position 66 to a proline. The proline should disrupt the potential α helix. Results from an import and fractionation experiment with this mutant (pr75A66P) revealed that if the region does form an α helix, it is likely not important to the targeting function of c75 (Figure 8D).

Although the mutations had no effect on targeting, they did affect the import rates. It is difficult to make precise conclusions about the differences in import rates because the differences are not large and there is an inherent variability among experiments. Four replications of a time course experiment were performed, and representative results are shown in Figure 9. The most obvious kinetic effects of the mutations were not on the accumulation of the i75 but rather on the subsequent processing of i75 to i275 and m75. i275 is a second size intermediate that is transiently observed during the import of pr75 (Tranel et al., 1995). Compared with the other mutations, the A66P mutation caused the most dramatic decrease in the rate of subsequent processing of i75. The most pronounced effect of the mut4 mutation seemed to be an increase in the accumulation of i275. The import kinetics of two other constructs, pr75A61R:A66R and pr75A66R:L72R, were also analyzed. Import rates of these constructs (data not shown) were similar to that seen for pr75A66R.

DISCUSSION

pr75 Contains a Bipartite Transit Peptide

There are two distinct targeting pathways for nuclear-encoded proteins of the chloroplastic outer envelope membrane. One pathway is transit peptide independent; the other is transit peptide dependent. The transit peptide–independent pathway, exemplified by OEP7 (E6.7) and OEP14 (OM14) (Salomon et al., 1990; Li et al., 1991), does not require ATP or chloroplastic proteins exposed to the cytosol. Thus, “import” of these proteins appears to consist of direct insertion of the proteins into the membrane.

There are two known proteins of the outer envelope membrane that follow the transit peptide–dependent pathway: OEP75 and OEP86 (Hirsch et al., 1994; Tranel et al., 1995). The import pathways of prOEP86 and pr75 are like those of stromal precursor proteins in that they require ATP and one or more envelope proteins exposed to the cytosol. The import pathway of prOEP86 does not appear to overlap with that of...
Further support for this conclusion comes from a representative experiment. The experiment was replicated four times. The results presented are the chloroplastic targeting domain of pr75 is inefficient but interchangeable with tSS. Although n75 appeared to have the same function as tSS, it was not as efficient as tSS in preserving the general chloroplastic protein import apparatus and initially follows the generalized import pathway. Consistent with this conclusion, pr75 was processed by the SPP.

The peptide that was removed from pr75 by SPP, n75, acted as a chloroplastic targeting domain and was functionally interchangeable with tSS. Although n75 appeared to have the same function as tSS, it was not as efficient as tSS in performing this function. For example, n75-mSS was imported with very low efficiency. However, import of tSS-n75 was no more efficient than the import of pr75. Thus, it does not appear that the chloroplastic targeting domain of pr75 is inefficient but rather that n75 does not represent the complete chloroplastic targeting domain of pr75. Further support for this conclusion comes from the observation that t75-mSS was efficiently imported. We used the SPP cleavage site of pr75 to subdivide the transit peptide into the n and c domains. This division is somewhat arbitrary in the sense that the functions of the two domains may overlap. The actual chloroplastic targeting domain of pr75 may consist of n75 plus the first few N-terminal residues of c75. Alternatively, n75 may contain all of the primary sequence information to act as a chloroplastic targeting domain, but c75 may assist in presenting this domain in a configuration that is recognized by the import apparatus. Regardless, the stromal processing site serves as a convenient division point of the functional domains of t75, and the experiments described above indicate that it is, to a first approximation, an accurate division point of the functional domains.

Until it is processed by the SPP, pr75 apparently follows the general chloroplastic protein import pathway. We provided evidence that diversion of pr75 from this pathway is accomplished by c75. Our evidence in support of this conclusion is twofold: (1) if c75 is deleted from pr75 or from tSS, m75, much of the imported m75 no longer cofractionates with envelope membranes but instead is imported into the stroma; and (2) if c75 is inserted between tSS and mSS, upon import, the major products do not cross the inner envelope membrane.

