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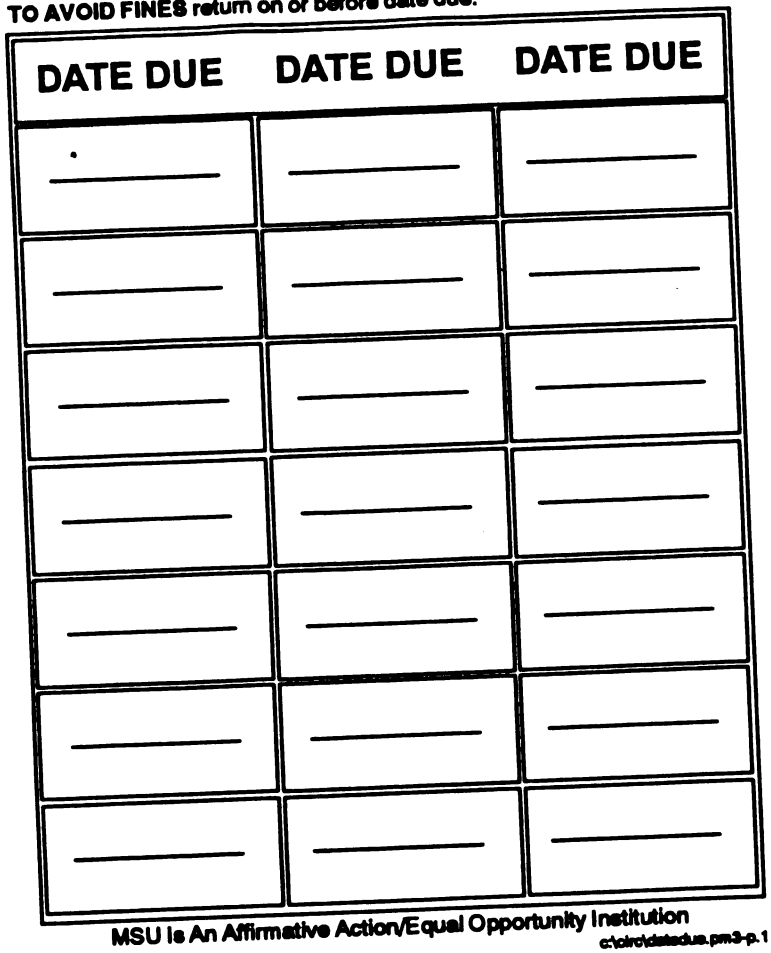

NOVEL TARGETING PATHWAY OF A COMPONENT OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS

By

Patrick J. Tranel

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ABSTRACT

NOVEL TARGETING PATHWAY OF A COMPONENT OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS

By

Patrick J. Tranel

Many chloroplastic proteins are encoded by nuclear genes and synthesized in the cytoplasm. Many of these proteins contain N-terminal transit peptides that act as intracellular targeting signals. A general protein import apparatus exists in the envelope of chloroplasts that functions to recognize and import these chloroplastic proteins. Previously known outer membrane proteins of the chloroplastic envelope do not have a transit peptide and do not use the general protein import apparatus in their targeting pathway. An outer envelope membrane protein of 75 kDa (OEP75) was identified previously as a component of the chloroplastic general protein import apparatus. A cDNA clone encoding OEP75 was isolated. Initial characterization of this clone and the encoded protein revealed that OEP75 was synthesized as a higher molecular weight precursor (prOEP75) containing an N-terminal transit peptide. prOEP75 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 (iOEP75) was also detected in chloroplastic envelopes isolated from young pea leaves. Import of prOEP75 was dependent on ATP and one or more surface-exposed proteinaceous components, and was competed by precursor to small subunit of ribulose-1,5-bisphosphate (prSS), a stromal-targeted protein. Experiments conducted with stromal extracts revealed that iOEP75 was produced 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-terminal and C-terminal domains. To determine the targeting functions of the two domains of the transit peptide and of the mature region of

prOEP75, deletion mutant constructs from prOEP75 and chimeric constructs between domains of prOEP75 and prSS were created. Analysis of these constructs by in vitro chloroplastic protein import assays revealed that the transit peptide of prOEP75 is bipartite in that the N-terminal and C-terminal portions contain chloroplast-targeting and intra-organellar targeting information, respectively. Preliminary experiments indicated that the novel import pathway of prOEP75 is conserved among different plant species.

To my family
and to Lea

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Chapter 1

INTRODUCTION

One of the distinguishing characteristics of plants is their ability to convert light energy into chemical energy. This process, photosynthesis, takes place within compartments of plant cells called chloroplasts. A chloroplast is one member of a group of organelles called plastids, that are specific to the plant kingdom. Plastids are thought to be descendants of a cyanobacterium that was engulfed by a host cell in an endosymbiotic event (Delwiche et al., 1995; Nelissen et al., 1995).

Different plastid types are specialized for different functions. The type of plastid that is in a particular cell depends upon where in the plant that cell is and that cell's developmental state (Possingham, 1980; Thomson and Whatley, 1980). For example, a leaf mesophyll cell will contain chloroplasts, specialized for conducting photosynthesis, whereas a tuber cell will contain amyloplasts, specialized for storing starch. Another example of a plastid type is a chromoplast, and its specialized function is to produce and store pigments. Plastids carry out a variety of processes in addition to their "specialized" process. Steps of amino acid assimilation, nitrogen fixation, and fatty acid synthesis take place within plastids (Emes and Tobin, 1993).

The different types of plastids are developmentally related. For example, the highly undifferentiated plastid type, the amoeboid plastid, may differentiate into a pregranal plastid and then into an etioplast and then into a chloroplast. A chloroplast may later develop into a senescent chloroplast or, alternatively, might dedifferentiate (e.g. in seeds) to an eoplast and then re-differentiate into an amyloplast (Thomson and Whatley, 1980). Although the exact inter-relatedness of all the different plastid types is unknown, it is clear that several plastid types have different developmental pathways that may be followed. Thus, plastids are appropriately named, not only because of their "plasticity" in function, but also because of their developmental "plasticity".

Plastids are semi-autonomous organelles within cells in that they are capable of undergoing division within the cell. Furthermore, plastids contain their own DNA and are capable of transcribing genes from their DNA and translating the resultant mRNA to

produce their own proteins. Despite these abilities, a plastid's genome encodes only a small subset of the proteins a plastid needs to carry out its many functions and, thus, plastid function is dependent upon proteins that are encoded by nuclear genes. These proteins are synthesized in the cytoplasm and must be transported into plastids.

It is this general subject, protein transport into plastids, that is the focus of my dissertation. Specifically, I have investigated a 75-kDa protein that is present in the outer membrane of the plastid's double-membrane envelope. This outer envelope membrane protein (OEP75) had been identified by a crosslinking strategy as a component of the machinery that facilitates the transport of cytoplasmically synthesized proteins into plastids (Perry and Keegstra, 1994). Because OEP75 itself is encoded by a nuclear gene, and for reasons that will be discussed later, its targeting pathway to the outer envelope membrane became of interest. Thus, the key question addressed by my dissertation is: How is OEP75 targeted to the outer envelope membrane?

Before addressing this question, I will provide an overview of protein import into plastids. In giving this overview, I will include the paradigm, that existed as I began my dissertation research, for how proteins of the plastid outer envelope membrane were targeted to that location. Transport of proteins into plastids has several parallels to transport of proteins into mitochondria. Furthermore, several envelope membrane proteins of the mitochondrial protein import machinery have been identified, and their targeting pathways have been investigated. Thus I will provide a brief overview of protein import into mitochondria and then discuss in some detail the targeting of envelope membrane proteins of the mitochondrial import machinery.

PROTEIN IMPORT INTO PLASTIDS

Although all plastid types must import proteins from the cytoplasm, this process has been studied the most by far in chloroplasts. My discussion will focus on data that were obtained from analysis of protein import into chloroplasts; however, this discussion is likely to be generally applicable to all plastid types. It is thought that the import process is very similar in all plastid types, although subtle differences probably exist (Fischer et al., 1994b; Wan et al., 1995).

Chloroplasts contain six distinct locations: thylakoid lumen, thylakoid membrane, stroma, inner membrane of the envelope, outer membrane of the envelope, and the space between the two envelope membranes (intermembrane space). Thus, protein transport into chloroplasts contains two levels of complexity. Not only must the proteins be targeted to the proper organelle, but they must also be targeted to the proper location within the organelle. With the exception of proteins of the outer envelope membrane, all known chloroplastic proteins encoded by nuclear genes are synthesized as higher molecular weight precursors (deBoer and Weisbeek, 1991; Theg and Scott, 1993). These precursor proteins contain a sequence of amino acids, termed a transit peptide, N-terminal to the mature protein, which is responsible for targeting a precursor protein to a chloroplast.

Transit peptides of stromal precursor proteins are removed by a stromal processing peptidase (VanderVere et al., 1995) upon translocation of the precursors across the chloroplastic envelope. Proteins destined for the thylakoid lumen contain a bipartite transit peptide consisting of N-terminal and C-terminal domains (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 stromal targeted precursors. Intra-organellar targeting information for these inner

envelope membrane and thylakoid membrane precursor proteins apparently resides within the mature proteins.

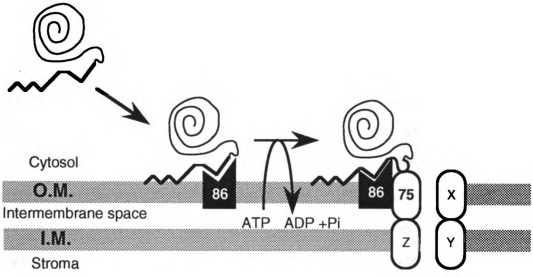
Import of transit-peptide bearing precursor proteins into chloroplasts is an energy dependent process. At least two separate steps in the import process can be distinguished, precursor binding and precursor translocation. Irreversible binding of a precursor to a chloroplast requires micromolar levels of nucleoside triphosphates (Olsen et al., 1989; Olsen and Keegstra, 1992) whereas translocation of a precursor across the envelope requires millimolar levels of ATP (Theg et al., 1989). One or more outer envelope membrane proteins mediate the binding step. Treatment of chloroplasts with thermolysin, a protease that does not breach the outer membrane boundary, greatly reduces subsequent precursor binding (Friedman and Keegstra, 1988).

Several additional proteins, collectively referred to as the "general protein import apparatus", mediate the import process. This protein import apparatus is "general" in the sense that most precursor proteins use the apparatus regardless of their final intra-organellar location (Perry et al., 1991). Three years ago when I began my dissertation research, there was little known about the composition of the general protein import apparatus. Perry and Keegstra (1994) had just recently identified two putative components, OEP75 and OEP86. Based on results from their cross-linking experiments, they devised a working model similar to the one presented in Figure 1.1.

Within the last three years, additional proteins of the import apparatus have been identified and cDNA clones encoding them have been obtained. Two recent reviews (Schnell, 1995; Gray and Row, 1995) have been written on the import components, and some of this information is discussed in later chapters. At this point, however, the reader should be aware of the general ensemble of proteins that are expected to be needed for the import process: cytosolic chaperones that maintain import competence of the precursor proteins, one or more receptors, channel-forming proteins in both the outer and inner envelope membranes, and stromal proteins to facilitate transport across the envelope and to

Figure 1.1. 1994 version of a working model of the general chloroplastic protein import apparatus.

OEP86 was postulated to be the receptor protein. OEP75 was thought to form at least part of the protein translocation channel in the outer envelope membrane. ATP-dependent interaction of the precursor with OEP75 was thought to cause the formation of an early translocation intermediate. O.M., outer envelope membrane; I.M., inner envelope membrane; X, Y, and Z, as yet unidentified components of the import apparatus. (Adapted from Perry and Keegstra, 1994.)


Cytosol
O.M.
Intermembrane space
I.M.
Stroma
86
86
75
X
Z
Y
ATP
ADP +Pi

process the translocated precursors.

TARGETING TO THE OUTER MEMBRANE OF THE CHLOROPLASTIC ENVELOPE

The transport of cytoplasmically synthesized proteins to the chloroplastic outer envelope membrane is quite different as compared to transport to other chloroplastic compartments. When I began my dissertation, cDNA clones had been reported for only two chloroplastic outer envelope membrane proteins, OEP7 (E 6.7) (Salomon et al., 1990) and OEP14 (OM14) (Li et al., 1991). The targeting pathways of OEP7 and OEP14 were investigated and found to be similar to each other. The import pathway of these outer envelope membrane proteins was different from that of other cytoplasmically synthesized chloroplastic proteins in three main ways: 1) they were not synthesized as higher molecular weight precursors, 2) import of these proteins was not dependent upon ATP, and 3) import of these proteins was not dependent upon a protease-susceptible outer envelope membrane component. Thus, the import pathway of these outer envelope membrane proteins is more accurately called an "insertion pathway" and likely does not involve the general chloroplastic protein import apparatus.

It was suspected that all chloroplastic outer envelope membrane proteins would follow the insertion pathway exemplified by OEP's 7 and 14. That is, all outer envelope membrane proteins would not be synthesized as higher molecular weight precursors and their insertion into the outer envelope membrane would proceed independent of ATP and a protease-susceptible receptor. To be sure, since beginning my dissertation, cDNA clones for additional proteins of the chloroplastic outer envelope membrane have been obtained and some of these fit that paradigm. OEP24 (omp24), OEP34 (IAP34), OEP44 (Com44),

and OEP70 (SCE70) (Fischer et al., 1994a; Kessler et al., 1994; Seedorf et al., 1995; Ko, et al., 1995; Ko et al., 1992) are all synthesized without a higher molecular weight precursor and do not strictly depend upon ATP nor a protease susceptible receptor for their insertion. ATP was shown to stimulate insertion of OEP24 and OEP34 (Fischer et al., 1994a; Seedorf et al., 1995); however, this point remains controversial (Kessler et al., 1994).

With the exception of OEP24, all of these newly identified outer envelope membrane proteins have been implicated as components of the general protein import apparatus. It will become clear in later chapters of this dissertation, however, that not all outer membrane components of the import apparatus have a targeting pathway that fits the targeting paradigm that existed when I began my dissertation.

BIOGENESIS OF THE IMPORT APPARATUS OF THE MITOCHONDRIAL ENVELOPE

Like chloroplasts, mitochondria contain their own genome but must import many proteins encoded by nuclear genes (Schatz, 1993). The protein import processes in chloroplasts and mitochondria have several common features. Most mitochondrial proteins encoded by nuclear genes are synthesized as higher molecular weight precursors. Import into the mitochondrial matrix requires that the precursor protein traverses a double membrane envelope. As in the case for translocation across the chloroplastic envelope, translocation across the mitochondrial envelope is ATP-dependent and mediated by a general protein import apparatus comprised of multiple protein components.

Like chloroplastic precursor proteins, mitochondrial precursor proteins are targeted to their respective organelle by an N-terminal stretch of amino acids. These analogous N-terminal targeting domains (referred to as 'transit peptides' and 'presequences' for

chloroplastic and mitochondrial precursors proteins, respectively) are similar in several respects (von Heijne et al., 1989). Neither transit peptides nor presequences have conserved primary structures; however, both of these targeting sequences are generally characterized by having few acidic residues and being enriched in serine and threonine. Lengths of both transit peptides and presequences vary greatly, although most are between 20 and 80 residues long (von Heijne et al., 1989).

There are some apparent differences between transit peptides and presequences. Compared to transit peptides, presequences are generally shorter, more highly enriched in arginine and alanine residues and not as highly enriched in serine and threonine residues. Also, typically the N-terminus of a mitochondrial presequence is predicted to from a positively charged, amphipathic alpha-helix (von Heijne et al., 1989).

Because of the many similarities between transit peptides and presequences and because of the variability among transit peptides and among presequences, one might expect mistargeting to occur in cells that contain both chloroplasts and mitochondria. That is, chloroplastic transit peptides might be recognized by mitochondria and vice versa. Although in general this does not seem to be the case (Boutry et al., 1987; Chaumont et al., 1990), examples of mistargeting in heterologous systems have been reported (Hurt et al., 1986; Huang et al., 1990). A transit peptide from a *Chlamydomonas reinhardtii* chloroplastic precursor protein targeted a passenger protein to yeast mitochondria (Hurt et al., 1986) and a presequence from a yeast mitochondrial precursor protein targeted a passenger protein to tobacco chloroplasts'(Huang et al., 1990). Furthermore, an example of mistargeting in a homologous system has been reported. Creissen et al., (1995) provided evidence that pea glutathione reductase is targeted to both chloroplasts and mitochondria by its native targeting sequence. In this case, dual targeting to chloroplasts and mitochondria is not "mistargeting" but rather is a mechanism of trafficking one protein to multiple cellular locations.

Thus, it is clear that targeting of precursor proteins to chloroplasts and mitochondria are analogous and similar processes. Interestingly, however, upon comparison of the protein import processes of both organelles on a molecular level, it appears that the general mechanisms of protein import may be quite different. As an illustration of this fact, with the exception of molecular chaperones, an import component has yet to be identified in one organelle that shows homology to an import component in the other organelle. Another important difference between transport into the two organelles is that transport into the mitochondrial matrix requires an electrochemical potential across the inner envelope membrane in addition to ATP (Schatz, 1993).

Several proteins of the mitochondrial import apparatus have been identified from yeast and *Neurospora crassa.* Several recent reviews have been written on the specific functions of these proteins and how these different proteins interact (Pfanner et al., 1994; Kübrich et al., 1995; Lil and Neupert, 1996). I will limit my discussion to a description of the import pathways of the protein components of the import apparatus, and only briefly mention the postulated function of each of these proteins. I will use the nomenclature prescribed by Pfanner et al. (1996). Briefly, outer and inner envelope membrane components are called TomXp and TimXp, respectively, where X is the molecular weight of the protein. Tom/Tim are abbreviations for "translocase of the outer/inner membrane of mitochondria".

Targeting of outer envelope membrane components

Of the known components of the mitochondrial import apparatus, the import of Tom70p has been studied the most. Tom70p is postulated to be the primary component of one of two receptor complexes (Lil and Neupert, 1996). Like all other known proteins of the

mitochondrial outer envelope membrane, Tom70p is encoded by a nuclear gene but is not synthesized as a higher molecular weight precursor.

Tom70p from yeast may be targeted to the outer membrane by the combination of an N-terminal matrix-targeting domain followed by a stop-transfer domain (Hase et al., 1984; Hurt et al., 1985; Nakai et al., 1989). If the sequence immediately C-proximal to the matrix-targeting domain (the putative stop-transfer domain) is disrupted, some of the resultant Tom70p is mistargeted to the matrix (Nakai et al., 1989). A conclusion from these studies is that Tom70p from yeast uses the general mitochondrial import apparatus for its targeting to the outer membrane (Shore et al., 1995). Results from studies conducted with *Neurospora* mitochondria revealed that antibodies against Tom20p, the primary component of the other receptor complex, inhibited import of *Neurospora* Tom70p (Sollner et al., 1990). Thus, there is evidence to suggest that both the yeast and *Neurospora* homologs of Tom70p engage the general mitochondrial protein import apparatus.

Recently, Schlossmann and Neupert (1995) reported that binding of Tom70p to the mitochondrial surface was not dependent on protease-susceptible surface components. (Both Tom20p and Tom70p are susceptible to protease that does not breach the mitochondrial outer envelope membrane boundary). Correct assembly of Tom70p into the envelope, however, was dependent on other outer envelope membrane proteins. Thus it is unclear whether the import pathway of Tom70p initially resembles that of matrix-targeted precursor proteins. It is likely, however, that the association of Tom70p with one or more other Tom proteins is a necessary part of its targeting pathway.

As mentioned previously, Tom20p is part of another receptor complex in the mitochondrial outer envelope membrane. Tom20p is found in association with Tom22p (Lil and Neupert, 1996). Treatment of mitochondria with protease to degrade surface proteins does not inhibit subsequent import nor assembly of Tom20p. Import of Tom20p appears to be mediated by its specific interaction with Tom40p (Schneider et al., 1991). Tom40p is thought to make up, at least in part, the pore in the outer envelope membrane

through which precursors pass. Although Tom40p is not susceptible to external protease, antibodies that recognize Tom40p bind to the surface of mitochondria from yeast. Incubation of yeast mitochondria with anti-Tom40p antibodies inhibits subsequent import of Tom20p (Schneider et al., 1991).

The import pathways of Tom22p and Tom40p are similar to each other. Import of Tom22p and Tom40p is greatly inhibited by pretreatment of mitochondria with protease. Furthermore, incubation of mitochondria with antibodies against either Tom20p or Tom70p inhibits subsequent import of Tom22p and Tom40p. Thus, Tom22p and Tom40p apparently require both of the primary receptor components, Tom20p and Tom70p, for import (Keil and Pfanner, 1993; Keil et al., 1993). Keil et al. (1993) argue that the requirement of both Tom20p and Tom70p for the import of Tom22p and Tom40p prevents "mistargeting by a self-amplification mechanism". For example, if Tom40p was mistargeted, subsequently Tom20p could be mistargeted followed by the mistargeting of precursor proteins recognized by Tom20p. Thus, the requirement of both receptors for import of Tom22p and Tom40p is a quality-control mechanism that increases targeting fidelity.

An additional outer envelope membrane protein of the mitochondrial import apparatus for which its import pathway has been addressed is Tom6p. It is thought to stabilize interactions between other Tom proteins (Alconada et al., 1995). Its import pathway does not require either receptor complex (Cao and Douglas, 1995). What mediates the targeting specificity for Tom6p is unknown; the targeting information, however, apparently resides within the C-terminus of Tom6p.

Targeting of inner envelope membrane components

Tim44p is a peripheral membrane protein of the inner membrane. It associates with Hsp70 on the matrix side of the membrane and facilitates import of precursors (Kronidou et al., 1994; Rassow et al., 1994; Berthold et al., 1995). Although a detailed analysis of Tim44p's import pathway has not been reported, Tim44p is synthesized with an N-terminal presequence.

