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A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway

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A chloroplastic outer envelope membrane protein of 75 kDa (OEP75) was identified previously as a component of the protein import machinery. Here we provide additional evidence that OEP75 is a component of protein import, present the isolation of a cDNA clone encoding this protein, briefly describe its developmental expression and tissue specificity, and characterize its insertion into the outer envelope membrane. OEP75 was synthesized as a higher molecular weight precursor (prOEP75) which bound to isolated chloroplasts in an *in vitro* import assay and subsequently was processed to the mature form (mOEP75). During this import assay, two proteins intermediate in size between prOEP75 and mOEP75 were detected. One of these intermediates was also detected in chloroplast envelopes isolated from young pea leaves. Binding and processing of prOEP75 required ATP and one or more surface-exposed proteinaceous components, and was competed by prSSU, a stromal-targeted protein. We propose that the N-terminus of the prOEP75 transit peptide acts as a stromal-targeting domain and a central, hydrophobic region of this transit peptide acts as a stop-transfer domain. A complex route of insertion and processing of prOEP75 may exist to ensure high fidelity targeting of this import component.

Key words: chloroplast/envelope membrane/plastid biogenesis/protein import/transit peptide

Introduction

Plastids are vital, plant-specific organelles that perform various biochemical processes. The predominant plastids in leaves are chloroplasts, which must undergo rapid biogenesis during leaf development. Because many chloroplastic proteins are nuclear encoded and synthesized in the cytoplasm, chloroplast biogenesis is dependent upon a protein import apparatus that recognizes these proteins and translocates them across the plastid's double-membrane envelope.

Many nuclear-encoded chloroplastic proteins, including those destined for the stroma, thylakoid membrane and thylakoid lumen, apparently use a common envelope import apparatus (for review, see de Boer and Weisbeek, 1991; Theg and Scott, 1993). Specific recognition of these proteins by that apparatus is achieved through an N-terminal transit peptide which is removed during or shortly after import. A similar, if not identical, import apparatus is thought to exist in all plastid types. To better understand plastid biogenesis, attempts have been made to identify components of the import apparatus, with a longer term goal of isolating these components and investigating their function at the molecular level. An additional question of interest in plastid biogenesis is how the components of the import apparatus themselves are targeted to and assembled within plastids.

A protein import apparatus analogous to that in chloroplasts exists in mitochondria (for review, see Baker and Schatz, 1991; Schatz, 1993). Like its chloroplastic counterpart, the mitochondrial import apparatus recognizes cytoplasmically synthesized preproteins via an N-terminal presequence and facilitates their translocation across the mitochondrial double-membrane envelope. Several outer membrane components of this import apparatus have been identified, and the question of how these components are directed to the mitochondria has been addressed (Söllner *et al.*, 1990; Schneider *et al.*, 1991; Keil and Pfanner, 1993; Keil *et al.*, 1993). Outer membrane components of the import apparatus characterized to date do not contain an N-terminal presequence. MOM19, the so-called 'master receptor', is targeted to mitochondria by its association with MOM38, which forms part of the 'general insertion site' (Schneider *et al.*, 1991). MOM72, the main receptor for the ATP/ADP carrier, uses MOM19 for its targeting (Söllner *et al.*, 1990). MOM38 uses both receptors, MOM19 and MOM72, for its targeting (Keil *et al.*, 1993). If MOM38 was mistargeted, MOM19 could be subsequently mistargeted followed by the mistargeting of precursor proteins recognized by MOM19. Thus, the requirement of both receptors for MOM38 import ensures high fidelity targeting of proteins that make up the import apparatus. MOM22, another component of the import complex, also requires both MOM19 and MOM72 for its import (Keil and Pfanner, 1993).

Four different chloroplastic outer membrane proteins, 6.7, 14, 70 and 24 kDa in molecular size, are like the mitochondrial outer membrane proteins in that they do not contain N-terminal transit peptides (Salomon *et al.*, 1990; Li *et al.*, 1991; Ko *et al.*, 1992; Fischer *et al.*, 1994). Unlike chloroplast-targeted, transit peptide-bearing proteins, these outer membrane proteins can be properly targeted to chloroplasts in the absence of outer membrane surface proteins (as determined by chloroplast pretreatment with protease) and thus apparently do not use the general import apparatus. These outer membrane proteins are of unknown function. Only recently have outer membrane protein components of the import apparatus been identified (Waegemann and Soll, 1991; Perry and Keegstra, 1994; Schnell *et al.*, 1994). With the isolation of cDNAs encoding these proteins, their targeting to the outer membrane can be addressed.

Using a chemical cross-linking strategy, two outer membrane proteins of 75 and 86 kDa were identified as components of the chloroplastic protein import apparatus (Perry and Keegstra, 1994). When precursor to the small subunit of ribulose bisphosphate carboxylase/oxygenase (prSSU) was covalently linked to a label-transfer reagent, APDP, and subsequently bound to the chloroplastic import apparatus, photo-activation of APDP resulted in specific labeling of the 75 and 86 kDa proteins. The precursor associated first with the 86 kDa protein and then, in an ATP-dependent step, with the 75 kDa protein. Association with the 75 kDa protein was correlated with the formation of an early translocation intermediate, in which the precursor was associated presumably with contact sites between the outer and inner membranes. It was suggested that the 86 kDa protein is a receptor and the 75 kDa protein is a general insertion site protein (Perry and Keegstra, 1994). Subsequent cross-linking studies were conducted in which precursor to the 23 kDa oxygen-evolving enhancer protein (prOEE23), a thylakoid lumen protein, was substituted for prSSU. Cross-linking with prOEE23 resulted in labeling of the 75 and 86 kDa proteins identical to that seen with prSSU, providing additional evidence that these two proteins were part of a general chloroplastic import apparatus (Tranel and Keegstra, unpublished data).

In this communication, we provide further evidence in support of the assignment of the 75 kDa protein as a component of the protein import apparatus and present the isolation of a cDNA clone encoding the precursor for this protein. Recently, a cDNA clone for the same precursor protein was obtained by Schnell *et al.* (1994). Analysis of the assembly of this precursor protein into the outer membrane indicates that it proceeds via a novel pathway.

Results

Antibodies against OEP75 inhibit protein import into chloroplasts

Using a cross-linking strategy, an abundant chloroplastic outer envelope membrane protein of 75 kDa (OEP75) was implicated as a component of the protein import machinery (Perry and Keegstra, 1994). To provide additional evidence that OEP75 is a component of the chloroplastic protein import machinery, we attempted to block import of a precursor protein with antibodies raised against OEP75. This strategy has been used successfully to provide evidence to support assignments of proteins as mitochondrial import components as well as gain insight into their functions (Vestweber *et al.*, 1989; Kiebler *et al.*, 1993; Moczko *et al.*, 1993). OEP75 was isolated from preparations of outer membrane proteins that were fractionated via SDS–PAGE. Antibodies were raised against the purified protein and IgG molecules were obtained. Low concentrations of these IgG molecules (<1 mg/ml) did not inhibit import of prSSU in an *in vitro* chloroplastic import assay (data not shown). Higher concentrations of IgG molecules aggregated the chloroplasts. Therefore, we prepared Fab fragments from the IgG molecules. When chloroplasts were pre-incubated with 5 mg/ml Fab fragments and subsequently incubated for 10 min with prOEE23, import of this precursor was reduced by 60% (Figure 1A). Under similar conditions, import of prSSU