Once diverted from the generalized import pathway, m75 must fold and assemble in the outer envelope membrane. The fact that m75 derived from n75-m75 or tSS-m75 was targeted to the stroma indicates that m75 does not contain intraorganellar targeting information. Why over half of the m75 derived from n75-m75 was recovered in the membrane fractions is unclear. Very little, if any, of this m75 was susceptible to trypsin, so apparently it was on the stromal side of the inner envelope membrane.

c75 evidently carries all of the intraorganellar targeting information to direct m75 to the outer envelope membrane. Thus, we refer to c75 as an outer membrane targeting domain; however, c75 may not specifically interact with the outer envelope membrane. In fact, we posit that c75 targets m75 to the outer membrane by interacting with the inner envelope membrane to prevent translocation into the stroma. Once translocation is halted, m75 is then able to assemble in the outer envelope membrane. If m75 is replaced with a non-membrane protein (e.g., mSS), then the protein is targeted to the intermembrane space. Exactly how c75 prevents translocation into the stroma remains to be determined. Mutagenesis analysis indicated that c75 most likely does not simply interact with membrane lipids via a hydrophobic region but rather that specific protein-protein interactions are probably involved. We determined that c75 has a strong affinity for the envelope because approximately half of the c75-mSS derived from either tSS-c75-mSS or t75-mSS was recovered in the membrane fractions.

Whether the c75-mSS recovered in the membrane fractions was associated with the outer or inner envelope membrane is not certain. It seems most likely that c75-mSS was associated with the inner envelope membrane because c75-mSS was protected from thermolysin but not trypsin and because the N terminus of its precursor was removed in the stroma.

**Several Steps for the Assembly of m75 in the Outer Envelope Membrane Have Yet to Be Elucidated**

Once translocation of pr75 is halted, the steps occurring to fold and assemble m75 in the outer envelope membrane are not known. The final topology of m75 in the membrane also remains to be determined. As previously discussed (Schnell et al., 1994; Tranel et al., 1995), m75 behaves as an integral membrane protein, with much of the protein apparently embedded within the membrane. m75 does not contain predicted membrane-spanning a helices and has been postulated to span the membrane with multiple amphipathic b strands. These b strands may come together to form a b-barrel structure, similar to that formed by porins (reviewed in Benz, 1994). pr75Δ51 was the only construct that we created containing only a portion of m75. Import and fractionation analyses of other constructs containing larger portions of m75 may be useful...
in determining which regions of m75 are needed to anchor it to the outer envelope membrane and in understanding the final topology of m75 in the membrane.

In addition to the specific details of how c75 functions and how m75 assembles in the outer envelope, the processing step of i75 to m75 is unclear. Both in vitro and in vivo, this step appears to be rate limiting (Tranel et al., 1995). Where in the chloroplast is the protease that performs this step and what regulates it? We modified the stromal processing assay in various ways in an attempt to obtain processing of pr75 to m75. For example, we included envelope vesicles with and without solubilization by Triton X-100, but processing was only to i75.

During the time course of in vitro import of pr75, i75 appears first, followed by a second size intermediate, i275, and finally by m75 (Tranel et al., 1995). i275, which migrates slightly more slowly than m75 during SDS-PAGE, is present only transiently and typically is not detected after a 30-min import reaction. Import of pr75mut4 resulted in the accumulation of a protein (designated i275mut4) with the same mobility as i275. Because of its transient nature during the import of pr75, we know little about i275 and are unsure of its physiological significance. pr75mut4 may be a useful tool to learn more about i275.