Tim17p and Tim23p are integral inner membrane proteins (Dekker et al., 1993; Jennifer et al., 1993; Maarse et al., 1994). These two proteins are thought to make up the protein translocation channel of the inner membrane (Kübrich et al., 1994 Blom et al., 1995). Unlike Tim44p, both Tim17p and Tim23p are synthesized in the cytoplasm without an N-terminal presequence. Nonetheless, the import of Tim17p and Tim23p is like that of other inner membrane proteins in that it is dependent on a membrane potential (Dekker et al., 1993; Maarse et al., 1994). Treatment of mitochondria with protease inhibited subsequent import of Tim17p, indicating that Tim17p uses either or both Tom20p and Tom70p for its import (Maarse et al., 1994).

STATEMENT OF PROBLEM AND ATTRIBUTION

Plastid function is dependent upon proteins that are encoded by nuclear genes and synthesized in the cytoplasm. These proteins must be targeted to plastids and subsequently sorted to their intra-plastidic location. Most of these proteins share a common step, translocation across the plastidic envelope, en route to their final location within a plastid. This common step is facilitated by a general protein import apparatus consisting of several protein components.

When I began my dissertation research, Perry and Keegstra (1994) had just recently obtained evidence implicating OEP75 and OEP86 as two of the components of this import apparatus. The initial goal of my dissertation research was to obtain cDNA clones encoding OEP75 and OEP86 and perform structure/function studies on the encoded proteins. Upon isolation and preliminary analysis of a cDNA clone encoding OEP75, we determined that OEP75 was encoded as a higher molecular weight precursor (prOEP75). This finding and subsequent characterization revealed that OEP75 had an unusual targeting pathway to the chloroplastic outer envelope membrane. Thus, the focus of my dissertation shifted from studying the function of the import apparatus to studying the biogenesis of the import apparatus.

Only since protein components of the import apparatus have been identified, and cDNA clones encoding them have been isolated, has it been possible to address how these proteins are targeted to and assembled in their proper location. Knowledge of this process, the biogenesis of the import apparatus, may provide important insights into the biogenesis of plastids.

Chapter 2 is a reproduction of a published manuscript [Tranel, P.J., Froehlich, J., Goyal, A., and Keegstra, K. (1995) EMBO J. 14:2436-2446] that describes the isolation of a cDNA clone encoding OEP75. Initial characterization of the cDNA clone and of the encoded protein and its import pathway is presented. I wrote this manuscript and was involved in all aspects of the experiments described. Arun Goyal assisted in isolation and sequencing of the cDNA clone and John Froehlich conducted the import assays. The work was performed in the laboratory of Ken Keegstra under his supervision.

Chapter 3 is a reproduction of a manuscript that I wrote and have submitted to The Plant Cell for publication. In this paper, the import pathway of OEP75 is further analyzed and specific targeting information within the protein is described. The work for this paper was performed in the laboratory of Ken Keegstra under his supervision. Ken and I are the authors of this manuscript.

Chapter 4 describes an investigation of the phenomenon that rabbit reticulocyte lysate enhances the import of OEP75. This phenomenon was initially discovered by John Froehlich and myself. Investigations beyond the discovery stage were conducted almost exclusively by myself. There are no immediate plans to publish this work outside of my dissertation.

The focus of Chapter 5 is on OEP75 homologs in other plant species. Information in this chapter suggests that the work conducted with the pea OEP75 may be generally applicable to other plant species. Most of the work in this chapter was conducted by myself, however John Froehlich did the maize import experiment. Some of the information in this chapter may form the basis for a future published manuscript, in which case I will be listed as a co-author.

Unanswered questions and future directions are discussed in Chapter 6.

REFERENCES

Alconada, A., Kübrich, M., Moczko, M., Hönlinger, A., and Pfanner, N. (1995). The mitochondrial receptor complex: the small subunit Mom8b/Isp6 supports association of receptors with the general insertion pore and transfer of preproteins. Mol. Cell. Biol. **15,** 6196-6205.

Blom, J., Dekker, P.J., and Meijer, M. (1995). Functional and physical interactions of components of the yeast mitochondrial inner-membrane import machinery (MIM). Eur. J. Biochem. **232,** 309-314.

Boutry, M., Nagy, F., Poulsen, C., Aoyagi, K., and Chua, N.H. (1987). Targeting of bacterial chloramphenicol acetyltransferase to mitochondria in transgenic plants. Nature **328,** 340-342.

Cai, D., Herrmann, R.G., and Klösgen, R.B. (1993). The 20 kDa apoprotein of the CP24 complex of photosystem II: an alternative model to study import and intra-organellar routing of nuclear-encoded thylakoid proteins. Plant J. **3,** 383-392.

Cao, W., and Douglas, M.G. (1995). Biogenesis of ISP6, a small carboxy-terminal anchored protein of the receptor complex of mitochondrial outer membrane. J. Biol. Chem. **270,** 5674-5679.

Chaumont, F., O'Riordan, V., and Boutry, M. (1990). Protein transport into mitochondria is conserved between plant and yeast species. J. Biol. Chem. 265, 16856-16862.

Creissen, G., Reynolds, H., Xue, Y., and Mullineaux P. (1995). Simultaneous targeting of pea glutathione reductase and of a bacterial fusion protein to chloroplasts and mitochondria in transgenic tobacco. Plant J. **8,** 167-175.

de Boer, A. and Weisbeek, P.J. (1991). Chloroplast protein topogenesis: import, sorting and assembly. Biochim. Biophys. Acta. **1071,** 221-253.

Dekker, P.J.T., Keil, P., Rassow, J., Maarse, A.C., Pfanner, N., and Meijer, M. (1993). Identification of MIM23, a putative component of the protein import machinery of the mitochondrial inner membrane. FEBS Lett. **330,** 66-70.

Delwiche, C.F., Kuhsel, M., and Palmer, J.D. (1995). Phylogenetic analysis of tufA sequences indicates a cyanobacterial origin of all plastids. Mol. Phylogenet. Evol. **4,** 110-128.

Emes, M.J., and Tobin, A.K. (1993). Control of metabolism and development in higher plant plastids. Int. Rev. Cytol. **145,** 149-216.

Emtage, J.L.T., and Jensen, R.E. (1993). MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. J. Cell Biol. **122,** 1003-1012.

Fischer, K., Weber, A., Arbinger, B., Brink, S., Eckerskorn, C. and Flügge, U. (1994a). The 24 kDa outer envelope membrane protein from spinach chloroplasts: molecular cloning, in vivo expression and import pathway of a protein with unusual properties. Plant Mol. Biol. **25,** 167-177.

Fischer, K., Weber, A., Brink, S., Arbinger, B., Schünemann, D., Borchert, S., Heldt, H.W., Popp, B, Benz, R., Link, T.A., Eckerson, C., and Flügge, U. (1994b). Porins from plants. J. Biol. Chem. **269,** 25754-25760.

Friedman, A.L., and Keegstra, K. (1988). Chloroplast protein import: quantitative analysis of precursor binding. Plant Physiol. **89,** 993-999.

Gray, J.C., and Row, P.E. (1995). Protein translocation across envelope membranes. Trends Cell Biol. **5,** 243-247.

Hand, J.M., Szabo, L.J., Vasconcelos, A.C., and Cashmore, A.R. (1989). The transit peptide of a chloroplast thylakoid membrane protein is functionally equivalent to a stromal-targeting sequence. EMBO J. **8,** 3195-3206.

Hase, T., Müller, U., Riezman, H., and Schatz, G. (1984). A 70-kD protein of the yeast mitochondrial outer membrane is targeted and anchored via its extreme amino terminus. EMBO J. **3,** 3157-3164.

Huang, J., Hack, E., Thornburg, R.W., and Myers, A.M. (1990). A yeast mitochondrial leader peptide functions in vivo as a dual targeting signal for both chloroplasts and mitochondria. Plant Cell **2,** 1249-1260.

Hurt, E.C., Müller, U., and Schatz, G. (1985). The first twelve amino acids of a yeast mitochondrial outer membrane protein can direct a nuclear-encoded cytochrome oxidase subunit to the mitochondrial inner membrane. EMBO J. **4,** 3509-3518.

Hurt, E.C., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J.D. and Schatz, G. (1986). The cleavable presequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria. EMBO J. **5,** 1343-1350.

Keil, P. and Pfanner, N. (1993). Insertion of MOM22 into mitochondrial outer membrane strictly depends on surface receptors. FEBS Lett., **321**, 197-200.

Keil, P., Weinzierl, A., Kiebler, M., Dietmeier, K., Söllner, T. and Pfanner, N. (1993). Biogenesis of the mitochondrial receptor complex. J. Biol. Chem., **268**, 19177-19180.

Kessler, F., Blobel, G., Patel, H.A. and Schnell, D.J. (1994). Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science **266,** 1035-1038.

Knight, J.S., and Gray, J.C. (1995). The N-terminal hydrophobic region of the mature phosphate translocator is sufficient for targeting to the chloroplast inner envelope membrane. Plant Cell **7,** 1421-1432.

Ko, K., Bornemisza, O., Kourtz, L., Ko, Z.W., Plaxton, W.C. and Cashmore, A.R. (1992). Isolation and characterization of a cDNA clone encoding a cognate 70-kDa heat shock protein of the chloroplast envelope. J. Biol. Chem. **267,** 2986-2993.

Ko, K., Budd, D., Chengbiao, W., Seibert, F., Kourtz, L., and Ko, Z.W. (1995). Isolation and characterization of a cDNA clone encoding a member of the Com44/Cim44 envelope components of the chloroplast protein import apparatus. J. Biol. Chem. **270,** 28601-28608.

Kronidou, N.G., Oppliger, W., Bolliger, L., Hannavy, K., Glick, B.S., Schatz, G., and Horst, M. (1994). Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. Proc. Natl. Acad. Sci. USA **91,** 12818-12822.

Kübrich, M., Dietmeier, K., and Pfanner, N. (1995). Genetic and biochemical dissection of the mitochondrial protein-import machinery. Curr. Genet. **27,** 393-403.

Kübrich, M., Keil, P., Rassow, J., Dekker, P.J.T., Blom, J., Meijer,M., and Pfanner, N. (1994). The polytopic mitochondrial inner membrane proteins MIM17 and MIM23 operate at the same preprotein import site. FEBS Lett. **349,** 222-228.

Li, H., Morre, T. and Keegstra, K. (1991). Targeting of proteins to the outer envelope membrane uses a different pathway than transport into chloroplasts. Plant Cell **3,** 709-717.

Li, H., Sullivan, T.D., and Keegstra, K. (1992). Information for targeting to the chloroplastic inner envelope membrane is contained in the mature region of the maize *Bt1*-encoded protein. J. Biol. Chem. **267,** 18999-19004.

Lill, R., and Neupert, W. (1996). Mechanisms of protein import across the mitochondrial outer membrane. Trends Cell Biol. **6,** 56-62.

Maarse, A.C., Blom, J., Keil, P., Pfanner, N., and Meijer, M. (1994). Identification of the essential yeast protein MIM17, an integral mitochondrial inner membrane protein involved in protein import. FEBS Lett. **349,** 215-221.

Nakai, M., Hase, T., and Matsubara, H. (1989). Precise determination of the mitochondrial import signal contained in a 70 kDa protein of yeast mitochondrial outer membrane. J. Biochem. **105,** 513-519.

Nelissen, B., Van de Peer, Y., Wilmotte, A., and De Wachter, R. (1995). An early origin of plastids within the cyanobacterial divergence is suggested by evolutionary trees based on complete 16S rRNA sequences. Mol. Biol. Evol. **12,** 1166-1173.

Olsen, L.J., and Keegstra, K. (1992). The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. J. Biol. Chem. **267,** 433-439.

Olsen, L.J., Theg, S.M., Selman, B.R. and Keegstra, K. (1989). ATP is required for the binding of precursor proteins to chloroplasts. J. Biol. Chem. **264,** 6724-6729.

Perry, S.E., Buvinger, W.E., Bennett, J., and Keegstra, K. (1991). Synthetic analogues of a transit peptide inhibit binding or translocation of chloroplastic precursor proteins. J. Biol. Chem. **266,** 11882-11889.

Perry, S.E., Keegstra, K. (1994). Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell **6,** 93-105.

Pfanner, N., Craig, E.A., and Meijer, M. (1994). The protein import machinery of the mitochondrial inner membrane. TIBS **19,** 368-372.

Pfanner, N., Douglas, M.G., Endo, T., Hoogenraad, N.J., Jensen, R.E., Meijer, M., Neupert, W., Schatz, G., Schmitz, U.K., and Shore, G.C. (1996). Uniform nomenclature for the protein transport machinery of the mitochondrial membranes. TIBS **21,** 51-52.

Possingham. J.V. (1980). Plastid replication and development in the life cycle of higher plants. Ann. Rev. Plant Physiol. **31,** 113-129.

Robinson, C., and Klösgen, R.B. (1994). Targeting of proteins into and across the thylakoid membrane - a multitude of mechanisms. Plant Mol. Biol. **26,** 15-24.

Salomon, M., Fischer, K., Flügge, U. and Soll, J. (1990). Sequence analysis and protein import studies of an outer chloroplast envelope polypeptide. Proc. Natl. Acad. Sci. **87,** 5778-5782.

Schatz, G. (1993). The protein import machinery of mitochondria. Protein Sci. **2,** 141-146.

Schlossmann, J., and Neupert, W. (1995). Assembly of the preprotein receptor MOM72/MAS70 into the protein import complex of the outer membrane of mitochondria. J. Biol. Chem. **270,** 27116-27121.

Schneider, H., Söllner, T., Dietmeier, K., Eckerskorn, C., Lottspeich, F., Trülzsch, B., Neupert, W. and Pfanner, N. (1991). Targeting of the master receptor MOM19 to mitochondria. Science **254,** 1659-1662.

Schnell, D.J. (1995). Shedding light on the chloroplast protein import machinery. Cell **83,** 521-524.

Seedorf, M., Waegemann, K. and Soll, J. (1995). A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J. **7,** 401-411.

Shore, G.C., McBride, H.M., Millar, D.G., Steenaart, N.A.E., and Nguyen,M. (1995). Import and insertion of proteins into the mitochondrial outer membrane. Eur. J. Biochem. **227,** 9-18.

Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N. and Neupert, W. (1990). A mitochondrial import receptor for the ADP/ATP carrier. Cell **62,** 107-115.

Theg, S.M., Bauerle, C., Olsen, L.J., Selman, B.R. and Keegstra, K. (1989). Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. J. Biol. Chem. **264,** 6730-6736.

Theg, S.M. and Scott, S.V. (1993). Protein import into chloroplasts. Trends in Cell Biol. **3,** 186-190.

Thomson, W.W., and Whatley, J.M. (1980) Development of nongreen plastids. Ann. Rev. Plant Physiol. **31,** 375-394.

VanderVere, P.S., Bennett, T.M., Oblong, J.E., and Lamppa, G.K. (1995). A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. Proc. Natl. Acad. Sci. USA **92,** 7177-7181.

von Heijne, G., Steppuhn, J. and Herrmann, R.G. (1989). Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. **180**, 535-545.

Wan, J., Blakeley, S.D., Dennis, D.T., and Ko, K. (1995). Import characteristics of a leucoplast pyruvate kinase are influenced by a 19-amino-acid domain within the protein. J. Biol. Chem. **270,** 16731-16739.

Chapter 2

A COMPONENT OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS IS TARGETED TO THE OUTER ENVELOPE MEMBRANE VIA A NOVEL PATHWAY

ABSTRACT

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.

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 to 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; Kiel 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 kDa, 14 kDa, 70 kDa, 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 kDa 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 kDa 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 eveidence 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.

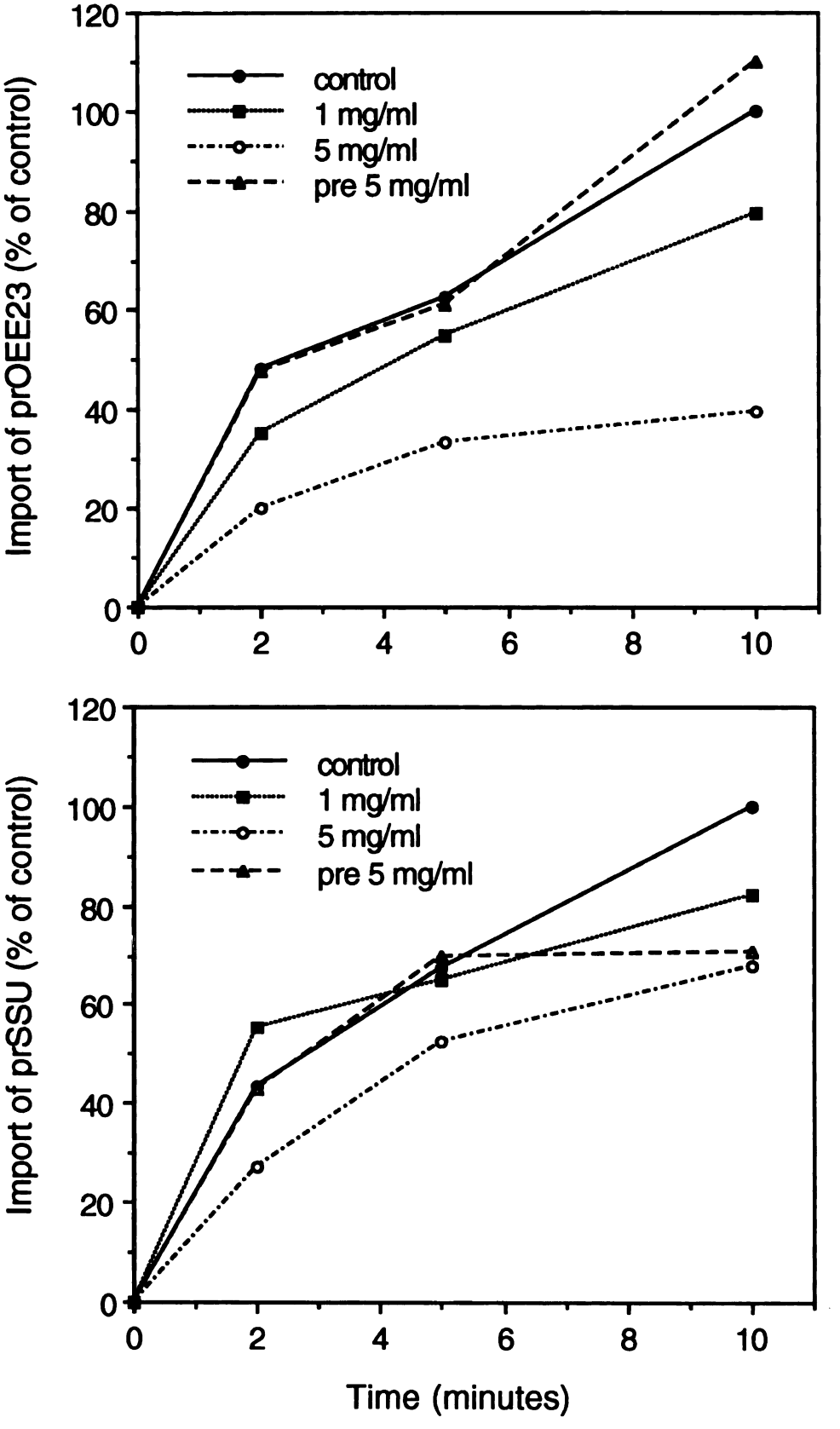
RESULTS

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., 1984; Moczko et al., 1993; Kiebler 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 2.1A). Under similar conditions, import of prSSU was reduced by 30% (Figure 2.1B). Fab fragments (5 mg/ml) isolated from pre-immune serum did not inhibit import of either precursor (Figure 2.1A and B). Taken together, the findings that OEP75 can be crosslinked 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.

Figure 2.1. 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 hour and then incubated with radiolabeled precursor under import conditions. After 2, 5 and 10 min, the reaction was stopped by the addition of 10-fold excess 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).


Import of prOEE23 (% of control)
120
100
80
60
40
20
0
control
1 mg/ml
5 mg/ml
pre 5 mg/ml
0
2
4
6
8
10
Import of prSSU (% of control)
120
100
80
60
40
20
0
control
1 mg/ml
5 mg/ml
pre 5 mg/ml
0
2
4
6
8
10
Time (minutes)

Isolation of cDNA encoding OEP75

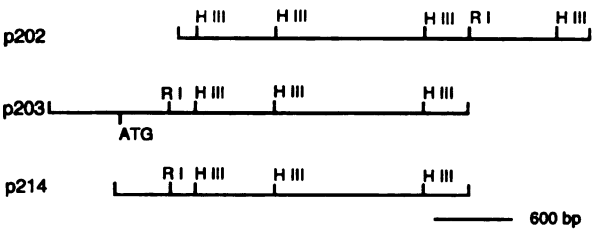
To obtain a cDNA clone for OEP75, an aliquot of the purified protein that was used for raising antibodies (described above) was subjected to tryptic digestion. Individual tryptic peptides were purified and their amino acid sequences determined. Peptide sequence data were used to design a degenerate oligonucleotide probe (see Material and Methods) that was then used to screen a pea cDNA library. Clone p202 was thus isolated and the nucleotide sequence of the insert was determined. The insert contained the adapter sequence, used for library construction, adjacent to an internal EcoRI site (Figure 2.2A), 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 less than 75 kDa, so we concluded that it was not a full-length clone. The 590 bp HindIII 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 2.2A). 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 2.2B. 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 2.2A), 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

Figure 2.2. Overview of cDNA clones (**A**) and deduced amino acid sequence of prOEP75 (**B**).

Locations of HindIII and EcoRI 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.

A



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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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 2.2B, 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 2.2B, boxed). Possibly 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, is rich in small aliphatic residues, and is rich 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 HindIII 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 2.3A, RNA isolated from very young leaves contained more message for prOEP75 than did RNA isolated from older leaves. Only a single transcript of ca. 3 kb was detected. This size agrees with the size of the cDNA isolated (clone p203, 3318 bp).