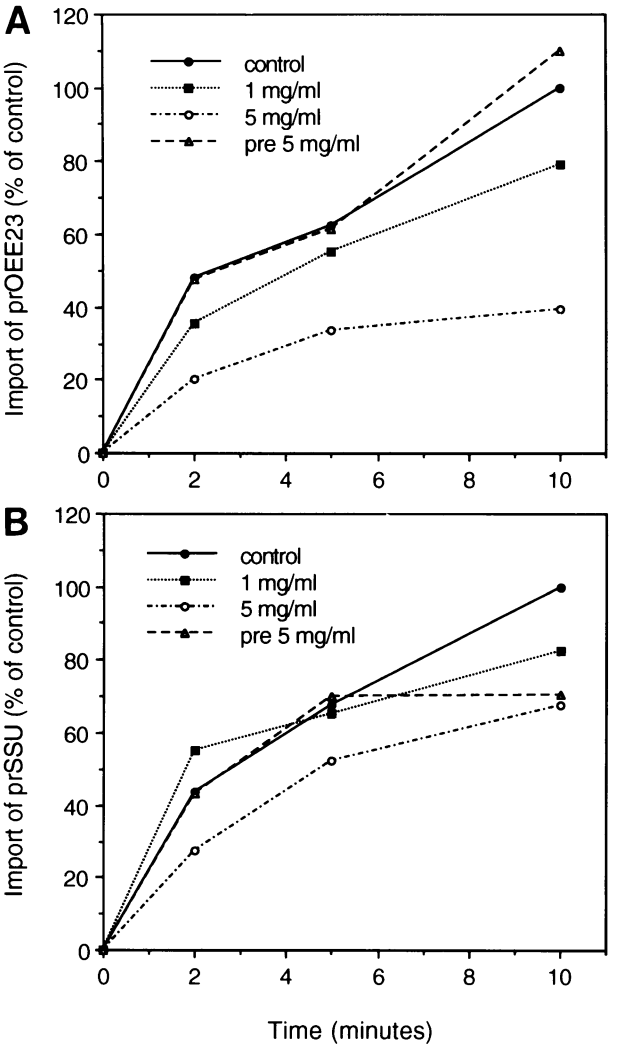

Fig. 1. Inhibition of protein import by Fab fragments derived from anti-OEP75 antibodies. Chloroplasts were pre-treated with Fab fragments in import buffer at 4°C for 1 h and then incubated with radiolabeled precursor under import conditions. After 2, 5 and 10 min, the reaction was stopped by the addition of a 10-fold excess of cold import buffer. Intact chloroplasts were repurified and analyzed by SDS–PAGE and autoradiography. Accumulation of mOEE23 (**A**) and mSSU (**B**) were quantitated with a phosphorimager (Molecular Dynamics).

was reduced by 30% (Figure 1B). Fab fragments (5 mg/ml) isolated from pre-immune serum did not inhibit import of either precursor (Figure 1A and B). Taken together, the findings that OEP75 can be cross-linked to a precursor protein (Perry and Keegstra, 1994) and that Fab fragments against OEP75 inhibit import of precursor proteins strongly suggest that OEP75 is a component of the import machinery.

Isolation of cDNA encoding OEP75

To obtain a cDNA clone for OEP75, an aliquot of the purified protein that was used for raising antibodies (described above) was subjected to tryptic digestion. Individual tryptic peptides were purified and their amino acid sequences determined. Peptide sequence data were used to design a degenerate oligonucleotide probe (see Materials and methods) that was then used to screen a pea cDNA library. Clone p202 was thus isolated and the

B

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 1 M R T S V I P N R L T P T L T T H P S R R R N D H I T T R T
31 S S L K C H L S P S S G D N N D S F N S S L L K T I S T T V
61 A V S S A A A S A F F L T G S L H S P F P N F S G L N A A A
91 G G G A G G G G G G S S S S G G G G G G W F N G D E G S F W
121 S R I L S P A R A I A D E P K S E D W D S H E L P A D I T V
151 L L G R L S G F K K Y K I S D I L F F D R N K K S K V E T Q
181 D S F L D M V S L K P G G V Y T K A Q L Q K E L E S L A T C
211 G M F E K V D M E G K T N A D G S L G L T I S F A E S M W E
241 R A D R F R C I N V G L M G Q S K P V E M D P D M S E K E K
271 I E F F R R Q E R E Y K R R I S S A R P C L L P T S V H E E
301 I K D M L A E Q G R V S A R L L Q K I R D R V Q S W Y H E E
331 G Y A C A Q V V N F G N L N T R E V V C E V V E G D I T K L
361 S I Q Y L D K L G N V V E G N T E G P V V Q R E L P K Q L L
391 P G H T F N I E A G K Q A L R N I N S L A L F S N I E V N P
421 R P D E M N E G S I I V E I K L K E L E Q K S A E V S T E W
451 S I V P G R G G R P T L A S L Q P G G T I T F E H R N L Q G
481 L N R S L T G S V T T S N F L N P Q D D L A F K M E Y A H P
511 Y L D G V D N P R N R T L R V S C F N S R K L S P V F T G G
541 P G V D E V P S I W V D R A G V K A N I T E N F S R Q S K F
571 T Y G L V M E E I I T R D E S N H I C S N G Q R V L P N G A
601 I S A D G P P T T L S G T G I D R M A F L Q A N I T R D N T
631 R F V N G T I V G S R N M F Q V D Q G L G V G S N F P F F N
661 R H Q L T V T K F L Q L M S V E E G A G K S P P P V L V L H
691 G H Y G G C V G D L P S Y D A F T L G G P Y S V R G Y N M G
721 E I G A A R N I L E L A A E I R I P I K G T H V Y A F A E H
751 G T D L G S S K D V K G N P T V V Y R R M G Q G S S Y G A G
781 M K L G L V R A E Y A V D H N S G T G A V F F R F G E R F *
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Fig. 2. Overview of cDNA clones (**A**) and deduced amino acid sequence of prOEP75 (**B**). Locations of *Hin*dIII and *Eco*RI restriction sites and the putative start codon are indicated in (A). In (B), the N-terminal residue of mOEP75 is circled; sequences obtained from tryptic peptides derived from the 75 kDa protein of the outer membrane are underlined; and a hydrophobic stretch of amino acids present in the transit peptide is boxed. The EMBL Nucleotide Sequence Databank accession number for clone p203 cDNA is X83767.

nucleotide sequence of the insert was determined. The insert contained the adapter sequence, used for library construction, adjacent to an internal *Eco*RI site (Figure 2A), suggesting that p202 contained two separate cDNAs. A sequence similarity search with sequences present in databases revealed that one of the two cDNAs was a portion of a cDNA encoding Clp protein (Moore and Keegstra, 1993). The other cDNA contained the sequence present in the oligonucleotide probe. *In vitro* transcription and translation of this cDNA yielded a protein of <75 kDa, so we concluded that it was not a full-length clone. The 590 bp *Hin*dIII fragment from p202 was used as a probe to re-screen the library for a full-length clone. This led to the isolation of p203 (Figure 2A). Sequence analysis indicated that p203 was identical to p202, except that p203 lacked the downstream Clp cDNA and p203 contained an additional 1051 bp at the 5′ end. The deduced amino acid sequence of the longest open reading frame encoded by p203 is given in Figure 2B. This amino acid sequence contains the peptides we identified by sequencing tryptic peptides derived from the 75 kDa outer membrane protein (underlined).