A more complete understanding of the pr75 import pathway may yield practical applications. The discovery that the N terminus of nuclear-encoded chloroplastic proteins serves as a chloroplast-targeting signal made it possible to direct recombinant heterologous proteins to the stroma (Van den Broeck et al., 1985). Most chloroplastic OEPs contain their targeting information within the mature portion of the protein. Thus, it is difficult to decipher what the targeting information is, and it is even more difficult to incorporate that information into a recombinant protein without altering that protein's function. Our findings indicate that it may be possible to use c75 as an intraorganellar targeting signal either to target recombinant membrane proteins to the chloroplast outer envelope membrane or to target recombinant soluble proteins to the intermembrane space.

The Import Pathway of pr75 Has Parallels with That of Some Mitochondrial Proteins

Of known mitochondrial outer envelope proteins, the import pathway of yTom70 (Mas70p; see Pfanner et al., 1996) has been the most studied (reviewed in Shore et al., 1995). Like all other known proteins of the mitochondrial outer envelope membrane, yTom70 is not synthesized as a higher molecular weight precursor. Nonetheless, yTom70 may engage the general mitochondrial import apparatus. The extreme N terminus (residues 1 to 12) of yTom70 acts as a weak mitochondrial targeting domain (Hase et al., 1984; Hurt et al., 1985). Immediately following this domain is a stop-transfer domain (Nakai et al., 1989). Disruption of the stop-transfer domain results in mis-targeting of some yTom70 to the matrix. Analogously, removal of c75 from pr75 resulted in targeting of m75 to the stroma.

NADH-cytochrome b5 reductase is present as two different isoforms in different locations of yeast mitochondria (Hahne et al., 1994). The higher and lower molecular weight isoforms are in the outer envelope membrane and the intermembrane space, respectively. A "leaky stop-transfer" mechanism has been proposed to account for the two isoforms. According to this model, the N terminus of NADH-cytochrome b5 reductase is a matrix targeting domain. Following this domain is a stop-transfer domain. Some of the protein follows an import pathway identical to that of yTom70, resulting in the higher molecular weight isoform in the outer envelope membrane. Some of the protein, however, "leaks" past the outer envelope membrane due to the matrix targeting domain overcoming the effects of the stop-transfer domain. The N terminus is then removed by inner membrane protease 1, resulting in the lower molecular weight isoform in the intermembrane space.

Both NADH-cytochrome b5 reductase and yTom70 are similar to pr75 in that they are targeted to the outer envelope membrane of their respective organelle by an N-terminal organellar targeting domain. This N-terminal domain is not removed in the case of yTom70. In the case of NADH-cytochrome b5 reductase, the N-terminal domain is removed only when the protein is targeted to the intermembrane space. Furthermore, the processing occurs in the intermembrane space. We know of only one protein, pr75, in either chloroplasts or mitochondria for which a processing event takes place in the stroma (or matrix), yet the final destination of the mature protein is the outer envelope membrane.

METHODS

In Vitro Transcription and Translation

Radiolabeled proteins were generated from cDNAs by using standard in vitro transcription and translation procedures (Bruce et al., 1994). For the determination of the stromal processing site in pr75, pr75 was labeled with 3H-leucine. 35S-Methionine was used to label pr75 for use in all of the other experiments and to label all of the other proteins. Radiochemicals were purchased from Du Pont-New England Nuclear, and all other reagents were purchased from Promega. Translation procedures were performed with nuclease-treated rabbit reticulocyte lysate by using the standard reaction suggested by the manufacturer, that is, 66% final concentration of lysate (v/v) and Mg2+ and K+ concentrations not being altered.

Stromal Processing Assay

The stromal processing assay was conducted essentially as described by Abad et al. (1989). Chloroplasts were resuspended at 0.5 mg of chlorophyll per mL in 5 mM Hepes-KOH, pH 8, and incubated on ice for 30 min. The lysed chloroplasts were centrifuged at 200,000 g for 30 min at 4°C. The resultant supernatant was the stromal extract. The precursor protein (75,000 dpm of pr75 or prSS) was incubated with or without 15 μL of stromal extract for 90 min at room temperature. The total reaction volume was 30 μL and contained 5 mM Hepes-KOH,
the sequence of a resultant mutant clone, the EcoRV fragment from the fourth and fifth codons for i75 by using the oligonucleotide 5'-GGA-

Preparation of Mutant and Chimeric cDNA Clones

Site-directed mutagenesis was performed using the method of Kunkel et al. (1987). cDNA inserts subjected to mutagenesis were sequenced by automated fluorescent sequencing by using the ABI (Foster City, CA) Catalyst 800 for Taq cycle sequencing and the ABI 373A sequencer for the analysis of products. Subcloning and other DNA manipulations were performed by using standard procedures (Maniatis et al., 1982).