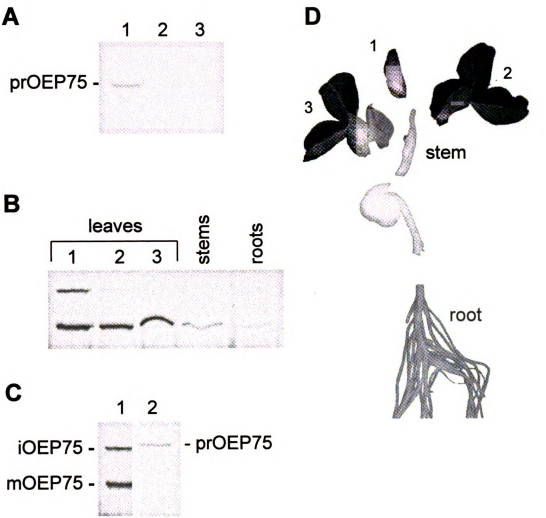
Western blot analysis of total protein extracts revealed that mOEP75 was present in leaves, stems, and roots (Figure 2.3B). In addition to mOEP75, a larger protein that reacts to anti-OEP75 antibodies was detected in young leaves. The abundance of this protein declined much more rapidly with leaf age than did mOEP75. Because this protein is intermediate in size between the precursor and mature forms of OEP75 (Figure 2.3C), 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 eight day old pea seedlings and separated into soluble, envelope, and thylakoid fractions as described in Material and

Figure 2.3. 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 HindIII fragment from p203. A weak signal could be detected in lanes 2 and 3 of the original blot. (**B**) One hundred micrograms 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 dpm translation product generated from p214 (prOEP75). Autoradiogram of lane 1 is shown in lane 2.


A
1 2 3
prOEP75 -
B
leaves
1 2 3
stems
roots
C
1 2
iOEP75 -
mOEP75 -
- prOEP75
D
1
2
3
stem
root

Methods. Analysis of the fractions by SDS-PAGE and immunoblotting with anti-OEP75 antibodies revealed that iOEP75 was localized to chloroplastic envelope membranes (Figure 2.4A). Furthermore, base extraction revealed that iOEP75 behaved as an integral membrane protein, similar to that seen for mOEP75 (Figure 2.4B).

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 2.5A). In addition to mOEP75, two additional proteins intermediate in size between prOEP75 and mOEP75 appeared during the timecourse. (The band detected at about 55 kDa was most likely 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 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 2.5A). 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 2.5A, 40 min time point), suggesting it was on a productive

Figure 2.4. Characterization of endogenous OEP75.

Chloroplasts were isolated from young, folded leaves from eight day old seedlings. (**A**) Chloroplasts were separated into stroma (S), envelope (E), or thylakoid (T) fractions. Twenty micrograms 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. Twenty micrograms of pellet (P) or supernatant (S) were analyzed. All samples were analyzed by SDS-PAGE and immunoblotting with anti-OEP75 antibodies.

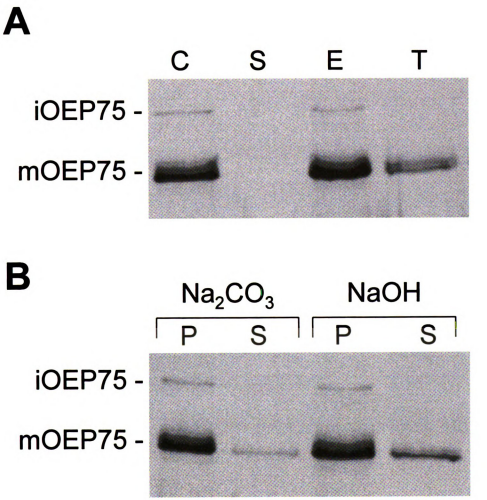
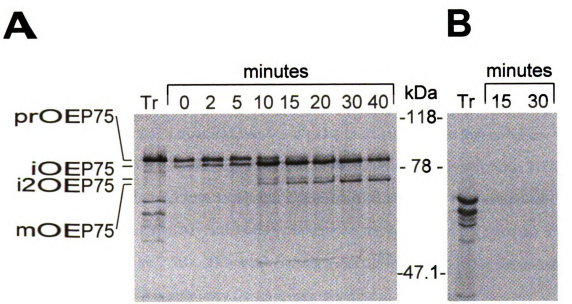

A
C
S
E
T
iOEP75 -
mOEP75 -
B
Na2CO3
NaOH
P
S
P
S
iOEP75 -
mOEP75 -

Figure 2.5. 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.


A
B
minutes
Tr
0 2 5 10 15 20 30 40
kDa
-118-
- 78 -
-47.1-
prOEP75
iOEP75
i2OEP75
mOEP75
minutes
Tr
15 30

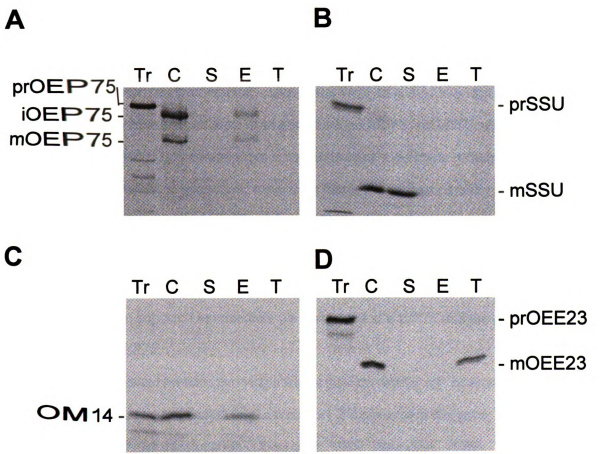
pathway to mOEP75. We can not eliminate the possibility, however, that i2OEP75 was degraded. iOEP75 was still present even after prolonged incubation. To test whether iOEP75 was en route to mOEP75, we attempted to chase it to the mature form. Chloroplasts were incubated with prOEP75 in an import assay 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 EcoRI 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 2.5B). 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 more directly address this point, 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 2.6), similarly to that seen with the endogenous proteins (Figure 2.4). Representative proteins of other chloroplastic compartments were

Figure 2.6. Fractionation of processed prOEP75 (**A**).

Radiolabeled precursor proteins were incubated with chloroplasts under import conditions. The import reaction was scaled up three-fold over the standard reaction, as described in Material 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-fourth 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.


A
B
Tr C S E T
prOEP75
iOEP75
mOEP75
Tr C S E T
- prSSU
- mSSU
C
D
Tr C S E T
OM14
Tr C S E T
- prOEE23
- mOEE23

included in the fractionation analysis as markers of the fractions (Figure 2.6). 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 2.7), 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 membrane or firmly held within the import complex. Again, newly imported mOEP75 and iOEP75 behaved similarly to the endogenous proteins (Figure 2.4), 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 2.8A), indicating that one or more exposed proteins on the outer membrane was required for its binding and

Figure 2.7. 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.

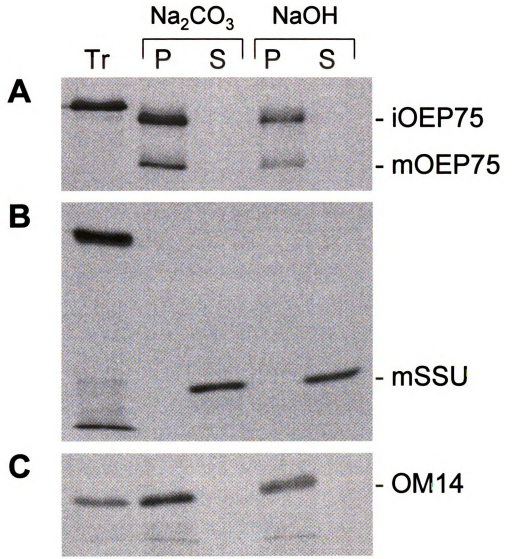
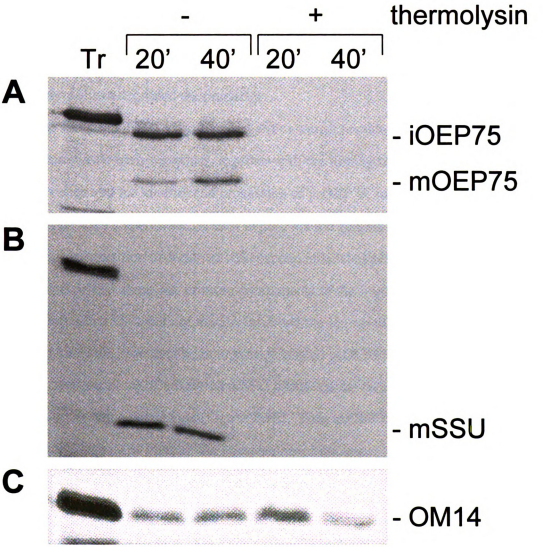

Na2CO3
NaOH
Tr
P
S
P
S
A
- iOEP75
- mOEP75
B
- mSSU
C
- OM14

Figure 2.8. 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.


-
+
thermolysin
Tr
20'
40'
20'
40'
A
- iOEP75
- mOEP75
B
- mSSU
C
- OM14

processing. This result was identical to that observed for prSSU, a representative stromal-targeted protein used as a control (Figure 2.8B). 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 2.8C). Thus the apparent lack of prOEP75 binding and import can not be attributed to the trivial explanation that residual thermolysin degraded the precursor.

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 2.9). Excess unlabeled prSSU, but not mSSU, inhibited the import of radiolabeled prOEP75 (Figure 2.9A 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 2.9E). Thus prOEP75 and prSSU likely 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 2.10). 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) that 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 2.10). When ATP

Figure 2.9. 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).

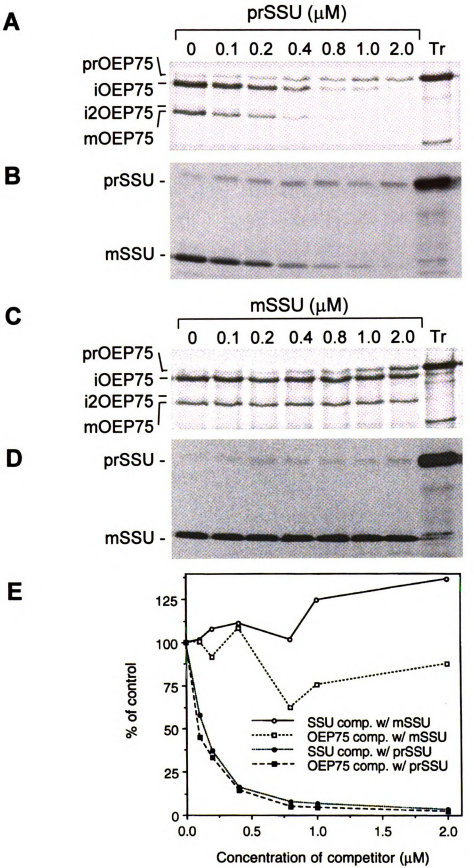
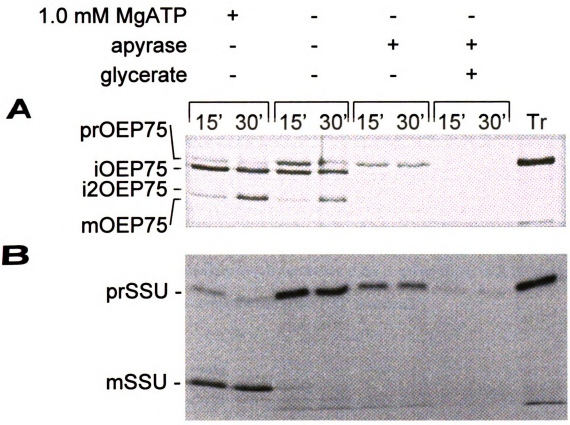

A
prSSU (μM)
0 0.1 0.2 0.4 0.8 1.0 2.0 Tr
prOEP75
iOEP75
i2OEP75
mOEP75
B
prSSU -
mSSU -
C
mSSU (μM)
0 0.1 0.2 0.4 0.8 1.0 2.0 Tr
prOEP75
iOEP75
i2OEP75
mOEP75
D
prSSU -
mSSU -
E
% of control
125
100
75
50
25
0
0.0 0.5 1.0 1.5 2.0
Concentration of competitor (μM)
SSU comp. w/ mSSU
OEP75 comp. w/ mSSU
SSU comp. w/ prSSU
OEP75 comp. w/ prSSU

Figure 2.10. 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 (ca. 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.


1.0 mM MgATP + - - -
apyrase - - + +
glycerate - - - +
A
15' 30' 15' 30' 15' 30' 15' 30' Tr
prOEP75
iOEP75
i2OEP75
mOEP75
B
prSSU
mSSU

was not exogenously added, ATP levels (ca. 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 2.10). 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 2.10). 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 fully understand 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 into chloroplasts of precursor proteins. 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, that is present in the import complex (Schnell et al., 1994; Kessler et al., 1994; Seedorf et al., 1995) have the consensus ATP/GTP binding domain, whereas OEP75 lacks such a domain. Thus ATP likely is not utilized by OEP75 but rather by OEP86 or OEP34. Possibly the ATP-dependent step causes a conformation change in either OEP86 or OEP34 that brings the bound precursor protein in contact with or 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 ß-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 freely pass (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 likely will 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 are 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 raise 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, about 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. Analogously, 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 than 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.

METHODS

Isolation of a cDNA clone encoding OEP75

A cDNA library was constructed from mRNA isolated from leaves of five and seven day-old pea (*Pisum sativum* var. *little marvel*) seedlings grown under a 12 hr light/12 hr dark cycle. A mixture of oligo dT and random primers were used in cDNA synthesis. cDNAs were cloned into the EcoRI 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, Dept. 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 HindIII 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 BstXI and XbaI. After exonuclease digestion, clones were ligated and transformed into *E. 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 2.9) 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 to 12 day old pea seedlings were isolated over Percoll gradients and resuspended in import buffer at 1 mg/ml chlorophyll. Translation mixture (ca. 5 x 10^5 dpm) 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 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 2.6), the import assay was scaled up three-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 M and 1.2 M sucrose solutions.

For extraction analysis (Figure 2.7) 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 2.3D. 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% nonfat 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 eight day old seedlings. Chloroplasts were

fractionated as described 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 HindIII 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 0.1x SSC at 65°C.

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REFERENCES

Baker, K.P. and Schatz, G. (1991). Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature **349**, 205-208.

Benz, R. (1994). Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. Biochim. Biophys. Acta. **1197**, 167-196.

Blobel, G. (1980). Intracellular protein topogenesis. Proc. Natl. Acad. Sci. **77**, 1496-1500.

de Boer, A. and Weisbeek, P.J. (1991). Chloroplast protein topogenesis: import, sorting and assembly. Biochim. Biophys. Acta. **1071**, 221-253.

Bruce, B.D., Perry, S., Froehlich, J. and Keegstra, K. (1994). In vitro import of proteins into chloroplasts. Plant Mol. Biol. Manual **J1**, 1-15.

Cline, K., Andrews, J., Mersey, B., Newcomb, E.H. and Keegstra, K. (1981). Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc. Natl. Acad. Sci. **78**, 3595-3599.

Cline, K., Werner-Washburne, M., Andrews, J. and Keegstra, K. (1984). Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. Plant Physiol. **75**, 675-678.

Dahlin, C. and Cline, K. (1991). Developmental regulation of the plastid protein import apparatus. Plant Cell **3**, 1131-1140.

Fischer, K., Weber, A., Arbinger, B., Brink, S., Eckerskorn, C. and Flügge, U. (1994). The 24 kDa outer envelope membrane protein from spinach chloroplasts: molecular cloning, in vivo expression and import pathway of a protein with unusual properties. Plant Mol. Biol. **25**, 167-177.

Friedman, A.L. and Keegstra, K. (1989). Chloroplast protein import: quantitative analysis of precursor binding. Plant Physiol. **89**, 993-999.

He, Z., Li, J., Sundqvist, C. and Timko, M.P. (1994). Leaf developmental age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum* L.) Plant Physiol. **106**, 537-546.

von Heijne, G., Steppuhn, J. and Herrmann, R.G. (1989). Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. **180**, 535-545.

Hirsch, S., Muckel, E., Heemeyer, F., von Heijne, G. and Soll, J. (1994). A receptor component of the chloroplast protein translocation machinery. Science **266**, 1989-1992.

Keegstra, K. and Yousif, A.E. (1986). Isolation and characterization of chloroplast envelope membranes. Meth. Enzymol. **118**, 316-325.

Keil, P. and Pfanner, N. (1993). Insertion of MOM22 into mitochondrial outer membrane strictly depends on surface receptors. FEBS Lett. **321**, 197-200,

Keil, P., Weinzierl, A., Kiebler, M., Dietmeier, K., Söllner, T. and Pfanner, N. (1993). Biogenesis of the mitochondrial receptor complex. J. Biol. Chem. **268**, 19177-19180.

Kessler, F., Blobel, G., Patel, H.A. and Schnell, D.J. (1994). Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science **266**, 1035-1038.

Kiebler, M., Keil, P., Schneider, H., van der Klei, I.J., Pfanner, N. and Neupert, W. (1993). The mitochondrial receptor complex: a central role of MOM22 in mediating preprotein transfer from receptors to the general insertion pore. Cell **74**, 483-492.

Ko, K., Bornemisza, O., Kourtz, L., Ko, Z.W., Plaxton, W.C. and Cashmore, A.R. (1992). Isolation and characterization of a cDNA clone encoding a cognate 70-kDa heat shock protein of the chloroplast envelope. J. Biol. Chem. **267**, 2986-2993.

Li, H., Morre, T. and Keegstra, K. (1991). Targeting of proteins to the outer envelope membrane uses a different pathway than transport into chloroplasts. Plant Cell **3**, 709-717.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Moczko, M., Gärtner, F. and Pfanner, N. (1993). The protein import receptor MOM19 of yeast mitochondria. FEBS Lett. **326**, 251-254.

Moore, T. and Keegstra, K. (1993). Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. Plant Mol. Biol. **21**, 525-537.

Olsen, L.J. and Keegstra, K. (1992). The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. J. Biol. Chem. **267**, 433-439.

Olsen, L.J., Theg, S.M., Selman, B.R. and Keegstra, K. (1989). ATP is required for the binding of precursor proteins to chloroplasts. J. Biol. Chem. **264**, 6724-6729.

Perry, S.E., Keegstra, K. (1994). Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell **6**, 93-105.

Salomon, M., Fischer, K., Flügge, U. and Soll, J. (1990). Sequence analysis and protein import studies of an outer chloroplast envelope polypeptide. Proc. Natl. Acad. Sci. **87**, 5778-5782.

Schatz, G. (1993). The protein import machinery of mitochondria. Protein Sci. **2**, 141-146.

Schneider, H., Söllner, T., Dietmeier, K., Eckerskorn, C., Lottspeich, F., Trülzsch, B., Neupert, W. and Pfanner, N. (1991). Targeting of the master receptor MOM19 to mitochondria. Science **254**, 1659-1662.

Schnell, D.J., Kessler, F. and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. Science **266**, 1007-1012.

Seedorf, M., Waegemann, K. and Soll, J. (1995). A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J. **7**, 401-411.

Selden, R.F. (1987). In Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.P. and Struhl, K. (ed.) Current Protocols in Molecular Biology. John Wiley and Sons, NY, Vol. 1., pp 4.9.1-4.9.4.

Soll, J. and Alefsen, H. (1993). The protein import apparatus of chloroplasts. Physiol. Plant. **87**, 433-440.

Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N. and Neupert, W. (1990). A mitochondrial import receptor for the ADP/ATP carrier. Cell **62**, 107-115.

Theg, S.M., Bauerle, C., Olsen, L.J., Selman, B.R. and Keegstra, K. (1989). Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. J. Biol. Chem. **264**, 6730-6736.

Theg, S.M. and Scott, S.V. (1993). Protein import into chloroplasts. Trends in Cell Biol. **3**, 186-190.

Vestweber, D., Brunner, J., Baker, A. and Schatz,G. (1989). A 42K outer-membrane protein is a component of the yeast mitochondrial protein import site. Nature **341**, 205-209.

Waegemann, K. and Soll, J. (1991). Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J. **1**, 149-158.

Werner-Washburne, M., Cline, K. and Keegstra, K. (1983). Analysis of pea chloroplast inner and outer envelope membrane proteins by two-dimensional gel electrophoresis and their comparison with stromal proteins. Plant Physiol. **73**, 569-575.

Chapter 3

A NOVEL, BIPARTITE TRANSIT PEPTIDE TARGETS OEP75 TO THE OUTER MEMBRANE OF THE CHLOROPLASTIC ENVELOPE

ABSTRACT

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-terminal 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 deletion mutant constructs from prOEP75 and chimeric constructs between domains of prOEP75 and precursor to 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-terminal and C-terminal portions contain chloroplast-targeting and intra-organellar 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 (for reviews see Archer and Keegstra, 1990; deBoer and Weisbeek, 1991; Theg and Scott, 1993). The transit peptides of precursors to stromal proteins are removed by a stromal processing peptidase upon translocation of the precursors across the chloroplastic envelope. Proteins destined for the thylakoid lumen contain a bipartite transit peptide consisting of N-terminal and C-terminal domains (reviewed by 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 stromal targeted precursors. Intra-organellar targeting information for these inner envelope membrane and thylakoid membrane precursor proteins apparently resides within the mature proteins. Of 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 pathways of these outer envelope membrane proteins is distinct from that of all 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 by Schnell, 1995; Gray and Row, 1995). At least five outer envelope membrane proteins (OEP's) have been implicated to play a role in the import process. OEP34 (IAP34) has GTPase activity, however its specific function is unknown (Kessler et al., 1994; Seedorf et al., 1995). OEP44 (Com44) was identified by chemical cross-linking and also is of unknown function (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 (Kessler et al., 1994; Hirsch et al., 1994). OEP's 34, 40 and 70 are like most chloroplastic outer envelope membrane proteins in that they are not synthesized as higher molecular weight precursors (Kessler et al., 1994; Seedorf et al., 1995; Ko, et al., 1995; Ko et al., 1992). Surprisingly, however, both OEP75 and OEP86 are synthesized as higher molecular weight precursors, containing N-terminal extensions (Schnell et al., 1994; Tranel et al., 1995; Hirsch et al, 1994; Kessler et al., 1994).