Surprisingly, the calculated molecular weight of the predicted protein encoded by p203 was 88.2 kDa, much larger than the expected size of 75 kDa, suggesting the presence of a cleavable transit peptide. *In vitro* transcription and translation of p214, a derivative of p203 with the 5′ untranslated region removed to increase translation efficiency (Figure 2A), yielded a protein that migrated between 85 and 90 kDa during SDS–PAGE. When incubated with intact chloroplasts under import conditions, this 88 kDa protein was processed to a protein that migrated at 75 kDa (described below), confirming the presence of a transit peptide. We thus designated the protein encoded by clone p203 as prOEP75 (precursor to OEP75). N-terminal sequencing of endogenous OEP75 indicated that the mature protein (mOEP75) began at the aspartate residue at position 132 (Figure 2B, circled) (J.Soll, personal communication). The calculated molecular weight of the resultant mature OEP75 is 75.0 kDa, agreeing well with its predicted size based on its migration during SDS–PAGE.

Comparison of the deduced amino acid sequence of prOEP75 with proteins present in the databases revealed that this protein was identical to IAP75. IAP75 was recently identified by Schnell *et al.* (1994) as a component of the chloroplastic protein import complex. There are no significant similarities within the mature region of this protein to any other proteins; however, the transit peptide showed sequence similarities to glycine-rich proteins. The similarities extended only through the glycine-rich regions of the transit peptide and may not have functional relevance. Hydropathy analysis of the mature region of prOEP75 indicated that it was a relatively hydrophilic protein with no obvious membrane-spanning domains (data not shown). Hydropathy analysis did reveal a long hydrophobic stretch of amino acids near the center of the transit peptide of prOEP75 (Figure 2B, boxed). It is possible that this hydrophobic region plays an important role in the targeting of OEP75 to the chloroplastic outer membrane.

prOEP75 is only the second of two outer membrane proteins from either chloroplasts or mitochondria characterized to date that is synthesized as a precursor form, the other being prOEP86 (Hirsch *et al.*, 1994). The transit peptides of prOEP75 and prOEP86 are similar in length to each other, but unusually long when compared to transit peptides of other chloroplastic proteins (von Heijne *et al.*, 1989; de Boer and Weisbeek, 1991). The transit peptide of prOEP75 is not acidic like that of prOEP86; rather, it is like other chloroplastic transit peptides in that it has few acidic residues, and is rich in small aliphatic residues and in hydroxylated amino acids.

Developmental regulation/tissue specificity of OEP75

If OEP75 is a component of the chloroplastic import machinery, its gene should be most actively expressed in tissues undergoing rapid plastid development. The 1139 bp *Hin*dIII fragment from p203 was used as a probe to detect mRNA encoding prOEP75 in total RNA from leaves of different developmental stages. As shown in Figure 3A,

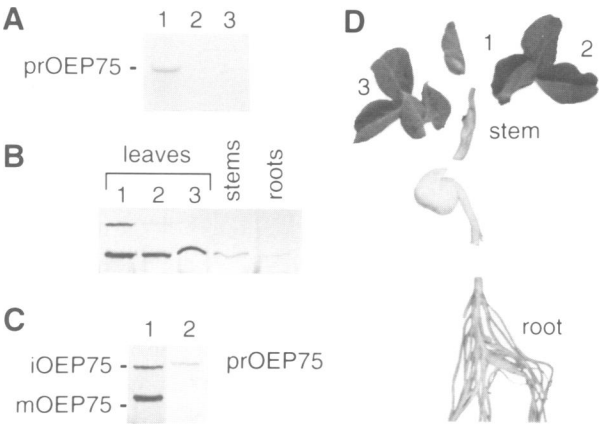

Fig. 3. Tissue-specific and developmental expression of OEP75. (**A**) 20 μg of total RNA from leaves of different developmental stages from 13 day-old plants (shown in **D**) were fractionated by electrophoresis, blotted and probed with an anti-sense RNA probe generated from the 1139 bp *Hin*dIII fragment from p203. A weak signal could be detected in lanes 2 and 3 of the original blot. (**B**) 100 μg of total protein from the different tissues were separated by SDS–PAGE, transferred onto a PVDF membrane and probed with anti-OEP75 antibodies. (**C**) Lane 1 is identical to lane 1 in (B), except that the protein sample was supplemented with 150 000 d.p.m. of translation product generated from p214 (prOEP75). An autoradiogram of lane 1 is shown in lane 2.

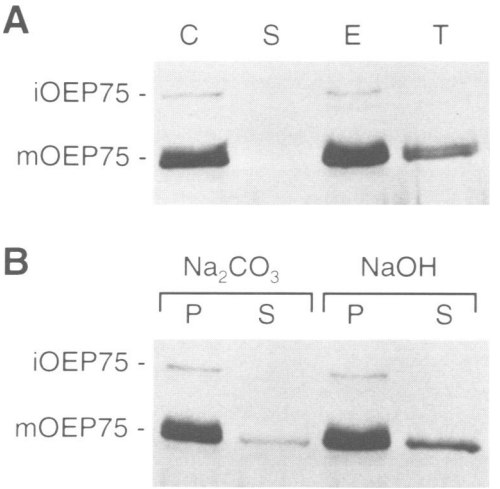

Fig. 4. Characterization of endogenous OEP75. Chloroplasts were isolated from young, folded leaves from 8 day-old seedlings. (**A**) Chloroplasts were separated into stroma (S), envelope (E) or thylakoid (T) fractions. 20 μg of whole chloroplasts (C) or chloroplastic fraction were analyzed. (**B**) Total chloroplastic membrane fraction was extracted with 100 mM sodium carbonate or 100 mM sodium hydroxide. 20 μg of pellet (P) or supernatant (S) were analyzed. All samples were analyzed by SDS–PAGE and immunoblotting with anti-OEP75 antibodies.

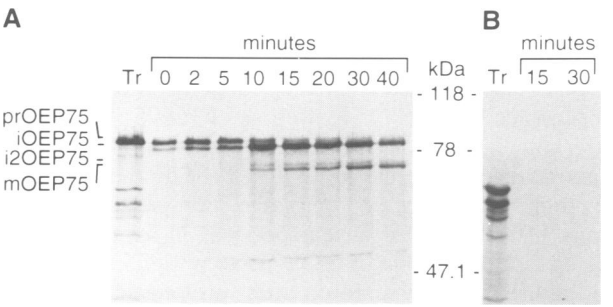

Fig. 5. Binding and processing of prOEP75 in an *in vitro* chloroplastic import assay. Radiolabeled prOEP75 (**A**) or a truncated construct (beginning with the first methionine residue in mOEP75) (**B**) was incubated with isolated chloroplasts at 25°C in the presence of 3 mM ATP for the times indicated. Chloroplasts were then repurified over 40% Percoll and analyzed by SDS–PAGE and fluorography. Tr, one-tenth volume of translation product added to the assay.

RNA isolated from very young leaves contained more message for prOEP75 than did RNA isolated from older leaves. Only a single transcript of ~3 kb was detected. This size agrees with the size of the cDNA isolated (clone p203, 3318 bp).

Western blot analysis of total protein extracts revealed that mOEP75 was present in leaves, stems and roots (Figure 3B). In addition to mOEP75, a larger protein that reacts to anti-OEP75 antibodies was detected in young leaves. The abundance of this protein declined much more rapidly with leaf age than did mOEP75. Because this protein is intermediate in size between the precursor and mature forms of OEP75 (Figure 3C), we designated it as iOEP75. A protein similar in size to iOEP75 was observed during *in vitro* import of prOEP75 (described below), and therefore we concluded that iOEP75 was derived from prOEP75.