Chimeric proteins containing portions of pr75 and prSS were obtained from in vitro transcription and translation of cDNA clones. These clones were obtained by subcloning portions of the parent cDNA clones encoding pr75 and prSS. The parent clones for pr75 and prSS were pET11-d-prSSU (Klein and Salvucci, 1992) and p214 (Tranel et al., 1995), respectively. To facilitate subsequent cloning steps, the cDNA insert from pET11-d-prSSU was excised from the vector by XbaI and EcoRV digestion and directionally cloned into pBluescript II SK+ (Stratagene) digested with XbaI and EcoRV. The resulting clone encoding prSS was designated p46.

Preparation of Mutant cDNA Clones Encoding pr75

p46 contains a unique Ball site that is located between the fourth and fifth codons for mSS. Site-directed mutagenesis was used to introduce a unique HpaI site into p214 between the fourth and fifth codons for m75. First, the Sacl-EcoRV fragment of p214 was cloned into pBluescript II SK+ digested with Sacl and EcoRV to generate clone p44. This was done so that after mutagenesis, only a small region would need to be sequenced. Mutagenesis was performed on single-stranded DNA obtained from p44 by using the oligonucleotide 5'-GGGCGAGCGCTGGAAACACGGACGGTGGT-3' were used, respectively. To generate subsequent cloning steps, the cDNA insert in the resultant clone (p56) encoded pr75, except that the fourth and fifth residues of m75 were changed from lysine to valine and from serine to asparagine, respectively. To generate cDNAs encoding either n75-mSS or tSS-i75, the Sacl-Ball fragment from p46 and the Sacl-EcoRV fragment from p72 were exchanged with each other.

Preparation of cDNA Clones Encoding n75-mSS, tSS-i75, tSS-c75-mSS, n75-m75, and i75

p52 was further mutagenized to introduce an Eco47III site between the fourth and fifth codons for i75 by using the oligonucleotide 5'-GGGATCCTCGGATCCATAGCGCTAGAAGAGGTGAC-3'. After verifying the sequence of a resultant mutant clone, the EcoRV fragment from p214 was cloned into the EcoRV site of p52. Restriction mapping was used to select a clone that contained the EcoRV fragment in the proper orientation. The insert in the resultant clone (p56) encoded pr75, except that the fourth and fifth residues of m75 were changed from lysine to valine and from serine to asparagine, respectively. To generate cDNAs encoding either i75-mSS or iSS-m75, the Sacl-Ball fragment from p46 and the Sacl-HpaI fragment from p56 were exchanged with each other.

Preparation of a cDNA Clone Encoding pr75A251

To obtain a cDNA encoding a C-terminal truncation of pr75, p214 was digested with NsiI, the protruding 3' overhang was removed, and the plasmid was religated. This manipulation changed the reading frame such that a stop codon was introduced after amino acid 250. Also, serine at position 247 was changed to a cysteine, methionine at 248 to an isoleucine, and leucine at 249 to asparagine. The valine at position 250 was unchanged. The protein encoded by this cDNA was designated pr75A251.

Mutagenesis of the Hydrophobic Domain within c75

Mutagenesis of the region encoding the hydrophobic domain of p75 was performed on the Sacl-EcoRI fragment of p214 subcloned into pBluescript II SK+ (Bruce et al., 1994). Chloroplasts from 8- to 12-day-old pea (Pisum sativum var Little Marvel) seedlings were isolated over Percoll gradients and resuspended in import buffer at 1 mg of chlorophyll per mL.