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 small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (prSS), a stromal targeted precursor protein (Hirsch et al., 1994).

In a previous communication (Tranel et al., 1995) we presented evidence suggesting 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 can be competed by prSS.

In this communication we provide further 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 the transit peptide of prOEP75 is bipartite: it contains a chloroplast-targeting domain and an envelope-targeting domain.

RESULTS

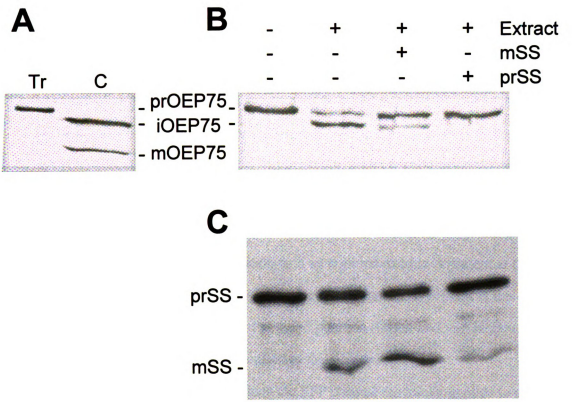
prOEP75 is processed to iOEP75 by the stromal processing peptidase

Upon incubation with isolated chloroplasts, prOEP75 is stepwise processed 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 3.1A. Previously (Tranel et al., 1995) we speculated that the N-terminus of the prOEP75 transit peptide was a chloroplast-targeting domain, and that during import it would be removed by the stromal processing peptidase (SPP) (VanderVere et al., 1995), generating iOEP75. To test this hypothesis, we incubated radiolabeled prOEP75 with stromal extract. As shown in Figure 3.1B, incubation of prOEP75 with stromal extract resulted in accumulation of iOEP75 but not mOEP75. We also performed the stromal processing assay with prSS (Figure 3.1C). prOEP75 and prSS were processed with similar efficiency. To determine if prSS and prOEP75 were processed by the same protease, we attempted to compete processing of prOEP75 by adding excess, unlabeled prSS. As can be seen in Figure 3.1B, addition of prSS greatly reduced the accumulation of iOEP75, indicating that its appearance was dependent on the SPP. mSS was not an efficient competitor of prOEP75 processing.

Based on molecular size determination from SDS-PAGE, three to four kilodaltons of the prOEP75 transit peptide were removed by the SPP. To determine the specific processing site we employed radiolabeled protein sequencing. prOEP75 was labeled by incorporation of ^{3}H-leucine and incubated with intact chloroplasts under standard import conditions. Under these conditions, 3 mM ATP, 30 min at room temperature, typically 10% to 20% of the added prOEP75 is processed to iOEP75, half of which is further processed to mOEP75. Resultant iOEP75 was isolated by SDS-PAGE, transferred onto a PVDF membrane and subjected to sequencing by Edmann degradation.

Figure 3.1. prOEP75 is processed to iOEP75 by the stromal processing peptidase.

(A) Radiolabeled prOEP75 translation product (Tr) was incubated with chloroplasts in 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 **(B)** or prSS **(C)** was incubated with or without stromal extract for 90 min at room temperature. Stromal extract was prepared as described in Methods. Unlabeled mSS or prSS (1.8 μM final concentration) was added as a competitor to reactions as indicated. Products of the reactions were analyzed by SDS-PAGE and fluorography.


A
Tr
C
prOEP75
iOEP75
mOEP75
B
- + + + Extract
- - + - mSS
- - - + prSS
C
prSS
mSS

The amount of radioactivity released after each cycle is presented in Figure 3.2A. A strong peak of radioactivity was released after the second cycle, followed by at least ten cycles in which no radioactive peaks were detected. There are four potential processing sites which could give rise to this sequence pattern. That is, there are four leucine residues, underlined in Figure 3.2B, that do not have another leucine residue immediately N-proximal to it nor within ten residues C-proximal to it. Of these four potential cleavage sites, however, the removal of three to four kilodaltons 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 17th 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 (V/I)-X-(A/C)↓A (Gavel and von Heijne, 1990). Additionally, arginine residues are common between 6 and 10 residues N-proximal to 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 the -1 position, and an arginine residue is present at the -7 position. In all previously known cases where a cysteine residue is present at the -1 position, an isoleucine residue is present at the -3 position (Gavel and von Heijne, 1990). The cleavage site of prOEP75 is an exception to this observation.

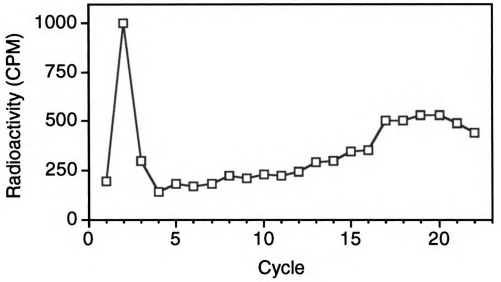
Having determined the site at which the SPP cleaved the transit peptide to generate iOEP75, we were able to subdivide prOEP75 into three regions, the N-terminal and the C-terminal regions of the transit peptide (n75 and c75, respectively) and the mature region (m75). A schematic showing these divisions and the nomenclature we use throughout the remainder of this manuscript is given in Figure 3.3. Having subdivided the transit peptide into two regions, we could begin to address the function of each region. To do so, we created deletion mutant constructs from prOEP75 and chimeric constructs between domains of prOEP75 and the transit peptide and mature regions of prSS (tSS and mSS,

Figure 3.2. The stromal processing peptidase cleaves prOEP75 between residues 35 and 36.

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

(B) Amino acid sequence of the prOEP75 transit peptide. All leucine residues are in bold; leucine residues that do not have another leucine immediately N-proximal to it nor within the ten residues C-proximal to it are underlined. ↓, the deduced stromal processing site.

A



B

```
  1 M R T S V I P N R L T P T L T T H P S R
 21 R R N D H I T T R T S S L K C↓H L S P S
 41 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
 81 P N F S G L N A A A G G G A G G G G G G
101 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
```

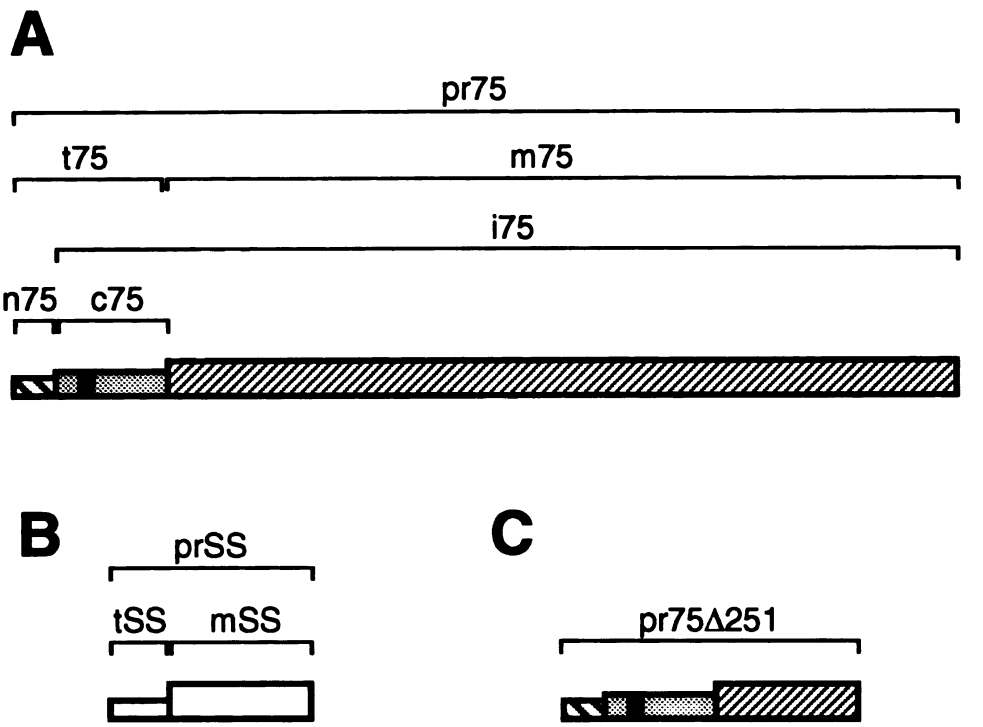
Figure 3.3. Schematic of prOEP75, prSS, and a C-terminal truncation of prOEP75, and the nomenclature for domains within the precursors.

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-terminus 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).


A
pr75
t75
m75
i75
n75
c75
B
prSS
tSS
mSS
C
pr75Δ251

respectively). To name these constructs, we simply listed, in order and joined by hyphen, 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. Using in vitro assays to analyze the import characteristics of these constructs, we were able to make inferences as to the function of each of the three regions of pr75 in its import pathway (described below).

n75 is a chloroplast-targeting domain

The fact that pr75 and prSS compete for import (Tranel et al. 1995) together with the finding that n75 is removed by the SPP, suggest that n75 functions as a typical chloroplastic transit peptide. That is, n75 directs the precursor to the general import apparatus. If this is true, then n75 and tSS should be functionally interchangeable. As shown in panels A and B of Figure 3.4, n75 can be functionally replaced with tSS. Upon incubation with chloroplasts under import conditions, tSS-i75 was processed to two major products which were the sizes expected for i75 and m75 (Figure 3.4A). When chloroplasts were lysed after the import reaction and separated into soluble, envelope and thylakoid fractions, the products were recovered in the envelope and thylakoid fractions. A nearly identical fractionation pattern was obtained for i75 and m75 when derived from pr75 (Figure 3.4B). The import efficiency of tSS-i75 was similar to that of pr75 (compare lane C of Figures 3.4A and 3.4B).

It is extremely difficult to isolate thylakoids that are not contaminated with envelope membranes. Thus we interpret the fractionation results from the pr75 and tSS-i75 import experiments to mean that in both cases, the products were targeted to the envelope membranes. The appearance of i75 and m75 in the thylakoid fraction was most likely due

Figure 3.4. n75 is functionally interchangeable with tSS.

Radiolabeled precursor was incubated with chloroplasts under import conditions. Intact chloroplasts were then re-purified over Percoll. Two-ninths of the reaction was analyzed directly (C, chloroplasts prior to fractionation). The remaining seven-ninths was 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, 1/45 **(A)**, **(B)**, **(D)**, **(E)** or 1/90 **(C)** the volume of translation product that was added to the initial reaction.

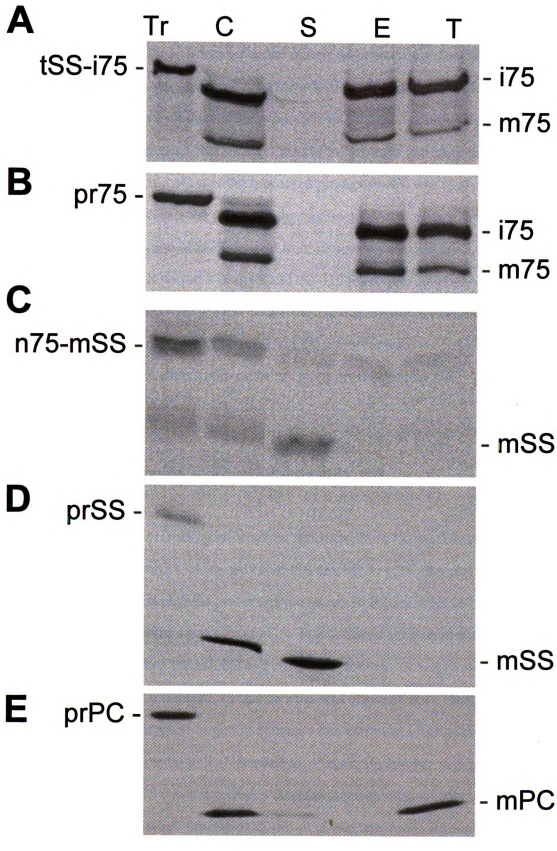
(A) Import and fractionation of tSS-i75.

(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).


A
Tr
C
S
E
T
tSS-i75 -
- i75
- m75
B
pr75 -
- i75
- m75
C
n75-mSS -
- mSS
D
prSS -
- mSS
E
prPC -
- mPC

to contamination of the thylakoids with envelope membranes. A protein targeted to the thylakoid lumen was recovered almost exclusively in the thylakoid fraction (Figure 3.4E).

Similar analysis was performed on the reciprocal construct, n75-mSS (Figure 3.4C). 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 of the processed product (mSS) was recovered in the soluble fraction. The fractionation pattern of the mature product was very similar to that produced upon fractionation of mSS derived from imported prSS (Figure 3.4D).

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 i75 to the import apparatus. Subsequent targeting information that directs m75 to the envelope must be within i75.

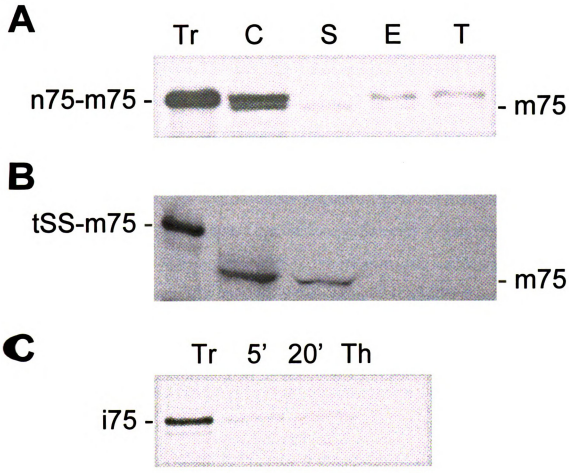
Intra-organellar targeting information is within c75

To determine if envelope targeting information resides within c75, we deleted this region from pr75 to obtain n75-m75. Also, we fused tSS directly to m75. Results from import and fractionation analysis of these precursors are shown in Figure 3.5A. n75-m75 was processed to a product of the same size as m75. Fractionation analysis revealed that the product was present in both the soluble and envelope fractions. n75-m75 was not efficiently imported nor processed, similar to what was observed for n75-mSS. Thus, n75 was not an efficient chloroplast-targeting domain when taken out of its native context, i.e., when c75 was not C-proximal to it. Nonetheless, it was apparent that without c75 a significant portion of m75 was not correctly targeted to the envelopes. The same conclusion was reached when the import of tSS-m75 was analyzed. As shown in Figure

Figure 3.5. c75 contains envelope-targeting information.

(A) and **(B)** Import and fractionation of n75-m75 **(A)** and tSS-m75 **(B)** as described in the legend to Figure 3.4. Tr, 1/90 **(A)** or 1/45 **(B)** volume of translation product added to the reaction.

(C) Import of i75. Radiolabeled i75 was incubated with chloroplasts under import conditions. After five min, chloroplasts from one-third of the reaction were re-purified over Percoll. The remaining chloroplasts were re-purified over Percoll after 20 min, half of which were then treated with thermolysin (0.2 mg/ml for 30 min on ice) and then pelleted. All samples were analyzed by SDS-PAGE and fluorography. Tr, 1/30 volume of translation product added to the reaction; Th, sample that was treated with thermolysin after import.


A
Tr
C
S
E
T
n75-m75 -
- m75
B
tSS-m75 -
- m75
C
Tr
5'
20'
Th
i75 -

3.5B, 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 the reproduction shown in Figure 3.5B. Comparison of Figures 3.5A and 3.5B with Figures 3.4B and 3.4A, respectively, reveals that c75 has a profound effect on the targeting of m75. We conclude that much of the envelope-targeting information within pr75 resides within c75.

The recovery of the products during fractionation of n75-m75 and tSS-m75 was inefficient. (Compare the ratios of product that was in the fractions versus in the whole chloroplast sample of Figures 3.5A and 3.5B to the same ratio in Figure 3.4D.) We suspected that if m75 was 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 appreciable loss of the product (data not shown). Another possibility was 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 upon acetone precipitation (data not shown). Thus, the results presented in Figures 3.5A and 3.5B 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 3.5C indicate that it can not. Upon incubation with chloroplasts, only a small percentage of i75 binds to the chloroplasts. The observed binding is probably due to non-specific association of i75 to the chloroplastic surface because the binding was not time dependent (compare 5 and 20 min incubations). Furthermore, all of the associated protein was susceptible to external protease, indicating that i75 was not incorporated into the 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, t75 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 intra-organellar targeting information.

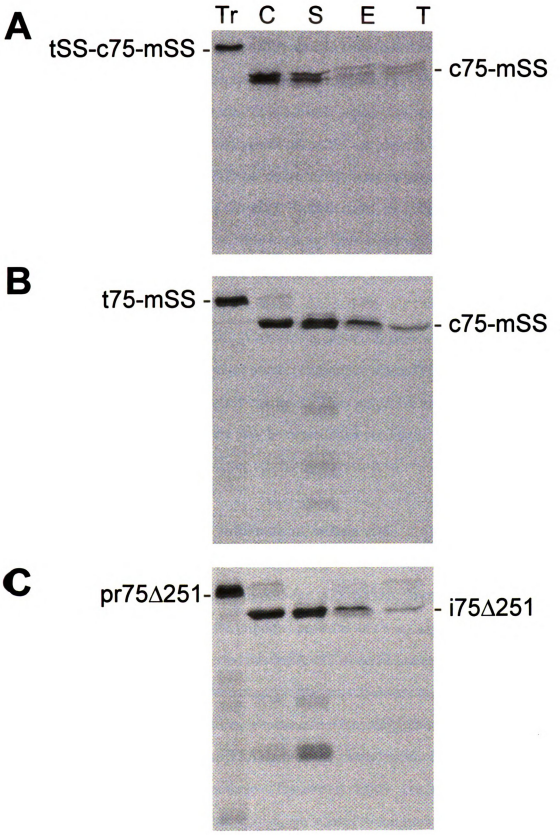
c75 can direct a passenger protein to the envelope

To more rigorously test whether c75 contains envelope-targeting information, we attempted to use this domain to target mSS to the envelope. The c75 domain was inserted into prSS to obtain tSS-c75-mSS. Results presented in Figure 3.6A reveal that, upon incubation with chloroplasts, tSS-c75-mSS was processed to two major products. These two products are similar in size and of the approximate size expected if tSS is removed. Likely, 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). Upon fractionation, both products behaved the same and are collectively referred to as c75-mSS. The majority of c75-mSS was recovered in the soluble fraction. Significantly, however, a portion of c75-mSS was recovered in the envelope and thylakoid fractions. We interpret this to mean that c75 successfully diverted some of the precursor from the general 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 3.6B 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, the majority of the product was in the soluble fraction but a portion was targeted to the envelopes. Why is all of the product not targeted to the envelope fraction? Specific envelope-targeting information may reside within m75.

Figure 3.6. c75 can target a passenger protein to the envelope.

(A), (B), and **(C)** Import and fractionation of tSS-c75-mSS **(A)**, t75-mSS **(B)**, and pr75Δ251 **(C)** as described in the legend to Figure 3.4. Tr, 1/45 volume of translation product added to the reaction.


A
Tr C S E T
tSS-c75-mSS -
- c75-mSS
B
t75-mSS -
- c75-mSS
C
pr75Δ251-
- i75Δ251

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 C-terminal truncation of pr75, schematically represented in Figure 3.3C, was constructed (see Methods). This precursor protein, designated pr75Δ251, contains the first 246 residues of pr75 followed by cysteine, isoleucine, asparagine, valine, and a stop codon. Results from fractionation analysis of pr75Δ251 are given in Figure 3.6C. The major product derived from pr75Δ251 was no more efficiently targeted to the envelope than was the major product derived from t75-mSS. In both cases, roughly two-thirds of the major product was recovered in the soluble fraction. Thus, either additional envelope-targeting information is present within residues 247-809 of m75, or i75Δ251 is not able to efficiently anchor itself in the envelope.

Import of t75-mSS and pr75Δ251 resulted in the accumulation of lower molecular weight products in addition to the major products described above (Figures 3.6B and 3.6C). The identity of these products is unknown. They may have resulted from subsequent processing of c75, or they may be degradation products.

Investigation of the targeting information within c75

As described above, removal of c75 from pr75 (i.e. n75-m75) or from tSS-c75-m75 greatly decreased the proportion of the imported products that were targeted to the envelopes. Furthermore, when inserted into prSS, c75 directed some of the imported products to the envelopes. From these observations, we concluded that c75 contains envelope-targeting information. Earlier, we speculated that a hydrophobic region within c75 (denoted schematically in Figure 3.3A) 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 intra-organellar targeting information, we wanted to determine if, 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 3.7A. As shown in Figure 3.7B, 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 3.7C). 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 alpha-helical structure (data not shown). To determine if this putative structure was 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 alpha helix. Results from an import and fractionation experiment with this mutant (pr75A66P) revealed that if the region does form an alpha helix, it is likely not important to the targeting function of c75 (Figure 3.7D).

Although the mutations did not have any 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 because of the inherent variability among experiments. Four replications of a time course experiment were performed and representative results are shown in Figure 3.8. The most obvious kinetic effects of the mutations were not on the accumulation of the i intermediate, but rather were on the subsequent processing of the i intermediate to the i_2 intermediate and the mature form. $i_2$75 is a second size intermediate that is transiently observed during import of pr75 (Tranel et al., 1995). Compared to the other mutations, the A66P mutation caused the slowest rate of subsequent processing of the i intermediate. The most pronounced effect of the mut4

Figure 3.7. Disruption of the hydrophobic region within c75 does not alter targeting.

(A) Hydrophobicity plots (Kyte and Doolittle, 1982; window size=18) for the transit peptides of pr75 and pr75mut4.

(B), (C), and **(D)** Import and fractionation of pr75mut4 **(B)**, pr75A66R **(C)**, and pr75A66P **(D)** as described in the legend to Figure 3.4. Tr, 1/45 volume of translation product added to the reaction.