Characterization of endogenous OEP75

Previous studies have indicated that OEP75 is a component of the outer envelope membrane (Werner-Washburne *et al.*, 1983; Perry and Keegstra, 1994). Recently, Schnell *et al.* (1994) provided evidence that OEP75 is integrally associated with this membrane. iOEP75 had not been observed previously so we addressed where it was located. Chloroplasts were isolated from young, folded leaves of 8 day-old pea seedlings and separated into soluble, envelope and thylakoid fractions, as described in Materials and methods. Analysis of the fractions by SDS–PAGE and immunoblotting with anti-OEP75 antibodies revealed that iOEP75 was localized to chloroplastic envelope membranes (Figure 4A). Furthermore, base extraction revealed that iOEP75 behaved as an integral membrane protein, similar to that seen for mOEP75 (Figure 4B).

Characterization of prOEP75 import into isolated chloroplasts

Previously characterized outer membrane proteins do not contain cleavable transit peptides (Salomon *et al.*, 1990; Li *et al.*, 1991; Ko *et al.*, 1992; Fischer *et al.*, 1994). The presence of a transit peptide on OEP75 suggested that it was targeted and inserted into the outer membrane through a novel route. To characterize this process, we conducted *in vitro* import experiments with prOEP75. When radiolabeled prOEP75 was incubated with chloroplasts in the presence of 3 mM ATP, the precursor was processed to mOEP75 in a time-dependent process (Figure 5A). In addition to mOEP75, two additional proteins intermediate in size between prOEP75 and mOEP75 appeared during the timecourse. (The band detected at ~55 kDa was most probably an artifact due to compression of radioactivity by the large subunit of ribulose bisphosphate carboxylase/oxygenase.) One of these intermediates was the same size as iOEP75 detected endogenously, and therefore was given

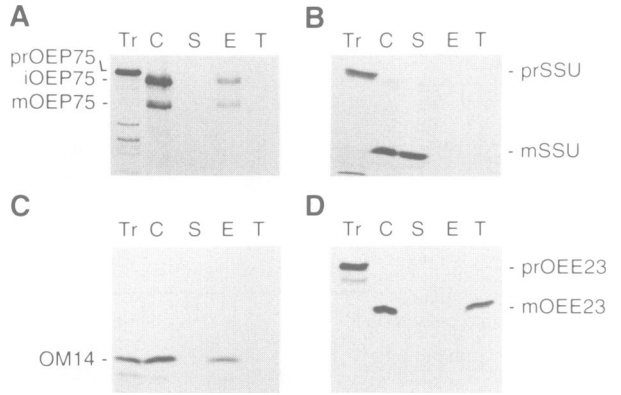

Fig. 6. Fractionation of processed prOEP75 (**A**). Radiolabeled precursor proteins were incubated with chloroplasts under import conditions. The import reaction was scaled up 3-fold over the standard reaction, as described in Materials and methods. Intact chloroplasts were then repurified over Percoll and one-third of the reaction was analyzed directly (C, chloroplasts prior to fractionation). The remaining two-thirds was lysed and fractionated. All of the resultant stroma fraction (S) and envelope fraction (E) and one-quarter of the resultant thylakoid fraction (T) were analyzed. All samples were anaylzed by SDS–PAGE and fluorography. (**B**) mSSU, (**C**) OM14 and (**D**) mOEE23 were used as markers of the stroma, envelope and thylakoid fractions respectively. Tr, one-thirtieth volume of translation product added to the assay.

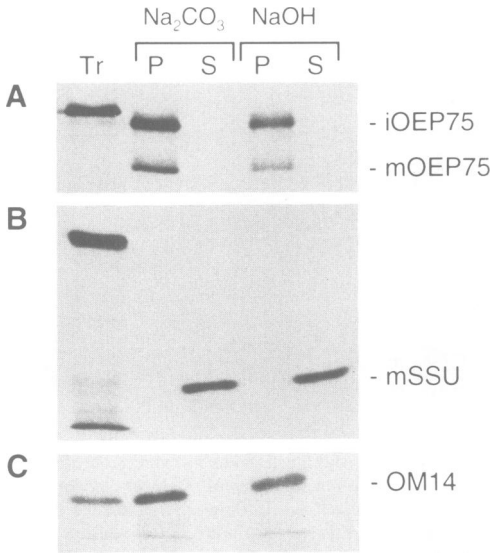

Fig. 7. Extraction of processed prOEP75 (**A**). After an import assay, repurified chloroplasts were extracted with 100 mM sodium carbonate or 100 mM sodium hydroxide and separated into pellet (P) and supernatant (S) fractions. (**B**) Extraction of a representative soluble protein (mSSU) and (**C**) an integral membrane protein (OM14). Samples were analyzed by SDS–PAGE and fluorography. Tr, one-tenth volume of translation product added to the assay.

the same name. We designated the second intermediate, which was slightly larger than mOEP75, as i2OEP75. iOEP75 appeared early in the reaction, followed by the appearance of mOEP75 as well as i2OEP75 (Figure 5A). Are both iOEP75 and i2OEP75 translocation/processing intermediates, en route to mOEP75, or do they represent aberrant processing or alternative, non-productive reactions? Their order of appearance was in agreement with a stepwise processing of prOEP75 to iOEP75 to i2OEP75 to mOEP75. Furthermore, after prolonged incubation under import conditions, i2OEP75 could no longer be detected (Figure 5A, 40 min time point), suggesting it was on a productive pathway to mOEP75. We cannot eliminate the possibility, however, that i2OEP75 was degraded. iOEP75 was still present even after prolonged incubation. To test whether iOEP75 was en route to mOEP75, we attempted to chase it to the mature form. Chloroplasts were incubated with prOEP75 in an import assay for 30 min, repurified over Percoll, then resuspended and incubated under import conditions for an additional 40 min, to allow iOEP75 to be processed further (chased). After this chase, iOEP75 was still present (data not shown). The fact that iOEP75 could not be chased to the mature form, together with the appearance of endogenous iOEP75 in young leaves but not in older leaves, may reflect its slow processing to the mature form. Alternatively, iOEP75 may have a function apart from mOEP75.

To determine if the transit peptide was necessary for targeting of OEP75 to chloroplasts, we subcloned the large *Eco*RI fragment from p203 into a transcription vector. The resultant clone is a truncated version of p214, in that it lacks the first in-frame methionine codon but contains the second in-frame methionine codon (residue 212). When this clone was subjected to *in vitro* transcription/translation and the resultant protein incubated with chloroplasts under import conditions, the protein did not associate with chloroplasts (Figure 5B). This result indicates that the transit peptide, or the extreme N-terminus of OEP75, is necessary for its proper targeting to chloroplasts.