For determination of the stromal processing site in p75, 1.2 x 10^6 cpm of [3H]leucine-labeled p75 was added to a 40-μL chloroplast sus-
pension in a final reaction volume of 350 μL. The final ATP concentration was 3 mM. Import was allowed to occur for 30 min at room temperature, after which intact chloroplasts were repurified through a cushion of 40% (v/v) Percoll in import buffer. The chloroplast pellet was lysed by resuspending it in 25 mM Hapes-KOH, pH 8, and incubating it on ice for 10 min. Membranes from the lysed chloroplasts were collected by centrifugation at 435,000g for 10 min at 4°C and then resuspended in 2 x sample buffer. The sample was subjected to SDS-PAGE and then blotted onto a ProBlott (Applied Biosystems, Foster City, CA) membrane. The membrane was exposed to film, and the region of the membrane corresponding to i75 was cut out.

For the import and fractionation experiments, 5 × 10^6 dpm of precursor protein (10^5 dpm for n75-mSS and n75-m75) was added to a 150-μL chloroplast suspension in a final reaction volume of 450 μL. The final ATP concentration was 3 mM. For import reactions with proteins containing m75, the final rabbit reticulocyte lysate concentration was 9% (v/v). (Previous experiments indicated that the optimal import of m75-bearing precursors occurred when rabbit reticulocyte lysate was present at this concentration.) Import was allowed to occur for 30 min at room temperature. After import, chloroplasts from 100 μL of the reaction were repurified through a cushion of 40% Percoll in import buffer, resuspended in 2 x electrophoresis sample buffer, and analyzed directly. Chloroplasts from the remaining 350 μL of the reaction were repurified and lysed as described above. The lysed chloroplasts were then separated by density centrifugation over sucrose step gradients into soluble, envelope, and thylakoid fractions, as described previously (Tranel et al., 1995). Samples were analyzed by SDS-PAGE and fluorography. Equivalent amounts (on a chloroplast basis) of soluble and envelope fractions and one-fourth the equivalent of the thylakoid fraction were analyzed.

Import reactions for the protease analysis were set up as described for the fractionation analysis. After import, chloroplasts were repurified through a cushion of 40% Percoll in import buffer, resuspended in import buffer, and divided into three aliquots. One aliquot was incubated for 60 min at 20°C in the presence of 25 μg of trypsin (11,400 BAEE units per mg; a BAEE unit is one unit of activity using Na-benzoyl-L-arginine ethyl ester as substrate) and 0.1 mM CaCl2. A second aliquot was incubated under the same conditions but without trypsin. The third aliquot was incubated 30 min at 4°C in the presence of 10 μg of thermolysin and 1 mM CaCl2. Final protease reaction volumes were 100 μL. The trypsin and thermolysin reactions were stopped by the addition of 150 μg of soybean trypsin inhibitor and 5 mM EDTA. The protease- and mock-treated samples were performed in the presence of soybean trypsin inhibitor (0.5 mg/mL) and EDTA (5 mM), respectively. Subsequent manipulations of the trypsin- and thermolysin-treated samples were performed in the presence of soybean trypsin inhibitor (0.5 mg/mL) and EDTA (5 mM), respectively. The protease/mock-treated chloroplasts were repurified through a cushion of 40% Percoll in import buffer and washed twice with 1 mL of import buffer. After the final wash, the chloroplast pellets were resuspended in 50 μL of electrophoresis sample buffer and boiled immediately. The chlorophyll concentration was determined by absorbance (A760), and equal quantities of chloroplasts (10 μg of chlorophyll) were analyzed by SDS-PAGE and fluorography. Quantitation of recovered products from the various import experiments was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Protein Sequencing

3H-leucine–labeled i75 was sequenced with an ABI-494 protein sequencer. Radioactivity released after each cycle was quantitated by liquid scintillation spectroscopy.

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