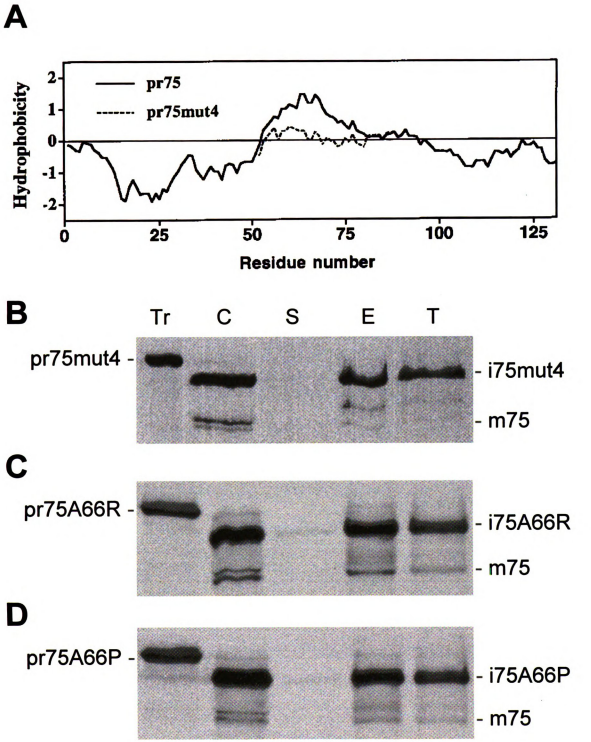
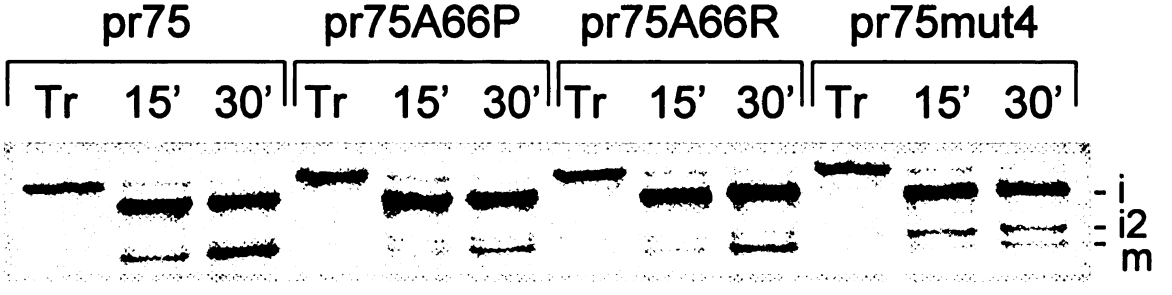

A
pr75
pr75mut4
Hydrophobicity
2
1
0
-1
-2
0
25
50
75
100
125
Residue number
B
Tr
C
S
E
T
pr75mut4 -
- i75mut4
- m75
C
pr75A66R -
- i75A66R
- m75
D
pr75A66P -
- i75A66P
- m75

Figure 3.8. Effects of disruption of the hydrophobic region within c75 on import kinetics.

Radiolabeled precursor protein was incubated with chloroplasts under import conditions. After 15 min and 30 min, chloroplasts from half of the reaction were re-purified over Percoll. Samples were analyzed by SDS-PAGE and fluorography. Tr, 1/20 volume of translation product added to the reaction. The experiment was replicated four times. The results presented are from a representative experiment.


pr75
pr75A66P
pr75A66R
pr75mut4
Tr
15'
30'
Tr
15'
30'
Tr
15'
30'
Tr
15'
30'
i
i2
m

mutation seemed to be an increase in the accumulation of the i_2 intermediate. 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

Three domains within pr75 sequentially carry out three steps in the import pathway

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 (E 6.7) and OEP14 (OM14) (Salomon et al., 1990; Li et al., 1991), does not require ATP nor chloroplastic proteins exposed to the cytosol. Thus, "import" of these proteins appears to consist of direct insertion of the protein into the membrane.

There are two known proteins of the outer envelope membrane that follow the transit peptide-dependent pathway, OEP75 and OEP86 (Tranel et al., 1995; Hirsch et al., 1994). The import pathways of prOEP86 and pr75 are like that 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 stromal precursor proteins, however, because import of prOEP86 cannot be competed by prSS (Hirsch et al., 1994). Furthermore, a chimeric construct containing the prOEP86 transit peptide fused to mSS was not imported into chloroplasts (E. Muckel and J. Soll, personal communication). In contrast to prOEP86, pr75 competes for import with prSS (Tranel et al. 1995), and a chimeric construct containing the pr75 transit peptide fused to mSS was imported into chloroplasts. Thus, pr75 apparently uses the general chloroplastic protein import apparatus. Consistent with this conclusion, pr75 was processed by the SPP.

The peptide that was removed from pr75 by SPP, n75, acted as a chloroplast-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 carrying out this function. For example, n75-mSS was imported with very low efficiency. However, import of tSS-

i75 was no more efficient than the import of pr75. Thus it does not appear that the chloroplast-targeting domain of pr75 is inefficient, but rather that n75 does not represent the complete chloroplast targeting domain of pr75. In support of this is the fact 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 chloroplast-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 chloroplast-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 is two-fold: 1) if c75 is deleted from pr75 or from tSS-i75, much of the imported m75 no longer co-fractionates with envelope membranes, but instead is present in the soluble fraction, 2) if c75 is inserted between tSS and mSS, upon import, a portion of the products co-fractionates with envelopes.

After being diverted from the general protein import pathway, m75 must be anchored and assembled in the outer envelope membrane. The final topology of m75 in the outer envelope membrane is unknown. pr75Δ251 was the only construct we created that contained only a portion of m75. Analysis of the import of other constructs that contain larger portions of m75 could provide further insight into the role of m75 in its targeting pathway. Additionally, such analysis may be useful in understanding the final topology of m75 in the outer envelope membrane.

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 alpha-helices, however, and has been postulated to span the membrane with multiple amphipathic beta-strands. These beta-strands may come together to form a beta-barrel structure, similar to that formed by porins (reviewed by Benz, 1994). Formation of such a tertiary structure may be necessary to firmly anchor m75 in the outer envelope membrane.

The formation of a membrane anchor of some kind by m75 appears to be important for its targeting to the outer envelope membrane. When t75 was fused to mSS, the product after import, c75-mSS, was not exclusively targeted to the envelope. Thus c75 is not sufficient for membrane targeting. Yet m75 alone, when targeted to the chloroplasts by n75 or tSS, was not exclusively targeted to the envelope. Furthermore, a truncated form of pr75, pr75Δ251, was inefficiently targeted to the envelope despite being very efficiently imported. One explanation for these observations is that c75 slows or opposes translocation of i75 across the envelope, allowing m75 to fold and assemble within the outer envelope membrane, and thereby anchor it to the membrane.

This model, in which c75 opposes translocation and m75 forms the membrane anchor, is consistent with other observations as well. For example, a greater proportion of imported m75 was recovered in the soluble fraction when m75 was fused to tSS rather than n75. Because tSS was more efficient than n75 as a chloroplast-targeting domain (discussed above), tSS would be expected to be more efficient at overcoming the effects of the opposing-translocation function of c75. Thus, when fused to tSS, m75 would have less time to assemble within the envelope during the import process.

A model in which c75 opposes translocation and m75 forms the membrane anchor also accounts for how c75 could contain all of the intra-organellar targeting information yet c75 inefficiently targets a passenger protein to the envelope. Because the function of c75

would only be to slow translocation, in the absence of an anchoring domain within the passenger protein, e.g. t75-mSS, the protein would eventually be imported into the stroma.

The specific mechanism by which c75 may act to oppose translocation remains unclear. We expected that the hydrophobic region within c75 would be involved with the targeting function of c75. However, our analysis indicated that neither the overall hydrophobicity nor the predicted alpha-helical structure of this region was important to its function.

It is not known where in the chloroplastic envelope c75 may be functioning. Assuming that i75 spans the envelope in an unfolded configuration during import, portions of c75 could be in both the inner and outer envelope membranes. Thus, it is possible that c75 could interact with proteins and/or lipids of either or both membranes. Also, it can not be ruled out that c75 interacts with intermembrane space proteins. Certainly, more detailed experiments are needed to fully understand how c75 functions.

Events involved with processing of i75 to m75 have yet to be elucidated

The specific details of how c75 functions and how m75 assembles to anchor itself in the outer envelope membrane remain unclear. An additional aspect of the prOEP75 import pathway that remains unclear is the processing of i75 to m75. Both in vitro and in vivo, this step appears to be a rate-limiting step (Tranel et al., 1995). Where in the chloroplast is the protease that carries out this step? 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 still saw processing only to i75.

During the time course of in vitro import of pr75, i75 appears first, followed by a second size intermediate, $i_2$75 and finally by m75 (Tranel et al. 1995). $i_2$75, which

migrates slightly slower 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 $i_2$75mut4) with the same mobility as $i_2$75. Because of its transient nature during import of pr75, we know little about $i_2$75 and are unsure of its physiological significance. pr75mut4 may be a useful tool to learn more about $i_2$75.

A more complete understanding of the prOEP75 import pathway may yield practical applications. The discovery that the N-terminus of nuclear-encoded chloroplastic proteins served as a chloroplast-targeting signal made it possible to direct recombinant proteins to the stroma (Van den Broeck et al., 1985). Most chloroplastic outer envelope membrane proteins contain their targeting information within the mature portion of the protein. Thus, it is difficult to decipher what the targeting information is, and 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 intra-organellar targeting signal to target recombinant proteins to the chloroplastic outer envelope membrane.

The import pathway of pr75 has parallels with that of some mitochondrial proteins

Of known mitochondrial outer envelope membrane proteins, the import pathway of Mas70p has been most studied (reviewed by Shore et al., 1995). Like all other known proteins of the mitochondrial outer envelope membrane, Mas70p is not synthesized as a higher molecular weight precursor. Nonetheless, Mas70p may engage the general mitochondrial import apparatus. The extreme N-terminus (residues 1-12) of Mas70p acts as a weak mitochondria-targeting domain (Hase et al., 1984; Hurt et al., 1985). Immediately C-proximal to this domain is a stop transfer domain (Nakai et al., 1989).

Disruption of the stop transfer domain results in mistargeting of some Mas70p to the matrix. Analogously, removal of c75 from pr75 resulted in targeting of some of the resultant m75 to the chloroplastic soluble fraction.

NADH-cytochrome b_5 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 b_5 reductase is a matrix-targeting domain. C-proximal to this domain is a stop-transfer domain. Some of the protein follows an import pathway identical to that of Mas70p, 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 b_5 reductase and Mas70p are like 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 Mas70p. In the case of NADH-cytochrome b_5 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. pr75, however, is the only protein that we know of 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.

Conclusions

Our results obtained from stromal processing assays and analysis of deletion and chimeric constructs provide direct evidence in support of our earlier hypothesis (Tranel et al. 1995) that a bipartite transit peptide targets OEP75 to the outer envelope membrane. Our current working model of the import pathway for pr75 can be divided into three main steps. Each of the three regions of pr75 (n75, c75, and m75) sequentially carries out one of these steps. In the first step, n75, functioning as a chloroplast-targeting domain, directs pr75 to the general import apparatus in the chloroplastic envelope. pr75 then translocates part way across the envelope, exposing n75 to the stroma whereupon it is removed by the SPP. The second step is carried out by c75. It opposes translocation and thereby prevents i75 from being fully imported into the stroma. The third step is carried out by m75. Although we do not think m75 contains specific targeting information, we think that it contains information needed for assembly in the outer envelope membrane. All three steps, chloroplast-targeting, opposition of translocation, and assembly, are necessary for efficient import of m75 to the outer envelope membrane.

METHODS

In vitro transcription and translation

Radiolabeled proteins were generated from cDNAs using standard in vitro transcription and translation procedures (Bruce et al., 1994). For determination of the stromal processing site in pr75, pr75 was labeled with ^{3}H leucine. ^{35}S methionine was used to label pr75 for use in all other experiments and to label all other proteins. Radiochemicals were purchased from DuPont-NEN, all other reagents were from Promega. 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.

Stromal processing assay

The stromal processing assay was conducted essentially as described (Abad et al., 1989). Chloroplasts were resuspended at 0.5 mg chlorophyll/ml in 5 mM Hepes/KOH (pH 8) and incubated on ice 30 min. The lysed chloroplasts were centrifuged at 200,000*g* for 30 min at 4°C. Resultant supernatant was the stromal extract. 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. Total reaction volume was 30 µl and contained 5 mM Hepes/KOH (pH 8). Some samples contained unlabeled prSS or mSS (1.8 µM final concentration). Unlabeled prSS and mSS were generated by overexpression in *E. coli* as described (Perry and Keegstra, 1994). Reactions were terminated by addition of 2x sample buffer and immediate heating to 100°C. Products of the reaction were analyzed by SDS-PAGE and fluorography.

Preparation of mutant and chimeric cDNA clones

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

Chimeric proteins containing portions of pr75 and prSS were obtained from in vitro transcription/translation of cDNA clones. These clones were obtained by subcloning portions of the parent cDNA clones encoding pr75 and prSS. The parent clones for prSS and pr75 were pET11d-prSSU (Klein and Salvucci, 1992) and p214 (Tranel et al., 1995), respectively. To facilitate subsequent cloning steps, the cDNA insert from pET11d-prSSU was excised from the vector by XbaI/EcoRV digestion and directionally cloned into pBluescript II SK+ (Stratagene) digested with XbaI/EcoRV. The resultant clone encoding prSS was designated p46.

Preparation of chimeric cDNA clones encoding t75-mSS and tSS-m75. p46 contains a unique BalI site, 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 SacI/EcoRV fragment of p214 was cloned into pBluescript digested with SacI/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 using the oligonucleotide 5'-GCGAATCCCAATCTTCGTTAACTGGTTCGTCGG-3'. The cDNA insert from a resultant mutant (p52) was sequenced to verify that no off-site mutations occurred. Then, 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 t75-mSS or tSS-m75, the SacI/BalI fragment from p46 and the SacI/HpaI fragment from p56 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 using the oligonucleotide 5'-GGAATCGTTGTTGTCGCCCATAGCGCTAGAAAGGTGAC-3'. After verifying the sequence of a resultant mutant clone, the EcoRV fragment from p214 was cloned into the EcoRV site of the mutant clone. A resultant clone, p72, contained the EcoRV fragment in the proper orientation and was identical to p56 except that the fourth, fifth, and sixth codons for i75 encoded for serine-alanine-methionine rather than the native proline-serine-serine. The substitution of methionine for serine was made so that translation could be initiated from this codon in the cDNA encoding i75 (see below). There were only minor differences, if any, between the rates of import and processing of pr75 and the precursor encoded by p72; and fractionation patterns obtained after import of these two precursors were indistinguishable (data not shown).

To generate cDNAs encoding either n75-mSS or tSS-i75, the SacI/BalI fragment from p46 and the SacI/Eco47III fragment from p72 where exchanged with each other. tSS-c75-mSS was obtained from a cDNA clone consisting of the Eco47III/HpaI fragment from p72 cloned into the BalI site of p46. A clone encoding n75-m75 was obtained by digesting p72 with Eco47III/HpaI and then isolating and re-ligating the plasmid A clone encoding i75 was obtained by blunt-ending and re-ligating p72 after first digesting with SacI/Eco47III.

Preparation of cDNA clone encoding pr75Δ251. 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 re-ligated. 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 a isoleucine, and leucine at 249 to asparagine. The valine at position 250 was unchanged. The protein encoded by this cDNA was designated pr75Δ251.

Mutagenesis of the hydrophobic domain within c75. Mutagenesis of the region encoding the hydrophobic domain of pr75 was performed on the SacI/EcoRI fragment of p214 subcloned into pBluescript. To change alanine at position 66 to a proline (A66P mutation) the oligonucleotide 5'-GGAAGAAAGCGGAAGCGGGAGCGCTGGAAACAGC-3' was used. Mutagenesis with this oligonucleotide also introduced an HaeII site which facilitated selection of mutants. To make an A66R mutation and a triple mutation, A61R:A66R:F71R, the oligonucleotides 5'-GGAAGAAAGCGGAAGCGCGAGCGCTGGAAACAGC-3' and 5'-GGAGGGAGCCGGTGAGGCGGAAAGCGGAAGCGCGAGCGCTGGAAACACGGACGGTGGT-3' were used, respectively. Both of these oligonucleotides also introduced an HaeII site. Mutagenesis performed with the latter oligonucleotide yielded clones which contained the HaeII site but did not contain all three amino acid codon conversions. A mutant clone was isolated, however, that encoded for the A61R and A66R mutations. This clone was subjected to another round of mutagenesis using the oligonucleotide 5'-GGGAGTGGAGGGAGCCCGTACGGAAGAAAGCG-3'. Mutagenesis with this oligonucleotide introduced an L72R mutation as well as a BsiWI restriction site for mutant selection. A resultant mutant contained an off-site mutation, P79T, in addition to the L72R mutation. Nonetheless, this mutant, which contained the codons encoding the following mutations: A61R, A66R, L72R, P79T (collectively designated as "mut4"), was selected for import analysis. The EcoRI fragment from p214 was ligated into the EcoRI site of each mutagenized clone. Resultant clones were screened by restriction mapping to identify and select those that contained the EcoRI fragment in the proper orientation.

Import assays

Import assays were conducted essentially as described previously (Bruce et al., 1994). Chloroplasts were from 8 to 12 day old pea (*Pisum sativum* var. *little marvel*) seedlings and were isolated over Percoll gradients and resuspended in import buffer at 1 mg chlorophyll/ml.

For determination of the stromal processing site in pr75, 1.2 x 10^6 cpm of ^{3}H leucine-labeled pr75 were added to 40 µl chloroplast suspension 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 time intact chloroplasts were re-isolated over 40% (v/v) Percoll in import buffer. The chloroplast pellet was lysed by resuspending in 25 mM Hepes/KOH (pH 8) and incubating on ice 10 min. Membranes from the lysed chloroplasts were collected by centrifugation at 435,000*g* for 10 min at 4°C and then resuspended in 2x sample buffer. The sample was subjected to SDS-PAGE and then blotted onto a ProBlott (Applied Biosystems) 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 x 10^5 dpm of precursor protein were added to 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 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 re-isolated over 40% Percoll in import buffer, resuspended in 2x sample buffer and analyzed directly. Chloroplasts from the remaining 350 µl of the reaction were re-isolated 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.

Protein sequencing

^{3}H 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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REFERENCES

Abad, M.S., Clark, S.E., and Lamppa, G.K. (1989). Properties of a chloroplast enzyme that cleaves the chlorophyll a/b binding protein precursor. Plant Physiol. **90,** 117-124.

Archer, E.K., and Keegstra, K. (1990). Current views on chloroplast protein import and hypotheses on the origin of the transport mechanism. J. Bioenerg. Biomembr. **22,** 789-810.

Archer, E.K., and Keegstra, K. (1993). Analysis of chloroplast transit peptide function using mutations in the carboxyl-terminal region. Plant Mol. Biol. **23,** 1105-1115.

Benz, R. (1994). Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. Biochim. Biophys. Acta **1197,** 167-196.

de Boer, A. and Weisbeek, P.J. (1991). Chloroplast protein topogenesis: import, sorting and assembly. Biochim. Biophys. Acta. **1071,** 221-253.

Bruce, B.D., Perry, S., Froehlich, J. and Keegstra, K. (1994). In vitro import of proteins into chloroplasts. Plant Mol. Biol. Manual **J1,** 1-15.

Cai, D., Herrmann, R.G., and Klösgen, R.B. (1993). The 20 kDa apoprotein of the CP24 complex of photosystem II: an alternative model to study import and intra-organellar routing of nuclear-encoded thylakoid proteins. Plant J. **3,** 383-392.

Fischer, K., Weber, A., Arbinger, B., Brink, S., Eckerskorn, C. and Flügge, U. (1994). The 24 kDa outer envelope membrane protein from spinach chloroplasts: molecular cloning, in vivo expression and import pathway of a protein with unusual properties. Plant Mol. Biol. **25,** 167-177.

Gavel, Y., and von Heijne, G. (1990). A conserved cleavage-site motif in chloroplast transit peptides. FEBS Lett. **261,** 455-458.

Gray, J.C., and Row, P.E. (1995). Protein translocation across envelope membranes. Trends Cell Biol. **5,** 243-247.

Hahne, K., Haucke, V., Ramage, L., and Schatz, G. (1994). Incomplete arrest in the outer membrane sorts NADH-cytochrome b_5 reductase to two different submitochondrial compartments. Cell **79,** 829-839.

Hand, J.M., Szabo, L.J., Vasconcelos, A.C., and Cashmore, A.R. (1989). The transit peptide of a chloroplast thylakoid membrane protein is functionally equivalent to a stromal-targeting sequence. EMBO J. **8,** 3195-3206.

Hase, T., Müller, U., Riezman, H., and Schatz, G. (1984). A 70-kD protein of the yeast mitochondrial outer membrane is targeted and anchored via its extreme amino terminus. EMBO J. **3,** 3157-3164.

Hirsch, S., Muckel, E., Heemeyer, F., von Heijne, G. and Soll, J. (1994). A receptor component of the chloroplast protein translocation machinery. Science **266,** 1989-1992.

Hurt, E.C., Müller, U., and Schatz, G. (1985). The first twelve amino acids of a yeast mitochondrial outer membrane protein can direct a nuclear-encoded cytochrome oxidase subunit to the mitochondrial inner membrane. EMBO J. **4,** 3509-3518.

Kessler, F., Blobel, G., Patel, H.A. and Schnell, D.J. (1994). Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science **266,** 1035-1038.

Klein, R.R., and Salvucci, M.E. (1992). Photoaffinity labeling of mature and precursor forms of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase after expression in *E. coli*.. Plant Physiol. **98,** 546-553.

Knight, J.S., and Gray, J.C. (1995). The N-terminal hydrophobic region of the mature phosphate translocator is sufficient for targeting to the chloroplast inner envelope membrane. Plant Cell **7,** 1421-1432.

Ko, K., Bornemisza, O., Kourtz, L., Ko, Z.W., Plaxton, W.C. and Cashmore, A.R. (1992). Isolation and characterization of a cDNA clone encoding a cognate 70-kDa heat shock protein of the chloroplast envelope. J. Biol. Chem. **267,** 2986-2993.