The finding that prOEP75 bound to chloroplasts in the *in vitro* import assay and was processed to the mature form as well as to an intermediate-sized protein of the same size as endogenous iOEP75 suggested that the assay was capable of reproducing proper insertion of mOEP75 into the outer membrane. To address this point more directly, we verified that processed prOEP75 fractionated with envelopes. prOEP75 was incubated with chloroplasts under import conditions. The intact chloroplasts were re-isolated, lysed and subjected to fractionation on sucrose gradients. Both mOEP75 and iOEP75 were found in the envelope fraction (Figure 6), similar to the situation seen with the endogenous proteins (Figure 4). Representative proteins of other chloroplastic compartments were included in the fractionation analysis as markers of the fractions (Figure 6). Results from cross-linking studies (Perry and Keegstra, 1994) indicated that precursor protein in association with OEP75 was present in contact sites, whereas the majority of OEP75 was present in the outer membrane fraction. Further studies will be necessary to determine what proportion of newly inserted mOEP75 is present in contact sites versus the outer membrane. Furthermore, investigation of the precise envelope location of the intermediates may provide insights into the details of the insertion and processing pathway of prOEP75.

To determine if imported mOEP75 and iOEP75 were peripherally or integrally associated with the outer membrane, chloroplasts were subjected to sodium carbonate and sodium hydroxide extraction after incubation with prOEP75. mOEP75 was resistant to extraction (Figure 7), indicating that it was an integral membrane protein. Similarly, iOEP75 was resistant to extraction, suggesting that it was at least partially assembled within the outer

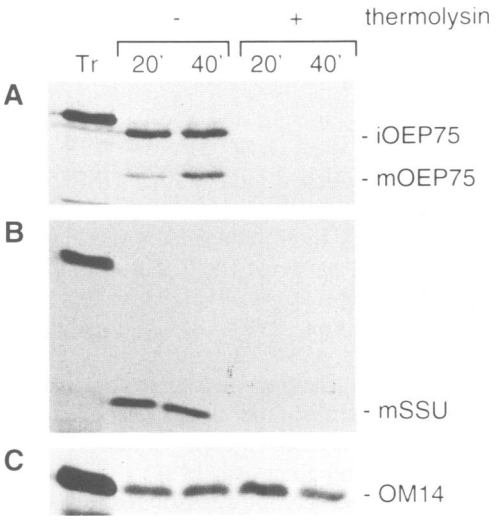

Fig. 8. Effect of removal of surface proteins on binding and processing of prOEP75. Chloroplasts were treated with thermolysin (0 or 200 μg/ml) at 4°C for 30 min, quenched with EDTA (5 mM final concentration), repurified over Percoll and washed twice with import buffer. Radiolabeled precursor, prOEP75 (**A**), prSSU (**B**) or OM14 (**C**) was then incubated with the pre-treated chloroplasts under import conditions for 20 or 40 min. Samples were analyed by SDS–PAGE and fluorography. Tr, one-tenth volume of translation product added to the assay.

membrane or firmly held within the import complex. Again, newly imported mOEP75 and iOEP75 behaved similarly to the endogenous proteins (Figure 4), confirming that the *in vitro* assay was reflecting the *in vivo* situation, as well as providing evidence that the imported intermediate we designated as iOEP75 was the same protein as endogenous iOEP75.

Chloroplastic proteins carrying a transit peptide require one or more protease-susceptible surface components (e.g. a receptor) (Friedman and Keegstra, 1986) and ATP for their binding and translocation (Olsen *et al.*, 1989; Theg *et al.*, 1989). We determined whether import of prOEP75 had these same requirements. To address the requirement of protease-susceptible surface components, chloroplasts were treated with thermolysin prior to an import assay. After thermolysin treatment, chloroplasts were repurified over Percoll and washed twice with import buffer. The chloroplasts were then resuspended in import buffer with 3 mM ATP and incubated with precursor protein. prOEP75 neither bound to nor was imported into chloroplasts pretreated with thermolysin (Figure 8A), indicating that one or more exposed proteins on the outer membrane was required for its binding and processing. This result was identical to that observed for prSSU, a representative stromal-targeted protein used as a control (Figure 8B). OM14, which is not synthesized in a precursor form, has previously been shown not to require a protease-susceptible surface component for its insertion into the outer membrane; furthermore, inserted OM14 is susceptible to thermolysin degradation (Li *et al.*, 1991). For these reasons, insertion of OM14 was used as a control to verify that thermolysin was completely removed from the external environment during chloroplast repurification (Figure 8C). Thus the apparent lack of prOEP75 binding and import cannot be attributed to the trivial explanation that residual thermolysin degraded the precursor.

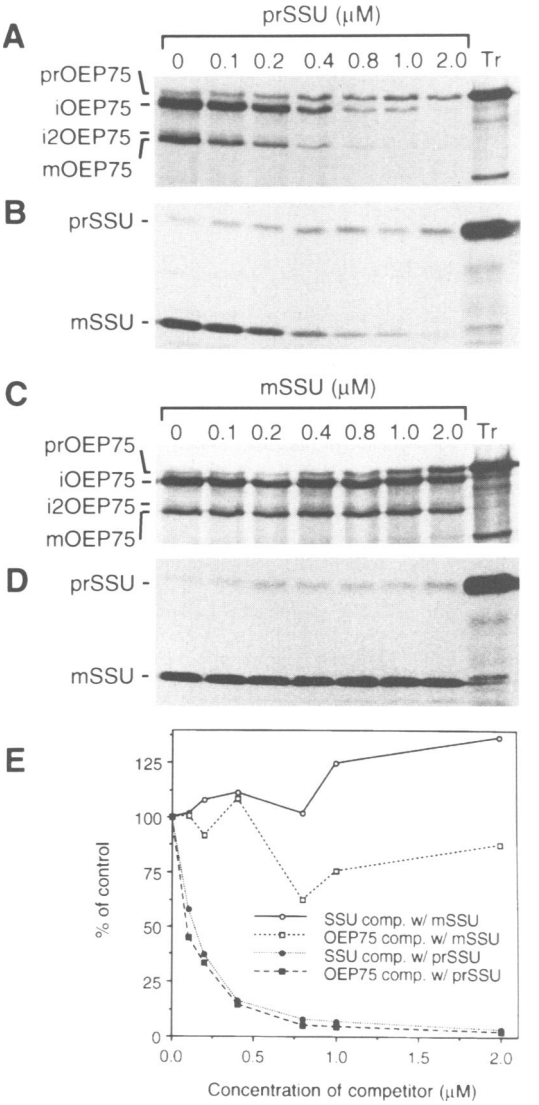

Fig. 9. Import competition experiments. Import assays were conducted with radiolabeled prOEP75 (**A** and **C**) or prSSU (**B** and **D**) in the presence of varying quantities of unlabeled prSSU (A and B) or mSSU (C and D). Samples were analyzed by SDS–PAGE and fluorography. Tr, one-tenth volume of translation product added to the assay. (**E**) Quantitation of mOEP75 (boxes) and mSSU (circles) when competed by prSSU (filled symbols) or mSSU (open symbols). Quantitation was performed with a phosphorimager (Molecular Dynamics).

The fact that OEP75 is synthesized with a transit peptide that has similarities to typical chloroplastic transit peptides, together with the finding that one or more surface proteins are required for binding and processing of prOEP75, raised the possibility that the insertion of prOEP75 may occur, at least in part, via the general chloroplastic import apparatus. A competition experiment with excess, unlabeled prSSU indicated that prOEP75 and prSSU share one or more components of the import machinery (Figure 9). Excess unlabeled prSSU, but not mSSU, inhibited the import of radiolabeled prOEP75 (Figure 9A and C). The decline in import of labeled prOEP75 in the presence of increasing concentrations of unlabeled prSSU paralleled the decline in import of labeled prSSU under the same conditions (Figure 9E). Thus prOEP75 and prSSU probably

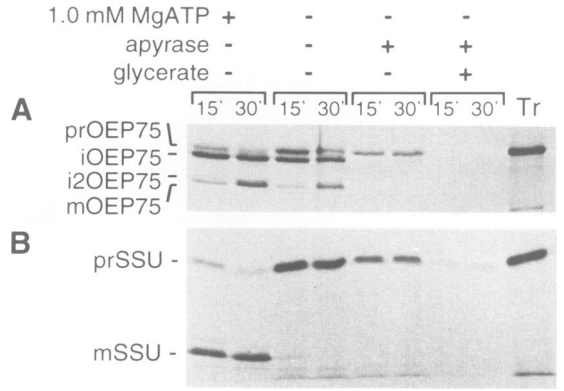

Fig. 10. ATP dependence of precursor import. Exogenous ATP was added to the import reaction or translation product and/or chloroplasts were treated prior to the import assay to manipulate the ATP concentrations. To remove ATP contributed by translation product (~50 μM), the translation mixture was treated with apyrase (50 U/ml) at 25°C for 15 min. To deplete chloroplasts of internal ATP, they were incubated with 10 mM glycerate at 25°C for 15 min. Incubation of chloroplasts with prOEP75 (**A**) or prSSU (**B**) was conducted at 25°C for 15 or 30 min. All incubations were in the dark. Samples were analyzed by SDS–PAGE and fluorography. Tr, one-tenth volume of translation product added to the assay.

have similar affinity for one or more of the components of the import machinery.

Although prOEP75 and prSSU seem to share a common import pathway at least in part, their ATP requirements are different (Figure 10). In a standard import assay, ATP is available to drive import from three different sources: exogenously added ATP, residual ATP from the *in vitro* translation system (wheat germ or rabbit reticulocyte lysate) which is added with the precursor protein, and ATP that is present in the stroma (Olsen and Keegstra, 1992). When the import assay was supplemented with 1 mM ATP, both prOEP75 and prSSU were readily processed to the mature form (Figure 10). When ATP was not exogenously added, ATP levels (~50 μM) were sufficient to drive prOEP75 processing to the mature form, although this step proceeded more slowly than when 1 mM ATP was present. ATP levels provided by the translation mixture were sufficient to support prSSU binding but, unlike that observed with prOEP75, were too low to support prSSU processing (import) (Figure 10). Apyrase does not penetrate the chloroplastic inner membrane and therefore can be used to remove external ATP contributed by the translation system. When external ATP was removed from the import assay by apyrase, prOEP75 still bound to chloroplasts but was not processed to the mature form (Figure 10). When glycerate was added to the import assay to deplete ATP from the stroma, prOEP75 no longer bound to chloroplasts. This result suggests that the binding observed in the presence of apyrase but in the absence of glycerate is due to an ATP-dependent protein–protein interaction rather than a non-specific interaction. Comparisons between the ATP requirements for binding and processing of prOEP75 and prSSU revealed that the ATP requirements for insertion of prOEP75 are similar to that needed for specific binding of prSSU. However, a more detailed investigation of the ATP requirements for prOEP75 binding and processing is necessary to understand fully the concentrations of ATP needed for these steps, and in which compartment(s) the ATP is utilized.

Discussion

Several lines of evidence indicate that OEP75 is a component of the chloroplastic protein import apparatus. Results obtained by three independent laboratories using different strategies have indicated that a 75 kDa protein of the outer membrane is involved in protein import (Waegemann and Soll, 1991; Soll and Alefsen, 1993; Perry and Keegstra, 1994; Schnell *et al.*, 1994). Results obtained from chemical cross-linking experiments revealed that a 75 kDa protein is either in direct contact or in close proximity to a precursor protein bound to the import apparatus (Perry and Keegstra, 1994). A 75 kDa protein was present in import complexes isolated by two different approaches (Waegemann and Soll, 1991; Soll and Alefsen, 1993; Schnell *et al.*, 1994). The cDNA we isolated encodes the same 75 kDa protein as does that isolated by Schnell *et al.* (1994). Peptide sequence data of the 75 kDa protein isolated by Soll and co-workers revealed that their protein also was identical (J.Soll, personal communication). Furthermore, we have shown that Fab fragments isolated from antiserum against OEP75 inhibited subsequent import of precursor proteins into chloroplasts. Taken together, these findings provide convincing evidence in support of the assignment of OEP75 as a component of the chloroplastic protein import apparatus.

Northern blot analysis revealed that mRNA encoding prOEP75 declined in abundance with leaf age. Results from studies by Dahlin and Cline (1991) indicated that the chloroplastic import apparatus was regulated such that chloroplasts with high demands for new proteins were more import competent. Thus, it is not surprising that expression of mRNA for an import component would be markedly higher in young leaves where rapid plastid biogenesis is occurring. Protein turnover would necessitate a functional import apparatus even in older leaves, and therefore some low level expression of genes for import components should be observed in these leaves. To determine how much protein is being imported in older, developed leaves, one could use the expression of a nuclear-encoded chloroplastic protein as a marker. Northern blot analysis revealed that mRNA encoding prSSU is most abundant in young, developing leaves and then declines with leaf age (He *et al.*, 1994), much like what we observed for prOEP75 mRNA. The abundance of prOEP75 mRNA declined with leaf age more rapidly, however, than did that of prSSU. This would be expected if OEP75 was a component of the apparatus that facilitated the import of prSSU, i.e. once the import apparatus is in place, production of precursor proteins could continue. Western blot analysis of total protein extracts revealed that mOEP75 was present in leaves, stems and roots. Root plastids are capable of importing proteins that normally function in chloroplasts (J.Davila-Aponte and K.Keegstra, unpublished data). Thus the presence of mOEP75 in root tissue is consistent with its proposed function as a component of the plastid import machinery.

What is the specific role of OEP75 in the import process? Results from the chemical cross-linking studies indicated that association of the precursor protein with

OEP75 required ATP (Perry and Keegstra, 1994). Both OEP86 and another outer membrane protein, OEP34, which is present in the import complex (Kessler *et al.*, 1994; Schnell *et al.*, 1994; Seedorf *et al.*, 1995) have the consensus ATP/GTP binding domain, whereas OEP75 lacks such a domain. Thus ATP is probably not utilized by OEP75 but rather by OEP86 or OEP34. It is possible that the ATP-dependent step causes a conformational change in either OEP86 or OEP34 that brings the bound precursor protein into contact with or in close proximity to OEP75. OEP75 was suggested earlier to be a component of the general insertion site (Perry and Keegstra, 1994). This suggestion was made on the basis that precursor protein first associated with the 86 kDa protein and subsequently associated with OEP75. Furthermore, OEP75 in association with precursor was predominantly present in contact sites between the outer and inner membranes.

We have shown that both endogenous and newly inserted mOEP75 is resistant to base extraction, suggesting that it is an integral membrane protein. This is in agreement with the tentative assignment of OEP75 as part of the general insertion site. The relative hydrophilicity of OEP75 raises questions as to its topology in the membrane. As discussed by Schnell *et al.* (1994), OEP75 may be embedded in the membrane via transmembrane β-strands, forming a channel. If OEP75 forms such a structure, it would lend support to its assignment as a pore through which precursor proteins pass. OEP75 is the most abundant protein of the chloroplastic outer membrane. The most abundant protein of the mitochondrial outer membrane is porin, which forms a general pore through which small molecules can pass freely (reviewed by Benz, 1994). Small molecules are also able to freely penetrate the chloroplastic outer membrane, however the protein through which the molecules pass has not been identified. Possibly OEP75 forms this pore and also constitutes the protein channel through which precursor proteins pass. Determination of the membrane topology of OEP75 will probably be critical to further understand its function in the protein import process.