Ko, K., Budd, D., Chengbiao, W., Seibert, F., Kourtz, L., and Ko, Z.W. (1995). Isolation and characterization of a cDNA clone encoding a member of the Com44/Cim44 envelope components of the chloroplast protein import apparatus. J. Biol. Chem. **270,** 28601-28608.

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154,** 367-382.

Kyte, J., and Doolittle R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157,** 105-132.

Li, H., Morre, T. and Keegstra, K. (1991). Targeting of proteins to the outer envelope membrane uses a different pathway than transport into chloroplasts. Plant Cell **3,** 709-717.

Li, H., Sullivan, T.D., and Keegstra, K. (1992). Information for targeting to the chloroplastic inner envelope membrane is contained in the mature region of the maize *Bt1*-encoded protein. J. Biol. Chem. **267,** 18999-19004.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Nakai, M., Hase, T., and Matsubara, H. (1989). Precise determination of the mitochondrial import signal contained in a 70 kDa protein of yeast mitochondrial outer membrane. J. Biochem. **105,** 513-519.

Perry, S.E., Keegstra, K. (1994). Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell **6,** 93-105.

Robinson, C., and Klösgen, R.B. (1994). Targeting of proteins into and across the thylakoid membrane - a multitude of mechanisms. Plant Mol. Biol. **26,** 15-24.

Salomon, M., Fischer, K., Flügge, U. and Soll, J. (1990). Sequence analysis and protein import studies of an outer chloroplast envelope polypeptide. Proc. Natl. Acad. Sci. **87,** 5778-5782.

Schnell, D.J. (1995). Shedding light on the chloroplast protein import machinery. Cell **83,** 521-524.

Schnell, D.J., Kessler, F. and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. Science, **266**, 1007-1012.

Seedorf, M., Waegemann, K. and Soll, J. (1995). A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J. **7,** 401-411.

Shore, G.C., McBride, H.M., Millar, D.G., Steenaart, N.A.E., and Nguyen,M. (1995). Import and insertion of proteins into the mitochondrial outer membrane. Eur. J. Biochem. **227,** 9-18.

Theg, S.M. and Scott, S.V. (1993). Protein import into chloroplasts. Trends in Cell Biol. **3,** 186-190.

Tranel, P.J., Froehlich, J., Goyal, A., and Keegstra, K. (1995). A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J. **14,** 2436-2446.

Van den Broeck, G., Timko, M.P., Kausch, A.P., Cashmore, A.R., Van Montagu, M., and Herrera-Estrella, L. (1985). Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase. Nature **313,** 358-363.

VanderVere, P.S., Bennett, T.M., Oblong, J.E., and Lamppa, G.K. (1995). A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. Proc. Natl. Acad. Sci. USA **92,** 7177-7181.

Wasmann, C.C., Reiss, B., and Bohnert, H.J. (1988). Complete processing of a small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from pea requires the amino acid sequence Ile-Thr-Ser. J. Biol. Chem. **263,** 617-619.

Wu, C., Seibert, F.S., and Ko, K. (1994). Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. J. Biol. Chem. **269,** 32264-32271.

Chapter 4

IMPORT OF prOEP75 IS STIMULATED BY A SMALL, HEAT-STABLE COMPOUND PRESENT IN RABBIT RETICULOCYTE LYSATE

ABSTRACT

OEP75 is an outer membrane protein of the chloroplastic envelope. It is encoded by a nuclear gene as a precursor protein (prOEP75) and has a novel import pathway. During analysis of prOEP75's import pathway, rabbit reticulocyte lysate was routinely included at a final concentration of 8% to 10% (v/v) in import reactions. When present in this concentration range, rabbit reticulocyte lysate greatly increased the amount of prOEP75 that was imported. Here, this phenomenon of prOEP75 import-stimulation by rabbit reticulocyte lysate is described. The import-stimulating factor is likely not a protein, as it is less than ten kilodaltons in size and is resistant to boiling. Further attempts to identify the import-stimulating factor revealed that EGTA stimulated import of prOEP75. EGTA alone was not as effective as rabbit reticulocyte lysate in stimulating import of prOEP75, however, and may not be the only active component in the lysate. Analysis of chimeric constructs indicated that the import stimulating factor acted on the mature domain of prOEP75, and not the transit peptide.

INTRODUCTION

In a standard chloroplastic protein import experiment, radiolabeled precursor protein is incubated with chloroplasts in the presence of ATP and import buffer (Bruce et al., 1994). Import buffer consists of 330 mM sorbitol in 50 mM Hepes/KOH (pH 8.0). Radiolabeled precursor protein is generated from in vitro transcription/translation or from overexpression in *E. coli*. Wheat germ extract or rabbit reticulocyte lysate is used for in vitro translation.

In previous import experiments with prOEP75 (e.g., Tranel et al., 1995), import reactions were adjusted so that the final concentration of rabbit reticulocyte lysate was 8% to 10% (v/v). This was done because rabbit reticulocyte lysate was found to stimulate import of prOEP75. The finding that rabbit reticulocyte stimulated import of prOEP75 is described in this chapter. Also described in this chapter is my investigation of the active ingredient in the rabbit reticulocyte lysate. This investigation was undertaken for two reasons. One reason was that identification of the active ingredient may provide insight into the import of prOEP75. The second reason was of more practical considerations: adding relatively large volumes of rabbit reticulocyte lysate to import reactions was expensive.

RESULTS AND DISCUSSION

Rabbit reticulocyte lysate stimulates the import of prOEP75

Results from initial import experiments with prOEP75 revealed that the proportion of added precursor that was imported was highly variable between experiments. For example, under standard incubation conditions (30 min incubation at room temperature in the presence of 3

mM ATP) anywhere from <1% to 20% of the added precursor was imported (data not shown). Also observed was the fact that prOEP75 produced in wheat germ extract was imported with extremely low efficiency whereas prOEP75 produced in rabbit reticulocyte lysate was much more efficiently imported (Figure 4.1). Note that for the experiment described in Figure 4.1 the final concentration of rabbit reticulocyte lysate and wheat germ extract in the import reactions were 11% and 1%, respectively. Thus the results presented in Figure 4.1 do not distinguish between whether the observed import stimulation was due to a component specific to rabbit reticulocyte lysate or due to the fact that more rabbit reticulocyte lysate was added. Nonetheless, these results illustrate the variability that was observed in prOEP75 import efficiency. Also, these results led me to suspect that a specific component in rabbit reticulocyte lysate stimulated import of prOEP75. [Different amounts of translation product typically are added in different experiments because the amount of added precursor is usually normalized to radioactivity (dpm), not to volume. Thus, the amount of added translation product is usually dependent upon the labeling efficiency of the particular translation reaction.]

An import experiment was performed in which an equal amount of radioactive prOEP75 was added to reactions that were supplemented with increasing amounts of rabbit reticulocyte lysate or wheat germ extract. As shown in Figure 4.2A, increasing amounts of mOEP75 were obtained with increasing concentrations of rabbit reticulocyte lysate, with maximum levels of mOEP75 occurring when the final concentration of rabbit reticulocyte lysate was between 9% and 11.5%. Higher concentrations of rabbit reticulocyte lysate, up to 16.5% had no additional effect on the import efficiency. Supplementing the reaction with wheat germ extract rather than rabbit reticulocyte lysate did not result in substantial increases in import efficiencies (Figure 4.2B). Thus it was concluded that the import-stimulating factor was present in rabbit reticulocyte lysate but not in wheat germ extract.

The results shown in Figure 4.2A indicated that the accumulation of iOEP75 was

Figure 4.1. Translation system affects import of prOEP75.

Chloroplastic protein import experiments were conducted essentially as described (Bruce et al., 1994; Tranel et al., 1995). Radiolabeled prOEP75 translation product (10^6 dpm) from rabbit reticulocyte lysate or wheat germ extract was added to chloroplasts (100 µg chlorophyll equivalent) in a final reaction volume of 300 µl. The volume of added translation product was 96 µl and 12 µl for rabbit reticulocyte lysate and wheat germ extract, respectively. After 30 min incubation at room temperature, the chloroplasts were repurified and the imported products (lanes labeled 'Import') were analyzed by SDS-PAGE and fluorography. The fluorographs shown are from 4-hr exposure (rabbit reticulocyte lysate) and 3 day exposure (wheat germ extract). Rabbit reticulocyte lysate was from Promega, wheat germ extract was prepared according to Anderson et al. (1983). iOEP75 and mOEP75 are the primary products obtained from imported prOEP75 (Tranel et al., 1995). Tr, one-tenth volume of translation product added to the import reaction.

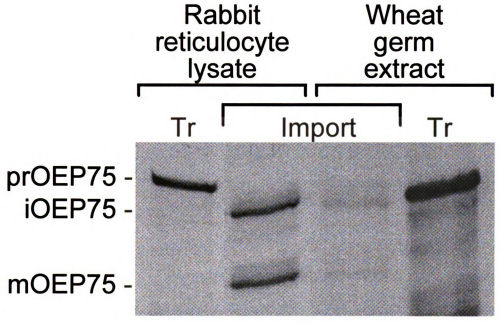
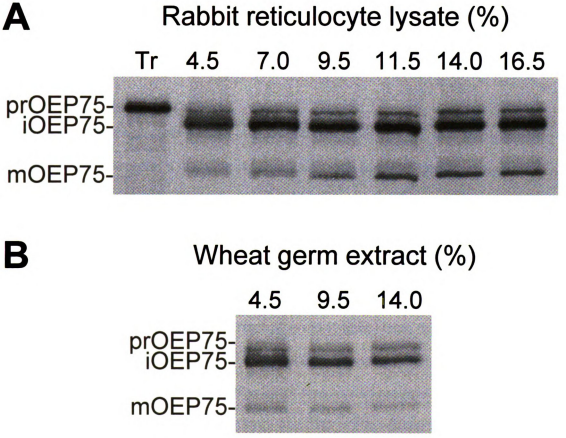

Rabbit reticulocyte lysate
Wheat germ extract
Tr
Import
Tr
prOEP75 -
iOEP75 -
mOEP75 -

Figure 4.2. Import of prOEP75 is dependent on the concentration of rabbit reticulocyte lysate.

Radiolabeled prOEP75 translation product (2.5 x 10^5 dpm) generated from rabbit reticulocyte lysate was added to chloroplasts (25 µg chlorophyll equivalent) in a final reaction volume of 75 µl. Reactions were adjusted with rabbit reticulocyte lysate **(A)** or wheat germ extract **(B)** to bring to the concentrations indicated. The reactions used for **(B)** contained 2.5% rabbit reticulocyte lysate in addition to the indicated concentrations of wheat germ extract. After 30 min incubation at room temperature, the chloroplasts were repurified and the imported products were analyzed by SDS-PAGE and fluorography. Tr, one-tenth volume of translation product added to the import reaction.


A
Rabbit reticulocyte lysate (%)
Tr
4.5
7.0
9.5
11.5
14.0
16.5
prOEP75-
iOEP75-
mOEP75-
B
Wheat germ extract (%)
4.5
9.5
14.0
prOEP75-
iOEP75-
mOEP75-

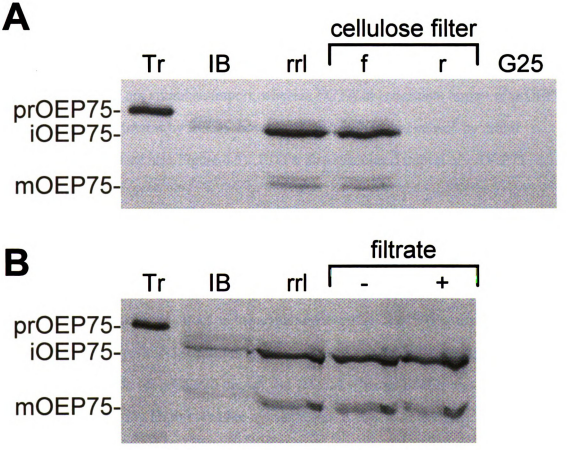
not enhanced by rabbit reticulocyte lysate. This was not a consistent observation from one experiment to the next. In some experiments, the accumulation of iOEP75 was strongly dependent on rabbit reticulocyte lysate (e.g., Figure 4.3, compare lanes 'IB' and 'rrl'). The correlation between concentration of rabbit reticulocyte lysate and accumulation of mOEP75, however, was consistently observed in all experiments and was further investigated (described below).

The import-stimulating factor in rabbit reticulocyte lysate is a small, heat-stable compound

I suspected that a protein was present in the rabbit reticulocyte lysate that was binding to prOEP75 and maintaining it in an import competent state (de Boer and Weisbeek, 1991). As a first attempt to identify the import-stimulating factor, I fractionated the rabbit reticulocyte lysate by filtration through a regenerated-cellulose filter (Millipore) which had a molecular weight cutoff of 10 kDa or by filtration through a G25 Sephadex (Sigma) column, which had a size exclusion limit of 5 kDa. I then assayed the filtrate and retentate from the cellulose filter and the eluate fraction (void volume) from the Sephadex column for ability to stimulate import of prOEP75. Results from this experiment, presented in Figure 4.3A, revealed that the import stimulation factor was a small molecule. The filtrate from the cellulose filter, but not the retentate from that filter nor the eluate from the G25 column, was able to stimulate import. Furthermore, the import efficiency when the filtrate from the cellulose filter was added was similar to that seen when an equivalent volume of non-fractionated rabbit reticulocyte lysate was added to the import reaction. Boiling the filtrate from the cellulose filter prior to adding it to the import reaction did not reduce its ability to stimulate import (Figure 4.3B).

Figure 4.3. Import-stimulating factor is small and heat-stable.

(A) Radiolabeled prOEP75 translation product (4 µl, 3.8 x 10^5 dpm) generated from rabbit reticulocyte lysate was added to 21 µl of either: import buffer (IB), 35% rabbit reticulocyte lysate (rrl), the filtrate (f) or retentate (r) fractions recovered after filtration of 35% rabbit reticulocyte lysate through a Millipore regenerated-cellulose filter with a molecular weight cutoff of 10 kDa, or the void volume eluate after centrifugation of 35% rabbit reticulocyte lysate through a G25 Sephadex matrix (G25). Import reactions were initiated by the addition of chloroplasts (33 µg chlorophyll equivalent). Final reaction volume was 100 µl. The translation product contained 35% rabbit reticulocyte lysate. Thus the final concentrations of rabbit reticulocyte lysate in the samples designated 'IB' and 'rrl' were 1.4% and 8.75%, respectively. After 30 min incubation at room temperature, the chloroplasts were repurified and the imported products were analyzed by SDS-PAGE and fluorography. Tr, one-tenth volume of translation product added to the import reaction. **(B)** The experiment described for **(A)** was repeated except that neither the retentate from the cellulose filter nor the G25 eluate was analyzed. Also, the filtrate from the cellulose filter was boiled (+) or incubated on ice (-) for 5 min prior to being mixed with the translation product.


A
cellulose filter
Tr
IB
rrl
f
r
G25
prOEP75-
iOEP75-
mOEP75-
B
filtrate
Tr
IB
rrl
-
+
prOEP75-
iOEP75-
mOEP75-

EGTA partially substitutes for rabbit reticulocyte lysate to stimulate import

The results presented in Figure 4.3 indicated that the import stimulation factor was a small, heat-stable compound, and therefore likely was not a protein. Small, heat-stable compounds present in the rabbit reticulocyte lysate but not in the wheat germ extract included hemin, EGTA, and millimolar levels of chloride ion (in the form of KCl) (Anderson et al., 1983; Promega). Thus I analyzed hemin, EGTA, and KCl for their abilities to stimulate import of prOEP75. Each component was added to a separate import reaction such that the final concentration of that component was the same as in a reaction that had a final rabbit reticulocyte lysate concentration of 9%. As shown in Figure 4.4, hemin and KCl did not stimulate import, whereas EGTA did stimulate import of prOEP75. The stimulation of import by EGTA, however, was less than that caused by rabbit reticulocyte lysate (see also Figure 4.5). EDTA also stimulated import of prOEP75 (data not shown).

I also assayed potassium phosphate for its ability to stimulate import because it had been shown to stimulate import of a chloroplastic inner envelope membrane protein (Hirsch and Soll, 1995). Potassium phosphate, at 80 mM, caused an increase in accumulation of iOEP75 but had little or no effect on the accumulation of mOEP75 (Figure 4.4B).

Titration experiments (data not shown) revealed that the optimum concentration of potassium phosphate for prOEP75 import was 20 mM whereas EGTA was as equally effective at stimulating import whether it was present at 50 μM or 400 μM. (In an import reaction containing 9% rabbit reticulocyte lysate, the EGTA concentration is 200 μM.) When both EGTA and potassium phosphate were added to an import reaction, import of prOEP75 was more efficient than when either of these components were added alone (Figure 4.5). Even with the combination of EGTA and potassium phosphate, however, accumulation of mOEP75 was less than that obtained by adding 9% rabbit reticulocyte lysate to the import reaction.

Figure 4.4. EGTA partially substitutes for rabbit reticulocyte lysate to stimulate import of prOEP75.

Radiolabeled prOEP75 translation product (4 µl, 3.8 x 10^5 dpm) generated from rabbit reticulocyte lysate was added to 21 µl of either: import buffer (IB), 35% rabbit reticulocyte lysate (rrl), 10 µM hemin, 35 mM KCl, 0.8 mM EGTA, or 386 mM potassium phosphate (KPi). [These concentrations of hemin, Cl^- and EGTA are the same as that present in 35% rabbit reticulocyte lysate (Promega)]. Import reactions were initiated by the addition of chloroplasts (33 µg chlorophyll equivalent). After 30 min incubation at room temperature, the chloroplasts were repurified and the imported products were analyzed by SDS-PAGE and fluorography. Tr, one-tenth volume of translation product added to the import reaction.

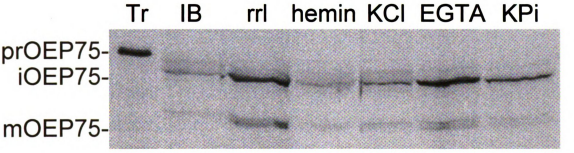
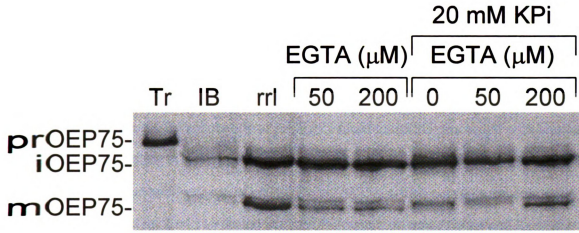

Tr
IB
rrl
hemin
KCl
EGTA
KPi
prOEP75-
iOEP75-
mOEP75-

Figure 4.5. Stimulation of prOEP75 import by EGTA and KPi.

Radiolabeled prOEP75 translation product (4 µl, 2.5 x 10^5 dpm) generated from rabbit reticulocyte lysate was added to chloroplasts (50 µg chlorophyll equivalent) in a final reaction volume of 150 µl. Reactions contained no additions (IB) a final concentration of 9% rabbit reticulocyte lysate (rrl) or the concentrations of EGTA and/or potassium phosphate (KPi) as indicated. After 30 min incubation at room temperature, the chloroplasts were repurified and the imported products were analyzed by SDS-PAGE and fluorography. Tr, one-tenth volume of translation product added to the import reaction.


20 mM KPi
EGTA (μM)
EGTA (μM)
Tr
IB
rrl
50
200
0
50
200
prOEP75-
iOEP75-
mOEP75-

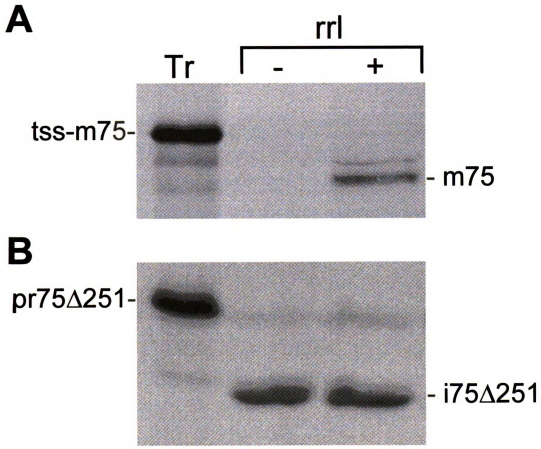
The import-stimulating factor acts on the mature domain of prOEP75

In efforts to understand the import pathway of prOEP75, chimeric proteins between prOEP75 and prSSU were created (Tranel and Keegstra, submitted). The import dependence on rabbit reticulocyte lysate of these chimeric precursor proteins is insightful. As shown in Figure 4.6A, import of a protein containing the transit peptide of prSS fused to mOEP75 was strongly dependent upon the presence of rabbit reticulocyte lysate in the import reaction. This implies that something about the mature region of prOEP75 makes its import dependent on rabbit reticulocyte lysate. As expected then, import of a chimeric protein containing the transit peptide of prOEP75 fused to mSSU proceeded with similar efficiencies regardless of the concentration of rabbit reticulocyte lysate (data not shown). Furthermore, a truncated version of prOEP75, pr75Δ251, which contains the complete transit peptide and the first 115 amino acids of the mature protein (Tranel and Keegstra, submitted), also was imported with similar efficiencies regardless of the concentration of rabbit reticulocyte lysate (Figure 4.6B). The primary product obtained from import of pr75Δ251 is i75Δ251, which is analogous to iOEP75 (Tranel and Keegstra, submitted). As described previously, accumulation of iOEP75 from imported prOEP75 occurred only occasionally under low concentrations of rabbit reticulocyte lysate. The import of pr75Δ251, however, always occurred with high efficiency even under very low concentrations of rabbit reticulocyte lysate (not all data shown).

Taken together, the results obtained from import analysis of pr75Δ251 and the chimeric constructs suggest that some region within the C-terminal two thirds of prOEP75 inhibits import of this precursor. Alternatively, some higher-order structure of prOEP75 is inhibitory to import, and at least part of the C-terminal two-thirds of this precursor participates in forming this structure. Possibly, formation of this structure is dependent upon cations. By chelating cations, EGTA prevents formation of this structure and thereby allows import of prOEP75 to occur. Because the import apparatus is dependent upon

Figure 4.6. Rabbit reticulocyte lysate affects the mature domain of prOEP75.