Whereas we found that anti-OEP75 Fab fragments inhibited subsequent import of precursor proteins, Hirsch *et al.* (1994) reported that anti-OEP86 Fab fragments, but not anti-OEP75 Fab fragments, inhibited subsequent import of prSSU. It is not surprising that a high concentration of anti-OEP75 Fab fragments is necessary to inhibit import. OEP75 is a major protein constituent of the outer membrane (Cline *et al.*, 1981; Keegstra and Yousif, 1986); furthermore, OEP75 seems to be relatively unexposed to the external environment. For instance, treatment of chloroplasts with thermolysin, which does not breach the outer membrane, causes only limited degradation of OEP75 (Cline *et al.*, 1984; data not shown). Thus, only a few epitopes of OEP75 should be accessible to external Fab fragments. Conversely, a large portion of OEP86 is exposed to the cytosol (Hirsch *et al.*, 1994). The reason we observed that anti-OEP75 Fab fragments inhibited import whereas Hirsch *et al.* did not may be attributed to differences in antisera, e.g. differences in quantity of IgG molecules against exposed epitopes of OEP75. Nonetheless, the fact that we were able to inhibit import of two different precursor proteins after incubation of chloroplasts with Fab fragments against OEP75 supports its assignment as a component of the chloroplastic import machinery. The fact that import seems to be more sensitive to anti-OEP86 Fab fragments than to anti-OEP75 fragments supports the speculation that OEP86 has a receptor-like role and OEP75 has a channel-like role in the import process.

Using *in vitro* import assays with isolated chloroplasts we were able to reproduce processing of prOEP75 and insertion of the mature form into chloroplastic outer membranes. The fact that OEP75 is synthesized as a higher molecular weight precursor and that two size intermediates are observed during *in vitro* import assays raises several questions regarding its targeting and insertion pathway. OEP75 and OEP86 (Hirsch *et al.*, 1994) are the only proteins from outer membranes of either chloroplasts or mitochondria characterized to date that are synthesized as precursor proteins. N-terminal sequence data of the endogenous proteins indicate that processing of the precursors occurs at the N-terminus, as seen for other chloroplastic precursor proteins. The transit peptides of prOEP75 and prOEP86 are similar in size to each other (~14 kDa) and about twice the size of most other chloroplastic transit peptides. In terms of amino acid composition, the transit peptide of prOEP75 is similar to other transit peptides. From this, one could speculate that it is targeted to chloroplasts through a route similar to that of other precursor proteins. This speculation is supported by the finding that prOEP75 not only requires surface-exposed proteinaceous components, but also competes with prSSU for one or more import components. prOEP86 does not compete with prSSU for its insertion (Hirsch *et al.*, 1994), suggesting that it is targeted by a different route. Although prOEP75 and prSSU compete for one or more components for import, their ATP requirements do not seem to be identical.

What is the significance of the size intermediates observed during *in vitro* import of prOEP75? A protein that was the same size as one of the intermediates (iOEP75) and that reacted to anti-OEP75 antiserum was present in young leaves. We concluded that iOEP75 detected *in vitro* was the same protein as the intermediate detected *in vivo* because both proteins were localized to chloroplastic envelopes and both were resistant to base extraction. The presence of endogenous iOEP75 in chloroplasts from young leaves, followed by its decline with leaf age, corresponds well with the abundance of the mRNA for prOEP75 in the same leaf samples. Analagously, during *in vitro* import, iOEP75 appears early in the reaction, followed by the appearance of mOEP75. Possibly iOEP75 is on a productive pathway to mOEP75, but its subsequent processing to the mature form is a regulated and rate-limiting step. OEP34 was shown to be associated with OEP75 (Seedorf *et al.*, 1995). Perhaps further processing of iOEP75 is dependent upon its interaction with OEP34. It is also possible that iOEP75 has a function different from that of mOEP75. Understanding the regulation of precursor processing to iOEP75 and mOEP75 should provide insights into whether iOEP75 represents a processing intermediate or has a functional role. The other intermediate we observed in our import assays, i2OEP75, was not detected after prolonged incubation under import conditions. Although this suggests that it was further

processed to the mature form, we cannot rule out the possibility that it was degraded.

If both intermediates are on a productive pathway to mOEP75, there must be multiple processing events. Precursor proteins destined for the thylakoid lumen contain a bipartite transit peptide (de Boer and Weisbeek, 1991). The N-termini of these bipartite transit peptides are chloroplast-targeting domains which are cleaved off in the stroma, and the C-termini are thylakoid-targeting domains which are cleaved off in the lumen. Possibly an analogous targeting system is in place for prOEP75. We propose that one portion of the transit peptide directs the precursor to the chloroplast, and additional domains of the transit peptide subsequently route the protein to the outer membrane. This would imply that prOEP75 is translocated through the general import apparatus and then subsequently sorted. The hydrophobic domain of the transit peptide may act as a stop-transfer domain (Blobel, 1980), anchoring the precursor in the envelope while the mature region of prOEP75 is assembled in the outer membrane. According to this model, the N-terminus of the transit peptide may be exposed to the stromal processing peptidase, whereupon iOEP75 is produced. Only after the mature region of iOEP75 is properly inserted into the membrane, and possibly associated with OEP34, is it further processed.

Why would such a complex targeting system exist for OEP75? Possibly a high fidelity targeting system such as that thought to function for proteins of the mitochondrial import complex also exists in chloroplasts. The targeting of prOEP86 to chloroplasts may be analogous to that of MOM19 to mitochondria in that neither requires a 'receptor' protein but rather directly associates with another component of the import apparatus. The finding by Hirsch *et al.* (1994) that OEP86 does not compete with prSSU for import is in agreement with this model. If targeting of OEP86 is dependent upon its association with OEP75, then mistargeting of OEP75 could lead to subsequent mistargeting of OEP86 followed by mistargeting of chloroplastic precursor proteins. Thus a complex system, which requires more than one processing enzyme for correct insertion of OEP75, would ensure high fidelity targeting of prOEP75 to chloroplasts, somewhat analogous to the targeting of MOM22 and MOM38. Certainly, further experiments are needed to more accurately describe the route of insertion for prOEP75 and the proteins involved in its processing.

Materials and methods

Isolation of a cDNA clone encoding OEP75

A cDNA library was constructed from mRNA isolated from leaves of 5 and 7 day-old pea (*Pisum sativum* var. *little marvel*) seedlings grown under a 12 h light/12 h dark cycle. A mixture of oligo(dT) and random primers was used in cDNA synthesis. cDNAs were cloned into the *Eco*RI site of the Lambda ZAP II vector (Stratagene).

Chloroplastic outer membranes were isolated as described (Keegstra and Yousif, 1986). Membrane proteins were separated by SDS–PAGE and surface stained with Coomassie Blue. The abundant 75 kDa protein, corresponding to the protein identified by chemical cross-linking (Perry and Keegstra, 1994), was cut out from the gel, electro-eluted, concentrated and digested with trypsin. Resultant peptides were separated on a microbore C18 reverse phase HPLC column and sequenced with an ABI-477 protein sequencer (Macromolecular Facility, Department of Biochemistry, Michigan State University). A degenerate oligonucleotide, AA(CT)ATGTT(CT)CA(AG)GTIGA(CT)CA(AG)GG, corresponding to the peptide NMFQVDQ was synthesized and 3′ end-labeled with digoxigenin-11-ddUTP (Boehringer Mannheim). This probe was used to screen the Lambda ZAP II library. Positive plaques were selected, purified by secondary and tertiary screening, and pBluescript II SK phagemid was excised using standard procedures (Stratagene). The 590 bp *Hin*dIII fragment of a partial, positive clone (p202) was subcloned into pBluescript II SK and used to make a random primed, digoxigenin-11-dUTP-labeled DNA probe (Boehringer Mannheim). This probe was used to re-screen the cDNA library. Phagemid containing positive cDNA inserts were obtained using Stratagene's protocols.

Generation of subclone for in vitro transcription/translation. Clone p214 was generated by exonuclease III digestion of p203 (Maniatis *et al.*, 1982). Prior to exonuclease digestion, p203 was digested with *Bst*XI and *Xba*I. After exonuclease digestion, clones were ligated and transformed into *Escherichia coli* strain DH5α. Clone p214 was selected by its ability to produce a protein of the expected size upon *in vitro* transcription and translation. To determine precisely the extent of exonuclease digestion, the 5′ end of p214 was sequenced. p214, which contains 14 bp 5′ to the first in-frame methionine codon of p203, was used for production of prOEP75 for import experiments.

DNA sequencing. Automated fluorescent sequencing was performed by our Plant Biochemistry Facility at Michigan State University using the ABI Catalyst 800 for *Taq* cycle sequencing and the ABI 373A Sequencer for the analysis of products. Both strands of p203 were sequenced.

Generation of proteins for import assays

Radiolabeled protein was generated from cDNAs using standard *in vitro* transcription and translation procedures (Bruce *et al.*, 1994). [^{35}S]-Methionine was used to label all proteins except prOEE23, which was labeled with [^{3}H]leucine. Radiochemicals were purchased from DuPont-NEN, all other reagents were from Promega. OM14, prSSU and prOEE23 were obtained from translation in wheat germ extract (Bruce *et al.*, 1994). All other translations were performed with nuclease-treated rabbit reticulocyte lysate using the standard reaction suggested by the manufacturer, i.e. 66% final concentration of lysate (v/v), and Mg^{2+} and K^{+} concentrations were not altered. Unlabeled prSSU and mSSU for use in competition experiments (Figure 9) were generated by overexpression in *E.coli* as described (Perry and Keegstra, 1994).

Import assays

Standard import assays were conducted as described previously (Bruce *et al.*, 1994). Briefly, chloroplasts from 9–12 day-old pea seedlings were isolated over Percoll gradients and resuspended in import buffer at 1 mg/ml chlorophyll. Translation mixture (~5×10^5 d.p.m.) and ATP (3 mM final concentration) were added to chloroplasts and incubated at 25°C for 30 min. Variations to this assay are given in the figure legends; however, in all cases except for the fractionation analysis, translation product was added to 50 μg chlorophyll and the final assay volume was 150 μl. For all assays with prOEP75, the final concentration of rabbit reticulocyte lysate was 8.9% (v/v). After incubation, intact chloroplasts were re-isolated over 40% Percoll (v/v) and analyzed by SDS–PAGE and fluorography.

For fractionation analysis (Figure 6), the import assay was scaled up 3-fold. After incubation with precursor protein, intact chloroplasts were repurified over Percoll and one-third of the reaction was analyzed directly. The remaining two-thirds was lysed hypotonically and fractionated as described (Perry and Keegstra, 1994) with the following modification: fractionation was performed with a sucrose step gradient consisting of 0.46 and 1.2 M sucrose solutions.

For extraction analysis (Figure 7) intact chloroplasts were re-isolated over Percoll after a standard import reaction. The intact chloroplasts were resuspended and incubated in 100 mM sodium hydroxide or 100 mM sodium carbonate for 30 min on ice and then separated into pellet and supernatant fractions. The pellet fraction was extracted again by resuspending and incubating it in sodium hydroxide or sodium carbonate and then pelleting. The supernatant from the extraction of the membrane pellet was combined with the supernatant from the extraction of the intact chloroplasts.

Antibodies

An aliquot of purified OEP75 that was used for peptide sequencing (see above) was injected into rabbits for the production of antibodies. TitreMax (Vaxcel Inc., Norcross, GA, USA) was used as an adjuvant. IgG and Fab fragments were isolated with immobilized protein A

and immobilized papain respectively, according to the manufacturer's recommendations (Pierce).

Western blotting

For detection of endogenous OEP75 in total protein extracts, 13 day-old pea seedlings were dissected as shown in Figure 3D. Tissue was ground under liquid nitrogen and total proteins were extracted with 0.15 M Tris–HCl (pH 6.8), 7.5% 2-mercaptoethanol, 3% SDS. Extracts were clarified by centrifugation and protein concentration determined with the Bradford protein assay (Bio-Rad). After separation by SDS–PAGE, proteins were transfered onto PVDF membranes (Immobilon P, Millipore), incubated with blocking buffer (TBS, 0.1% Tween 20, 1% non-fat dry milk) and then incubated in fresh blocking buffer supplemented with antiserum (1:2000 final dilution). Washing was carried out with 0.1% Tween 20 in TBS. Primary antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit antibody (Kirkegaard and Perry, Gaithersburg, MD, USA). Secondary antibody was detected with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim). To verify that equal quantities of proteins were analyzed by Western blotting, duplicate samples were analyzed by Coomassie staining after separation by SDS–PAGE.

Further characterization of endogenous OEP75 was conducted using chloroplasts isolated from young, folded leaves from 8 day-old seedlings. Chloroplasts were fractionated as described above under 'Import assays'. Resistance to base extraction was determined by first isolating total chloroplastic membranes and then extracting this fraction with 100 mM sodium hydroxide or 100 mM sodium carbonate. Membranes were extracted twice and supernatant fractions from the two extractions were combined. All samples were analyzed by SDS–PAGE and immunoblotting with anti-OEP75 antibodies as described above.

Northern blotting

The same tissue that was used for analysis of endogenous OEP75 protein by Western blotting was used for RNA analysis by Northern blotting. Total RNA was extracted from the tissues by vortexing with hot (100°C) extraction buffer (0.2 M sodium borate, 30 mM EDTA, 1% SDS, 1% deoxycholate, 2% PVP-40, 10 mM DTT). The resultant mixture was clarified by centrifugation, extracted with phenol and RNA was precipitated by addition of LiCl (2 M final concentration). The RNA pellet was dissolved in water and precipitated again with 0.2 M potassium acetate in 70% ethanol. The pellet was dried, dissolved in water and the RNA concentration was determined by absorbance at 260 nm. Twenty micrograms of total RNA were separated on 1.2% agarose under denaturing conditions (Selden, 1987) and blotted onto positively charged nylon membranes (Boehringer Mannheim). To verify that equal quantities of RNA were analyzed, the RNA was stained with ethidium bromide and visualized under ultraviolet light. The RNA blot was probed with a digoxigenin-labeled anti-sense RNA probe synthesized from a 1139 bp *Hin*dIII fragment from p203 subcloned into pBluescript II SK. Hybridization and detection was performed as described by Boehringer Mannheim. Hybridization was at 65°C; washing was conducted with 0.1× SSC at 65°C.

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