Radiolabeled translation product (3 µl, 5 x 10^5 dpm) containing tSS-m75 **(A)** or pr75Δ251 **(B)** from rabbit reticulocyte lysate was incubated with chloroplasts (50 µg chlorophyll equivalent) in a final reaction volume of 150 µl. Import reactions were not (-) or were (+) supplemented with rabbit reticulocyte lysate to bring the final concentration to 1% or 10%, respectively. After 30 min incubation at room temperature, the chloroplasts were repurified and the imported products were analyzed by SDS-PAGE and fluorography. tSS-m75 is a chimeric construct containing the transit peptide of prSS fused to the mature domain of prOEP75; pr75Δ251 contains the complete transit peptide of prOEP75 and the first 115 amino acids of the mature protein (Tranel and Keegstra, submitted). Tr, one-tenth volume of translation product added to the import reaction.


A
rrl
Tr
-
+
tss-m75-
- m75
B
pr75Δ251-
- i75Δ251

cations (primarily magnesium) for function (Olsen and Keegstra, 1992), chelation of cations by EGTA should also have an inhibitory effect on import. Rabbit reticulocyte lysate may have stimulated import greater than did EGTA alone because rabbit reticulocyte lysate also contains cations (e.g., magnesium) that have a greater effect on enhancing import than on enhancing folding of prOEP75. The fact that different cations may be both necessary and inhibitory for import of prOEP75 may explain why EGTA stimulated import of prOEP75 over a wide range of concentrations (described above). What role, if any, cations play in folding of prOEP75 either in vitro or in vivo remains to be determined.

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John Froehlich aided in the initial discovery that rabbit reticulocyte lysate stimulated the import of prOEP75.

REFERENCES

Anderson, C.W., Straus, J.W., and Dudock, B.S. (1983). Preparation of Cell-Free Protein-Synthesizing System from Wheat Germ. Meth. Enzymol. **101,** 635-644.

Bruce, B.D., Perry, S., Froehlich, J. and Keegstra, K. (1994). In vitro import of proteins into chloroplasts. Plant Mol. Biol. Manual **J1**, 1-15.

de Boer, A. and Weisbeek, P.J. (1991). Chloroplast protein topogenesis: import, sorting and assembly. Biochim. Biophys. Acta. **1071,** 221-253.

Hirsch, S., and Soll, J. (1995). Import of a new chloroplast inner envelope protein is greatly stimulated by potassium phosphate. Plant Mol. Biol. **27,** 1173-1181.

Olsen, L.J., and Keegstra, K. (1992). The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. J. Biol. Chem. **267,** 433-439.

Tranel, P.J., Froehlich, J., Goyal, A., and Keegstra, K. (1995). A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J. **14,** 2436-2446.

Tranel, P.J., and Keegstra, K. (submitted). A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope.

Chapter 5

OEP75 IS PRESENT IN SEVERAL PLANT SPECIES AND MAY HAVE A CONSERVED IMPORT PATHWAY

ABSTRACT

OEP75 is an outer envelope membrane component of the chloroplastic protein import apparatus in pea. OEP75 is synthesized as a higher molecular weight precursor (prOEP75) that is stepwise processed to an intermediate (iOEP75) and then to the mature protein (mOEP75). To determine if homologs to pea OEP75 are present in other plant species, immunoblot analysis was conducted with antibodies raised against pea OEP75. An OEP75 homolog was detected in each dicot species analyzed. In addition to mOEP75, a putative homolog to iOEP75 was detected in protein from young sunflower leaves. Although a monocot OEP75 was not detected by immunoblot analysis, a partial-length cDNA encoding a putative rice OEP75 was identified. A near full-length cDNA encoding *Arabidopsis* OEP75 was also identified and was sequenced. The mature domains of pea and *Arabidopsis* OEP75 are 81% identical at the amino acid level. Results from preliminary analysis suggested that the primary structure of rice OEP75 has not been as highly conserved. Pea prOEP75 was imported and stepwise processed to iOEP75 and mOEP75 in maize chloroplasts. Results from this in vitro experiment, together with the finding of a putative iOEP75 homolog in young sunflower leaves, suggest that the novel prOEP75 import pathway described for pea exists in other plant species.

INTRODUCTION

OEP75 was identified in pea as a putative channel protein through which precursor proteins are translocated across the outer membrane of the chloroplastic envelope (Perry and Keegstra, 1994; Schnell et al., 1994; Tranel et al., 1995). OEP75 is encoded by a nuclear gene as a higher molecular weight precursor, prOEP75, and is targeted to the chloroplastic outer envelope membrane by a novel pathway (Tranel et al., 1995, Tranel and Keegstra, submitted). prOEP75 is processed to an intermediate, iOEP75, by the stromal processing peptidase and subsequently assembled and processed to the mature form, mOEP75.

Prior analysis of the import pathway of OEP75 was restricted to one plant species. Both the chloroplasts used for import assays and the cDNA clone encoding prOEP75 were from pea. Thus, experiments were conducted to determine if the conclusions drawn from these studies were restricted to pea, or if the conclusions were generally applicable to other plant species.

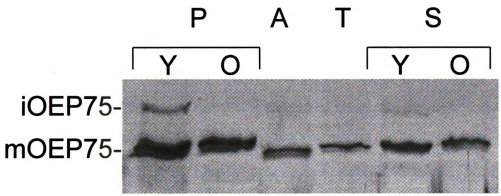
RESULTS AND DISCUSSION

OEP75 is present in several plant species

I first investigated whether a homolog to pea OEP75 was present in other plant species. Total protein was extracted from leaves of pea, *Arabidopsis*, tobacco, sunflower, maize, and barley seedlings. Protein samples were fractionated by SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was then performed with antibodies against pea OEP75. As shown in Figure 5.1, proteins similar in size to pea mOEP75 were detected in all of the dicot species analyzed. No proteins were detected from the monocot

Figure 5.1. mOEP75 and iOEP75 are not unique to pea.

Total protein was isolated from leaves of pea (P) (*Pisum sativum*), *Arabidopsis* (A) (*Arabidopsis thaliana*), tobacco (T) (*Nicotiana tabacum*), or sunflower (S) (*Helianthus annus*). Pea and sunflower seedlings were 11 days old; *Arabidopsis* and tobacco seedlings were 22 days old. For pea and sunflower, protein was isolated separately from the youngest leaves (Y) and the older leaves (O). 100 μg of protein was fractionated by SDS-PAGE, transferred onto a PVDF membrane (Immobilon-P, Millipore) and immunoblotted with antiserum raised against pea OEP75 (Tranel et. al., 1995). Bound antibodies were detected with alkaline phosphatase-conjugated secondary antibody and BCIP/nBT as described (Tranel et al., 1995).


P
A
T
S
Y
O
Y
O
iOEP75-
mOEP75-

species that reacted with the immune but not the pre-immune serum (data not shown).

If protein from young pea leaves is analyzed by immunoblotting, iOEP75 can be detected (Figure 5.1, lane P_Y; Tranel et al., 1995). Young sunflower leaves contained a protein that reacted to anti-OEP75 antibodies and that had a similar apparent molecular weight as did the pea iOEP75 (Figure 5.1, lane S_Y). This protein, presumably the sunflower homolog to pea iOEP75, was not detected in old leaves (Figure 5.1, lane S_O). Similarly, pea iOEP75 was not detected in old leaves (Figure 5.1, lane P_O). An iOEP75 homolog was not detected in protein isolated from young tobacco leaves (data not shown).

Although a monocot homolog for OEP75 was not detected by immunoblotting, likely this protein is present in monocots. A cDNA clone encoding a portion of a putative rice homolog to OEP75 has been obtained from the rice expressed sequence tags (EST) sequencing project (Sasaki et al., 1994) (discussed below). There are a few possible explanations why OEP75 was not detected in barley and maize, but the most likely explanation is that the antibodies raised against pea OEP75 did not have sufficient cross-reactivity to the monocot OEP75. Similarly, the fact that an iOEP75 homolog was not detected in protein from young tobacco leaves does not mean that iOEP75 was not present. The combination of poor cross-reactivity of the anti-OEP75 antibodies and low abundance of iOEP75 may have accounted for the lack of its detection.

Comparison of the primary structures of prOEP75 homologs

Databases of cDNA clones were routinely searched (Newman et al., 1994) for OEP75 homologs in an effort to obtain primary structure information of these homologs. No full-length cDNA clones encoding homologs to pea prOEP75 have yet been reported. A nearly full-length clone, however, which likely encodes all of the mature domain of *Arabidopsis* prOEP75, has been obtained through the *Arabidopsis* EST sequencing project (Höfte et al.,

1993). I obtained this cDNA clone (Genbank accession number Z29123) from the *Arabidopsis* Biological Resource Center and have sequenced the insert. The deduced amino acid sequence of the longest ORF of the cDNA insert was aligned with the pea prOEP75 amino acid sequence and is presented in Figure 5.2.

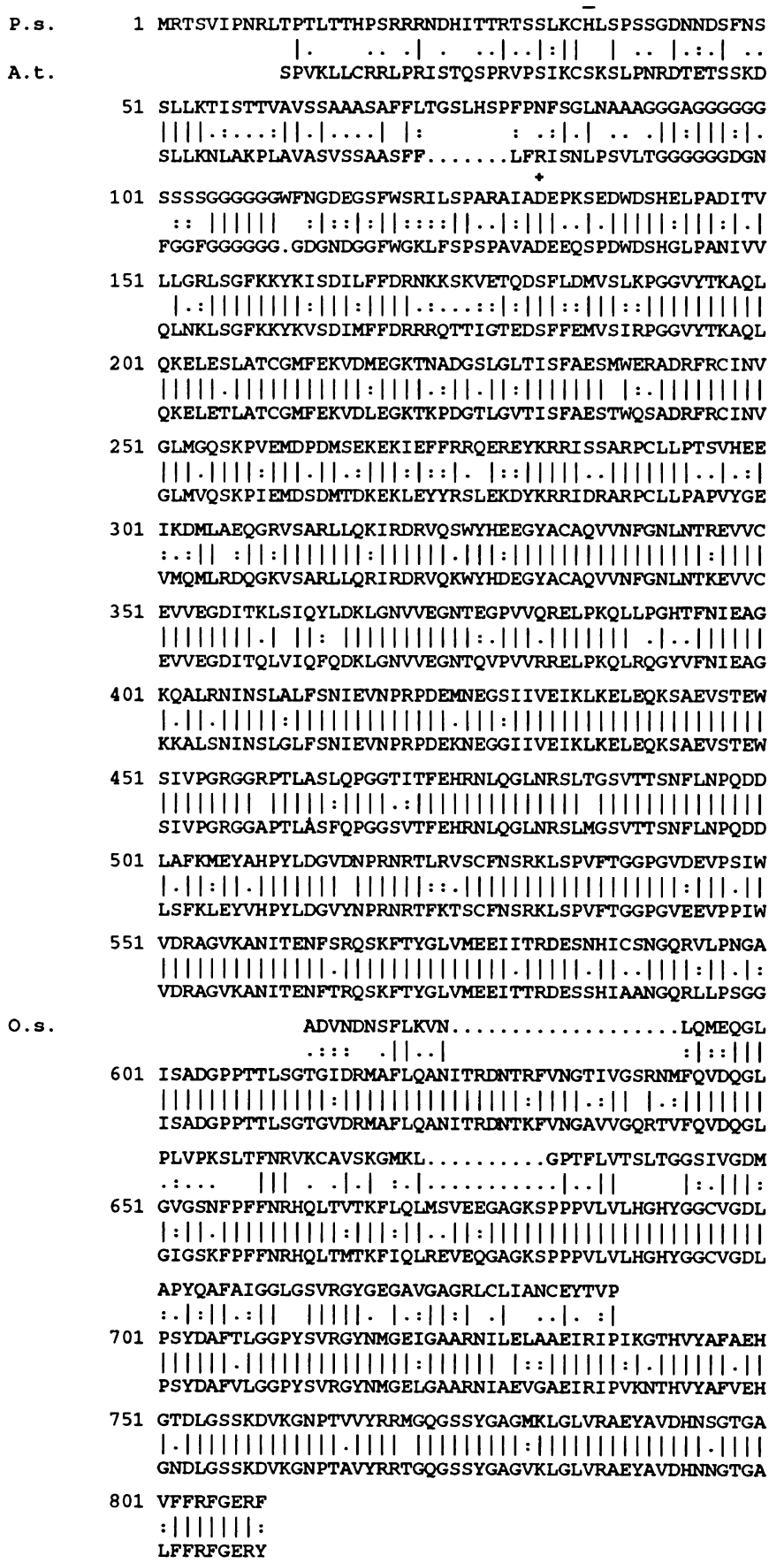
There are several unusual properties of clone Z29123: part of the vector sequence is missing, the cDNA encoding prOEP75 is in the wrong orientation, and poly A+ regions are present at both ends of the insert (data not shown). Thus, in addition to other cloning artifacts, clone Z29123 contains two separate cDNAs fused together. Comparison of the complete insert to known cDNA sequences indicates that the cDNA present at the 5' end of the insert encodes a homolog to a ribosomal protein (data not shown). Because the primary structure of the pea and *Arabidopsis* prOEP75 transit peptides apparently are not highly similar, it is difficult to determine precisely where within the prOEP75 transit peptide-encoding region of clone Z29123 is the cDNA fusion.

Despite the cloning artifacts in clone Z29123, it is possible to compare the sequence similarities within the mature domains of the encoded pea and *Arabidopsis* prOEP75 proteins. The mature domains of the pea and *Arabidopsis* prOEP75 precursor proteins are highly conserved. The amino acid sequence identity is 81%. At the nucleotide level, the sequences of the regions encoding mOEP75 are 75% identical.

As mentioned above, in addition to the cDNA clone encoding an *Arabidopsis* homolog, a cDNA clone encoding a portion of a putative rice homolog of OEP75 was identified (Genbank accession number D46516). Although I have not sequenced the insert from this cDNA clone, analysis of the sequence data obtained by the Rice Genome Research Program (Sasaki et al., 1994) suggests that the cDNA encodes approximately 150 amino acids of the extreme C-terminus of prOEP75. Over a stretch of approximately 100 amino acids, with the insertion of gaps, the sequence is 38% identical to pea prOEP75 (Figure 5.2). If in fact this cDNA clone encodes a portion of prOEP75, it is interesting that the degree of conservation is probably much less than that observed for the *Arabidopsis*

Figure 5.2. Sequence alignment of prOEP75 homologs.

The deduced amino acid sequences from cDNA clones encoding all or part of prOEP75 from pea (P.s.), *Arabidopsis* (A.t.), or rice (O.s.) (*Oryza sativa*) were aligned by the BestFit algorithm (Genetics Computer Group, 1994). Genbank accession numbers for the cDNA clones are: pea, X83767 (Tranel et al., 1995); *Arabidopsis*, Z29123 (Höfte et al., 1993); rice, D46516 (Sasaki et al., 1994). A line and a plus are placed above the first residue of iOEP75 and mOEP75, respectively, in the pea homolog (Tranel et al., 1995; Tranel and Keegstra, submitted).


P.s.
A.t.
O.s.

homolog. The large difference in sequence identity between the dicot and monocot species could explain why OEP75 was not detected in the monocot species by immunoblotting.

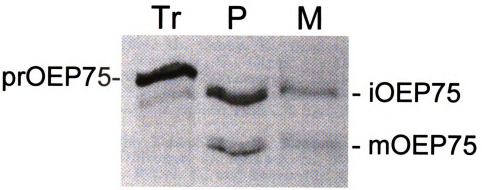
Pea prOEP75 is imported and processed by maize chloroplasts

The fact that OEP75 could be detected in different plant species and the finding that a putative iOEP75 was present in at least one other plant species suggested that OEP75 may have a conserved import pathway. To test whether the OEP75 import pathway is conserved among species, we attempted to import pea OEP75 into maize chloroplasts. Upon incubation of pea prOEP75 with maize chloroplasts under import conditions, the precursor was imported and processed to two major products (Figure 5.3). The products were the sizes expected for iOEP75 and mOEP75, (compare Figure 5.3, lanes P and M) indicating that the machinery needed for import and processing of prOEP75 is conserved among plant species.

Import of typical transit peptide-bearing precursors can occur in heterologous systems (Bruce et al., 1994). That is, chloroplasts from one species can import and process a precursor from another species. Because the N-terminus of the prOEP75 transit peptide is a chloroplast-targeting domain, i.e., a "typical" transit peptide, (Tranel et al., submitted), it is not surprising that prOEP75 was imported by maize chloroplasts and processed to iOEP75. However, prOEP75 is the only known example of a precursor protein that undergoes at least two processing steps en route to the outer envelope membrane. Thus, it is remarkable that processing of iOEP75 to mOEP75 also occurred in maize chloroplasts. This finding, together with the immunological detection of a putative sunflower homolog of iOEP75, provides strong correlative evidence that the OEP75 import pathway is conserved among plant species.

Figure 5.3. Pea prOEP75 is imported and processed by maize chloroplasts.

Chloroplasts were isolated from leaves of 10 day old pea seedlings and from leaves within the whorl of 5 day old maize (Zea mays) seedlings. Pea chloroplasts were isolated as described (Bruce et al., 1994). For the isolation of maize chloroplasts, the pea chloroplast isolation procedure was followed except that chloroplasts were isolated over Percoll gradients at 2,600*g* rather than at 8,000*g*. Radiolabeled prOEP75 was incubated with pea (P) or maize (M) chloroplasts at room temperature as described (Tranel et al., 1994), with the addition that the import reactions were illuminated at ca. 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. After incubation for 30 minutes, chloroplasts were re-purified over Percoll and import products were analyzed by SDS-PAGE and fluorography. Tr, one-tenth volume of translation product added to the import reaction.


Tr
P
M
prOEP75-
- iOEP75
- mOEP75

ACKNOWLEDGMENT

John Froehlich performed the import experiment with maize chloroplasts.

REFERENCES

Bruce, B.D., Perry, S., Froehlich, J. and Keegstra, K. (1994). In vitro import of proteins into chloroplasts. Plant Mol. Biol. Manual **J1**, 1-15.

Genetics Computer Group. (1994). Program Manual for the Wisconsin Package, Version 8. Genetics Computer Group, 575 Science Drive, Madison, Wisconsin.

Höfte, H., Desprez, T., Amselem, J., Chiapello, H., Caboche, M., Moisan, A., Jourjon, M.F., Charpenteau, J.L., Berthomieu, P., Guerrier, D., Giraudat, J., Quigley, F., Thomas, F., Yu, D.Y., Mache, R., Raynal, M., Cooke, R., Grellet, F., Delseny, M., Parmentier, Y., Marcillac, G., Gigot, C., Fleck, J., Philipps, G., Axelos, M., Bardet, C., Tremousaygue, D., and Lescure, B. (1993). An inventory of 1152 expressed sequence tags obtained by partial sequencing of cDNAs from *Arabidopsis thaliana*. Plant J. **4,** 1051-1061.

Newman, T., de Bruijn, F.J., Green, P., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Thomashow, M., Retzel, E., and Somerville, C. (1994). Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. Plant Physiol. **106,** 1241-1255.

Perry, S.E., Keegstra, K. (1994). Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell **6,** 93-105.

Sasaki, T., Song, J., Koga-Ban, Y., Matsui, E., Fang, F., Higo, H., Nagasaki, H., Hori, M., Miya, M., Murayama-Kayano, E., Takiguchi, T., Takasuga, A., Niki, T., Ishimaru, K., Ikeda, H., Yamamoto, Y., Mukai, Y., Ohta, I., Miyadera, N., Havukkala, I., and Minobe, Y. (1994). Toward cataloguing all rice genes: large-scale sequencing of randomly chosen rice cDNAs from a callus cDNA library. Plant J. **6,** 615-624.

Schnell, D.J., Kessler, F., and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. Science, **266**, 1007-1012.

Tranel, P.J., Froehlich, J., Goyal, A., and Keegstra, K. (1995). A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J. **14,** 2436-2446.

Tranel, P.J., and Keegstra, K. (submitted). A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope.

Chapter 6

FUTURE DIRECTIONS

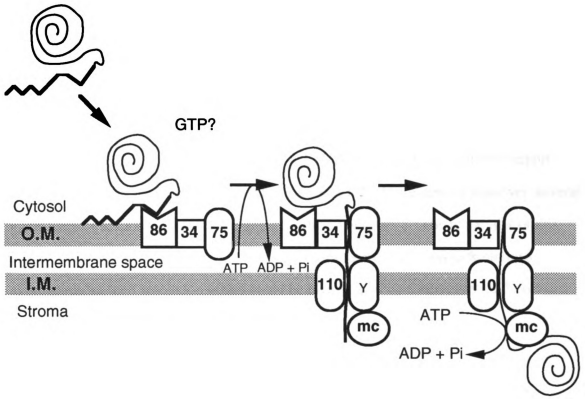
A working model of the general chloroplastic protein import apparatus is presented in Figure 6.1. Comparison of this model with the model present in Chapter 1 reveals that, in the three years since I began my dissertation research, there have been several advances in our knowledge of the general chloroplastic protein import apparatus. Several proteins are now implicated as components of this import apparatus, and cDNA clones encoding many of these proteins have been isolated (reviewed by Schnell, 1995; Gray and Row, 1995). We still know very little about the functions of the individual proteins and how the proteins interact to import a precursor protein. To be sure, some of the implicated proteins may later be shown not to play a role in protein import and additional components likely will be identified.

When I began my dissertation research, Perry and Keegstra (1994) had just recently obtained evidence implicating OEP75 and OEP86 as components of the import apparatus. The initial goal of my dissertation research was to obtain cDNA clones encoding OEP75 and OEP86 and perform structure/function studies on the encoded proteins. Upon isolation and preliminary analysis of a cDNA clone encoding OEP75, we determined that OEP75 was encoded as a higher molecular weight precursor (prOEP75). There is only one other known chloroplastic outer envelope membrane protein (OEP86) (Hirsch et al., 1994) and no known mitochondrial outer envelope membrane proteins that are synthesized as higher molecular weight precursor proteins. We suspected that the N-terminal extension of prOEP75 was a targeting sequence, and thus were curious about the prOEP75 import pathway. Our characterization of the prOEP75 import pathway is presented in Chapter 2. We found that the import pathway of prOEP75 was similar to that of other transit-peptide bearing precursor proteins. This finding led to the unexpected suggestion that prOEP75 might use the general chloroplastic protein import apparatus, and further stimulated our interests in understanding the import pathway of prOEP75. Thus, the import pathway of prOEP75 became the focus of my dissertation.

Further analysis of prOEP75's import pathway, presented in Chapter 3, revealed

Figure 6.1. 1996 version of a working model of the general chloroplastic protein import apparatus.

(Compare with the model presented in Figure 1.1.) OEP86 is still the most likely candidate for the receptor (Perry and Keegstra, 1994; Hirsch et al., 1994; Kessler et al., 1994). As discussed in some detail in Chapter 2, OEP75 is still thought to form part or all of the channel through which precursor proteins translocate across the outer envelope membrane. OEP34 likely is a component of the import apparatus, however, its specific function is unknown (Kessler et al., 1994; Seedorf et al., 1995). IEP110 is the most likely candidate for an inner envelope membrane component of the import apparatus (Schnell et al., 1994; Lübeck et al., submitted). Several proteins that may function as molecular chaperones have also been implicated to play a role in precursor protein import (Schnell et al., 1994; Ko et al., 1992; E. Nielsen, unpublished data). (Only one such molecular chaperone is indicated.) Import may require GTP in addition to ATP, although the role of GTP is unclear (Kessler et al., 1994; Hirsch et al., 1994; Seedorf et al., 1995). Likely, some components of the import apparatus have yet to be identified (represented by component 'Y' in the inner envelope membrane). O.M., outer envelope membrane; I.M., inner envelope membrane; mc, molecular chaperone.


GTP?
Cytosol
O.M.
86
34
75
Intermembrane space
ATP
ADP + Pi
I.M.
110
Y
mc
Stroma
ATP
ADP + Pi

that the N-terminus of the prOEP75 transit peptide, n75, behaved as a typical stromal-targeting domain and was removed by the stromal processing peptidase. The C-terminal portion of the prOEP75 transit peptide, c75, contained information for targeting the protein to the outer envelope membrane. Chapter 4 describes another intriguing aspect of prOEP75 import: it is greatly stimulated by a small, heat-stable compound present in rabbit reticulocyte lysate. Chapter 5 discusses preliminary evidence indicating that the unusual targeting pathway of OEP75 has been conserved among different plant species. The existence of an unusual targeting pathway for OEP75 is especially intriguing when one keeps in mind the fact that OEP75 itself is a likely component of the chloroplastic protein import apparatus.

The data presented in the preceding chapters of this dissertation provide an extensive analysis of the import pathway of OEP75. A model of the prOEP75 import pathway, based on these data, is presented in Figure 6.2. There remain, however, several unanswered questions regarding the import pathway of OEP75. The remainder of this chapter addresses some of these questions. Whereas some questions lack obvious approaches for their resolution others could be investigated by relatively straightforward procedures.

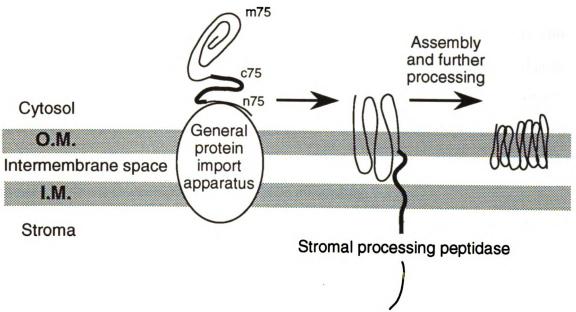
UNANSWERED QUESTIONS

Function of c75

One question regards the function of c75. As described in Chapter 3, c75 may oppose translocation into the stroma and thereby allow m75 to assemble in the outer envelope membrane. If so, c75 must be interacting with intermembrane space protein(s) or, more

Figure 6.2. Working model of the prOEP75 import pathway.

prOEP75 contains three domains: the N-terminus (n75) and the C-terminus (c75) of the transit peptide and the mature protein (m75). n75 acts as a typical chloroplast-targeting domain in that it is recognized by the general protein import apparatus and subsequently removed by the stromal processing peptidase. c75 prevents complete translocation of the precursor across the chloroplastic envelope. After m75 is assembled, c75 is removed by an unidentified peptidase, generating functional OEP75. Although the general protein import apparatus likely functions in several steps of the prOEP75 import process, it is shown only in the first step so that subsequent events could be visualized more clearly. O.M., outer envelope membrane; I.M., inner envelope membrane.


m75
c75
n75
Assembly
and further
processing
Cytosol
O.M.
Intermembrane space
I.M.
Stroma
General
protein
import
apparatus
Stromal processing peptidase

likely, with proteins and/or lipids in either of the envelope membranes. To address where c75 functions, one could extend the fractionation experiments with some of the constructs described in Chapter 3. For example, upon fractionation of imported t75-mSS, roughly one-third of the imported protein was recovered in the envelope fraction. It would be straightforward to further separate the envelope fraction, via sucrose gradient centrifugation, into outer and inner envelope membranes. Although this fractionation procedure results in inner envelope membranes that are contaminated with outer envelope membranes, one could tentatively conclude that c75 is interacting with the outer envelope membrane if the imported protein is recovered in the outer membrane fraction. A potential problem is that, if c75 is functioning in the outer envelope membrane, the imported protein may be in contact sites rather than in free outer envelope membranes. Since the contact sites would be present in the inner envelope membrane fraction, the imported protein would be recovered with the inner membranes. Thus, if the imported protein is recovered with the inner membrane fraction, the results could be misleading.

In addition to where c75 functions, it is unclear precisely how c75 functions. Site-directed mutagenesis experiments, described in Chapter 3 failed to implicate a hydrophobic region within c75 as a necessary element for c75 function. Unfortunately, the primary structure of the complete transit peptide of prOEP75 from another plant species is not known. Comparison of the c75 regions among different homologs of prOEP75 may provide clues as to the specific targeting information that is present within this domain.

One of the reasons that full-length cDNAs encoding prOEP75 homologs have not been identified from EST sequencing projects may be that the transit peptides are not well conserved. This is the case with other chloroplastic transit peptides (von Heijne et al., 1989). Based on the analysis of a nearly full-length *Arabidopsis* homolog of prOEP75, presented in Chapter 5, it in fact appears that the prOEP75 transit peptide is not highly conserved. Nonetheless, it may be conserved enough to allow some comparisons to be made. For example, in pea prOEP75 there is an intriguing stretch of amino acids within

c75 in which 23 consecutive residues consist only of alanine, glycine, and serine. Although the cDNA encoding the *Arabidopsis* prOEP75 homolog is not full-length, it apparently encodes a homologous region in which 15 of 23 residues are glycine (see Figure 5.2). One speculation is that this region may be a "spacer" region. The presence of such a spacer region may be important because it would allow the N-terminus of prOEP75 to protrude into the stromal space where it is removed while keeping the mature domain in the outer envelope membrane. A "spacer" function has also been ascribed to a $(Glu\text{-}Pro)_n$-$(Lys\text{-}Pro)_m$ repeat in TonB (Larsen et al., 1993). In the case of TonB, the putative spacer region allows the protein to span the *E. coli* periplasmic space.

Stroma versus intermembrane space

Upon import and fractionation of the C-terminal deletion construct of prOEP75 and of some chimeric constructs between prOEP75 and prSS, imported products were recovered in both soluble and envelope fractions (Chapter 3). For example, upon import and fractionation of t75-mSS, the primary product, c75-mSS, was recovered in soluble and envelope fractions. The soluble fraction not only contains stromal proteins but also might contain intermembrane space proteins. This raises the question of whether soluble c75-mSS, for example, was in the stroma or the intermembrane space. Being able to make this distinction would add to our understanding of the prOEP75 import pathway by providing clues as to how c75 functions.

Dorne et al. (1985) showed that phospholipase C could be used to selectively hydrolyze the major polar lipid of the outer membrane, phosphatidylcholine. Thus, I attempted to use a combination of phospholipase C and thermolysin to degrade intermembrane space proteins but not stromal proteins. These attempts, however, were unsuccessful. Others (Cline et al., 1984; Marshall et al., 1990; Schnell et al., 1994; Wu et

al., 1994; Scott and Theg, 1996) have used a combination of trypsin and chymotrypsin to selectively degrade intermembrane space proteins but not stromal proteins. My initial attempts at using this strategy were unsuccessful. This strategy probably could be used, however, to determine if imported c75-mSS is in the stroma or intermembrane space. Apparently, the key to the success of this technique is the liberal use of protease inhibitors both to stop the protease reaction and in all subsequent sample manipulations (unpublished observations).

Assembly and topology of OEP75

Little is known about the assembly and final topology of OEP75. Although OEP75 appears to be deeply embedded within the outer envelope membrane, specific fragments can be generated by mild protease treatments (Schnell et al., 1994; unpublished data). Furthermore, as shown in Chapter 2, OEP75 is accessible to antibodies. Thus, OEP75 is not completely embedded within the outer envelope membrane. Some efforts are underway in Keegstra's lab to use proteases in conjunction with anti-OEP75 antibodies to investigate the topology of OEP75.

Another potentially useful approach to address the topology of OEP75 would be to expand upon the import of a C-terminal deletion construct of prOEP75 presented in Chapter 3. Other deletion constructs could be prepared that contained increasing portions of the C-terminus of prOEP75. Additionally, one could prepare deletion constructs that lack varying portions of the N-terminus of the mature domain (resulting in the transit peptide being fused to the C-terminus of mOEP75). Import and fractionation analysis of these deletion constructs may lead to the identification of regions within mOEP75 that are essential for anchoring the protein in the outer envelope membrane. Simply knowing how

much of the mature domain is needed for complete targeting to the outer envelope membrane would be useful.

Although I mentioned in Chapter 3 that the formation of a complex tertiary structure consisting of several beta-strands may be necessary to anchor OEP75 in the outer envelope membrane, it is possible that a specific stretch of amino acids may anchor OEP75. If such a stretch of amino acids existed and was identified, this knowledge could form the basis of a model for the topology of OEP75. Regardless of how the topology of OEP75 is elucidated, it seems to be an important question for understanding both the targeting and function of OEP75.

Conservation of prOEP75 import pathway among plant species

Preliminary evidence, presented in Chapter 5, indicates that the prOEP75 import pathway is conserved among plant species. Pea prOEP75 is processed to iOEP75 and mOEP75 by maize chloroplasts. Direct evidence for conservation of the prOEP75 import pathway could be provided by the reciprocal experiment. That is, one could determine if maize prOEP75 (or other homolog) is processed to iOEP75 and mOEP75 by pea chloroplasts. As mentioned previously, however, a full-length clone encoding a prOEP75 homolog is unavailable at present, and may not be identified from EST sequencing projects.

In addition to using full-length clones encoding homologs to the pea prOEP75 to verify conservation of the import pathway, these clones could provide clues to structure and function of OEP75 and its transit peptide (described previously). Thus, it seems appropriate to make an effort to obtain cDNA clones encoding homologs of the pea prOEP75. One could use partial-length cDNA clones from other plant species as homologous probes to obtain full-length clones encoding prOEP75. As described in

Chapter 4, a putative, partial-length cDNA clone for rice OEP75 has already been identified. Alternatively, one could use heterologous cloning strategies.

Developmental regulation

As described in Chapter 2, mOEP75 was detected in whole protein extracts from leaves, stems, and roots. The plastid import apparatus is thought to be similar in all plastid types. The fact that mOEP75 was present in roots supports this idea. However, there is some evidence that there are slight variations in the import apparatus in different plastid types (Fischer et al., 1994; Wan et al., 1995). Now that antibodies and cDNA clones exist for OEP75 and other proteins of the import apparatus, one could began more thorough developmental analysis of the import apparatus by asking when and where these proteins are expressed. Likely, this type of work will soon be underway in Keegstra's lab.

Interesting questions that could be asked include: Are all the import components expressed in different plant tissues (different plastid types)? Are there temporal differences in the expression of the different components? Are there different isoforms of the different components? Are the components encoded by single genes, or members of a large gene family?

Processing of iOEP75 to mOEP75

As discussed in Chapter 2, processing of iOEP75 to mOEP75 was a slow step both in vitro and in vivo. In fact, even after prolonged incubation of in vitro reactions, iOEP75 was present. Where is the protease that processes iOEP75 to mOEP75 and what regulates this step? Is processing to i2OEP75 an obligate step en route to mOEP75? It is possible that

iOEP75 must associate with other import components before it is fully processed to mOEP75. Maybe, as discussed in Chapter 2, the association of iOEP75 with another import component is a quality control step that ensures high fidelity targeting of OEP75. Maybe iOEP75 has a functional role apart from mOEP75.

Relevant to the preceding questions and speculations, an interesting observation is that varying either the number of prOEP75 molecules or the number of chloroplasts in an in vitro import reaction does not alter the ratio of radiolabeled iOEP75 to radiolabeled mOEP75 (unpublished data). This implies that a certain fraction of prOEP75 is destined to be fully processed to mOEP75 whereas some will be processed only to iOEP75.

Incubation of other chloroplastic precursor proteins results in only a portion (10% to 50%) of the added precursor being imported (de Boer and Weisbeek, 1991; Bruce et al., 1994). The fact that only a portion of added precursor is imported is attributed to the "import competence" of the precursor. Import incompetent precursors are thought to be aggregated or misfolded such that they can no longer be recognized or imported by the chloroplastic protein import machinery . Molecular chaperones that likely maintain the import competence of precursor proteins are present in in vitro translation systems. When precursor proteins are obtained from over-expression in *E. coli*, typically the precursor is isolated and purified in a strong denaturant. Upon dilution out of the denaturant, the precursor loses import competence in a time dependent fashion (de Boer and Weisbeek, 1991; Hurley, 1993).

The import competence of prOEP75 likely explains why only a portion of added prOEP75 is imported. More importantly, however, import competence may also explain why, even after prolonged incubation of the import reaction, only a portion of the imported protein is fully processed to mOEP75. It would be interesting to determine if the percentage of over-expressed prOEP75 that is processed to iOEP75 and mOEP75 is different from that obtained from in vitro translated prOEP75. Unfortunately, it has not yet been possible to obtain prOEP75 by over-expression in *E. coli* (J. Davila-Aponte,

unpublished data). If the ratio of iOEP75 to mOEP75 can not be altered by altering the import competence of the precursor, then it is tempting to speculate that a portion of the precursor is processed only to iOEP75 because iOEP75 has a function apart from mOEP75.

Dependence of prOEP75 import on rabbit reticulocyte lysate

Another interesting phenomenon that may involve import competence is the dependence of prOEP75 import on rabbit reticulocyte lysate. As described in Chapter 4, EGTA (or EDTA) could partially substitute for rabbit reticulocyte lysate to stimulate import of prOEP75. Possibly EGTA chelates cations which enhance misfolding of prOEP75 into an import incompetent state. Again, purified over-expressed prOEP75 may be useful to address this question by providing a more defined system, i.e., one could more easily regulate the concentrations of various cations in the import reaction.

If cations cause folding of prOEP75, which in turn reduces its import competence, then it is interesting to speculate that cations may have a physiologically significant role in the prOEP75 import pathway. Maybe, in vivo, cations interact with iOEP75 to facilitate folding and assembly of the mature domain, and promote further processing of iOEP75 to mOEP75.

Unusual import pathways

The import pathways of several putative components of the chloroplastic protein import apparatus have now been addressed. OEP34 (IAP34) (Kessler et al., 1994; Seedorf et al., 1995), OEP44 (Com 44) (Ko et al., 1995) and OEP70 (SCE70) (Ko et al., 1992) follow

the targeting paradigm for outer envelope membrane proteins that existed when I began my dissertation research. That is, these proteins are not synthesized as higher molecular weight precursors and do not require ATP nor an outer envelope membrane surface protein for their import. Similarly, the import pathway of IEP110, a putative inner envelope membrane import component appears not to be unusual, i.e., it is similar to that of known inner envelope membrane proteins (Lübeck et al., submitted).

In contrast to the above mentioned import components, the import pathways of OEP75 (this dissertation) and OEP86 (Hirsch et al., 1994) are unusual. The similarities and differences between the import pathways of OEP75 and OEP86 are discussed in Chapter 3. To briefly summarize, although both are synthesized with N-terminal targeting sequences, the import pathway of OEP86, unlike that of OEP75, apparently does not overlap with that of other transit peptide-bearing precursor proteins. Furthermore, whereas processing of prOEP86 apparently takes place by an outer-envelope associated peptidase (Hirsch et al., 1994) and is not processed by the stromal processing peptidase (unpublished data) prOEP75 is processed to iOEP75 by the stromal processing peptidase (Chapter 3). It is possible, however, that subsequent processing of iOEP75 to mOEP75 may occur by the same peptidase that processes prOEP86. Nonetheless, there appear to be at least three pathways for targeting a protein to the outer membrane of the chloroplastic envelope: a transit peptide independent pathway and two transit peptide dependent pathways. Why two separate transit peptide dependent pathways exist is intriguing.

Two explanations for the novel pathway exhibited by OEP75 were discussed in Chapters 2 and 3. To briefly summarize, the OEP75 import pathway may have arisen as a quality control mechanism to prevent mistargeting of an import component to the wrong organelle. Alternatively, the OEP75 import pathway may be a conservative sorting pathway, analogous to that of some thylakoid lumen proteins, reflecting the evolutionary origin of OEP75. OEP86 apparently does not follow a conservative sorting pathway.

Thus, of the two hypothesis mentioned above, only the "quality control" hypothesis could account for the origin of the OEP86 import pathway.

I do not think the tools are yet available to methodically address the question of why the novel import pathways of either OEP75 or OEP86 exist. Possibly, the different pathways merely reflect different solutions that plant cells have developed for an intra-organellar protein targeting problem.

REFERENCES

Bruce, B.D., Perry, S., Froehlich, J. and Keegstra, K. (1994). In vitro import of proteins into chloroplasts. Plant Mol. Biol. Manual **J1**, 1-15.

Cline, K., Werner-Washburne, M., Andrews, J., and Keegstra, K. (1984). Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. Plant Physiol. **75**, 675-678.

de Boer, A. and Weisbeek, P.J. (1991). Chloroplast protein topogenesis: import, sorting and assembly. Biochim. Biophys. Acta. **1071**, 221-253.

Dorne, A., Joyard, J., Block, M.A., and Douce, R. (1985). Localization of phosphatidylcholine in outer envelope membrane of spinach chloroplasts. J. Cell Biol. **100**, 1690-1697.

Fischer, K., Weber, A., Brink, S., Arbinger, B., Schünemann, D., Borchert, S., Heldt, H.W., Popp, B, Benz, R., Link, T.A., Eckerson, C., and Flügge, U. (1994). Porins from plants. J. Biol. Chem. **269**, 25754-25760.

Gray, J.C., and Row, P.E. (1995). Protein translocation across envelope membranes. Trends Cell Biol. **5**, 243-247.

Hirsch, S., Muckel, E., Heemeyer, F., von Heijne, G. and Soll, J. (1994). A receptor component of the chloroplast protein translocation machinery. Science **266**, 1989-1992.

Hurley, M. L. (1993). Characterization of an overexpressed chloroplast precursor, prSSU. M.S. Thesis, University of Wisconsin-Madison.

Kessler, F., Blobel, G., Patel, H.A. and Schnell, D.J. (1994). Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science **266**, 1035-1038.

Ko, K., Bornemisza, O., Kourtz, L., Ko, Z.W., Plaxton, W.C. and Cashmore, A.R. (1992). Isolation and characterization of a cDNA clone encoding a cognate 70-kDa heat shock protein of the chloroplast envelope. J. Biol. Chem. **267**, 2986-2993.

Ko, K., Budd, D., Chengbiao, W., Seibert, F., Kourtz, L., and Ko, Z.W. (1995). Isolation and characterization of a cDNA clone encoding a member of the Com44/Cim44 envelope components of the chloroplast protein import apparatus. J. Biol. Chem. **270**, 28601-28608.

Larsen, R.A., Gwendolyn, E.W., and Postle, K. (1993). The conserved proline-rich motif is not essential for energy transduction by *Escherichia coli* TonB protein. Mol. Microbiol. **10**, 943-953.

Lübeck, J., Soll, J., Akita, M., Nielsen, E., and Keegstra, K. (submitted). Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane.

Marshall, J.S., DeRocher, A.E., Keegstra, K., and Vierling, E. (1990). Identification of heat shock protein hsp70 homologue in chloroplasts. Proc. Natl. Acad. Sci. USA **87,** 374-378.

Perry, S.E., Keegstra, K. (1994). Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell **6,** 93-105.

Schnell, D.J. (1995). Shedding light on the chloroplast protein import machinery. Cell **83,** 521-524.

Schnell, D.J., Kessler, F. and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. Science **266,** 1007-1012.

Scott, S.V., and Theg, S.M. (1996). A new chloroplast protein import intemediate reveals distinct translocation machineries in the two envelope membranes: energetics and mechanistic implications. J. Cell Biol. **132,** 63-75.

Seedorf, M., Waegemann, K. and Soll, J. (1995). A constituent of the chloroplast import complex represents a new type of GTP-binding protein. Plant J. **7,** 401-411.

von Heijne, G., Steppuhn, J. and Herrmann, R.G. (1989). Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. **180,** 535-545.

Wan, J., Blakeley, S.D., Dennis, D.T., and Ko, K. (1995). Import characteristics of a leucoplast pyruvate kinase are influenced by a 19-amino-acid domain within the protein. J. Biol. Chem. **270,** 16731-16739.

Wu, C., Seibert, F.S., and Ko, K. (1994). Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. J. Biol. Chem. **269,** 32264-32271.