The outer envelope protein OEP86 functions as a receptor for precursor proteins in the chloroplastic import machinery. In contrast to most other organellar outer membrane proteins it is synthesized as a precursor polypeptide (preOEP86) in the cytosol and is post-translationally targeted to the organelles. PreOEP86 is targeted to and productively inserted into the chloroplastic outer envelope mediated by a bipartite signal consisting of the presequence and the COOH terminus of the precursor protein. The cleavable presequence alone does not seem to contain sufficient information to target preOEP86 without the COOH terminus or a hybrid protein consisting of the presequence of preOEP86 and the mature form of the small subunit of ribulose bisphosphate carboxylase to intact chloroplasts. The presequence seems to be required to maintain preOEP86 in an integration competent state, whereas interaction of preOEP86 with chloroplasts is accomplished by a short sequence of amino acids in the COOH-terminal portion of the mature protein. The COOH-terminal portion of preOEP86 contains enough information to also direct mature OEP86 into the outer envelope membrane of pea chloroplasts. However, mature OEP86 enters the productive folding pathway much less efficiently than preOEP86. The COOH terminus of preOEP86 not only serves as a membrane anchor but seems to be required for a productive translocation through an interaction with other outer envelope proteins. Although the binding was ATP-dependent, productive folding was not. PreOEP86 seems to follow a unique road into the chloroplastic outer envelope.

The vast majority of mitochondrial and chloroplastic protein constituents are nuclear encoded, synthesized in the cytosol, and post-translationally imported into the organelles (1, 2). In general, proteins are made as precursors with NH2-terminal transit sequences, which contain the information necessary for targeting to the proper organelle and in many cases also for routing inside the organelle (1). In contrast, nuclear encoded polypeptide constituents of the chloroplastic outer envelope membranes are generally not synthesized as larger precursor proteins but contain internal targeting information (3–6). This insertion process does not require the hydrolysis of ATP or protease-sensitive chloroplast surface components (3). In some cases ATP seems to stimulate insertion (5, 7). In contrast, import of precursor proteins into chloroplasts requires the hydrolysis of ATP at several steps during the process and involves protease-sensitive chloroplast surface components (1, 2, 8).

Several constituents of the precursor protein import machinery of the chloroplastic outer envelope membrane have been identified recently, namely the outer envelope proteins of 86, 75, 44, and 34 kDa (OEP86, OEP75, OEP44, and OEP34) (9–15). Biochemical data suggest that OEP86 functions as a receptor for precursors (12), whereas OEP75 could form the central protein conducting channel of the outer envelope translocase (11). OEP86 and OEP75 are both synthesized with NH2-terminal cleavable presequences (12, 15). To date OEP86 and OEP75 are the only known proteins of an organellar outer membrane, which are made with a presequence. The presequence of preOEP75 contains typical features of stroma directig envelope transfer domains of “normal” chloroplastic precursors and biochemical evidence indicated that preOEP75 uses components of the general chloroplast protein import machinery (15). In contrast to stroma targeting signals, the presequence of preOEP86 is unusually long (15 kDa) and highly negatively charged. The productive translocation of preOEP86 into the chloroplastic outer envelope is dependent on ATP and requires protease-sensitive chloroplast surface components but does not use the general import machinery (12). These data indicate that a unique pathway has developed for the translocation of the protein import receptor into the chloroplastic outer envelope membrane.

In this study we have analyzed the role of the cleavable presequence of preOEP86 and that of internal sequences for targeting and insertion into the chloroplastic outer envelope membrane. Surprisingly the presequence of preOEP86 alone does not contain sufficient targeting information for chloroplasts but a productive translocation process requires a bipartite signal that is present in the presequence as well as in the COOH terminus of preOEP86. Our data suggest further that preOEP86 interacts initially with proteinaceous chloroplast surface components in an ATP-dependent step. The COOH terminus of preOEP86 seems to contain two domains, one that serves as a membrane anchor and one that is necessary for productive folding.

Materials and Methods

Construction of preOEP86 and mOEP86 for in Vitro Transcription-Translation

The cDNA coding for preOEP86 in the vector pET17b (12) was recloned into pET24c (Novagen, Madison, WI) in two steps after restriction with NdeI and XhoI resulting in preOEP86-pET24c. To obtain a cDNA coding for mOEP86, which could be used for in vitro transcription and translation, an additional NdeI site was introduced at the processing site of preOEP86 by in vitro mutagenesis. The primers used for the PCR1 were 5’-CGCCTCCCAATCATATGGCTCC-3’ (forward) and 5’-CGC-
CATATTGGTGACCC-3' (reverse). The PCR product was cloned into preOE86-pET24c after restriction with NdeI, resulting in mOE86pET24c. The DNA sequence was controlled by sequencing (16). The in vitro mutation results in the addition of a methionine NH$_2$-terminal of the sequence Ala-Pro-Ser of OE86 (12).

**Construction of Different Fusions from preOE86, mOE86, and SSU**

pre86SSU—A SphI site was introduced into preOE86-pET17b by in vitro mutagenesis using the following primers for the PCR 5′-GCTC-TACGATGCAAGGAGC-3′ (reverse) and T7 universal primer (forward). The PCR product was subcloned into the vector pGEM5Zf(+) (Stratagene, La Jolla, CA) after restriction with PstI and HindIII. The resulting clone pre86SSU-pGEM5Zf(+) was used to clone pre86SSU after restriction with PstI and HindIII into the vector pET24c (Novagen), resulting in clone pre86SSU-pET24c. The coding sequence of the resulting clone pre86SSU-pET24c was verified by sequencing. It contained amino acids 1–149 of preOE86 and the entire coding sequence of mature SSU.

SSU-86—A SphI/SacI fragment from preSSU-pSP64 (17) was ligated into pGEM5Zf+(Promega, Madison, WI) resulting in the clone preSSU-pGEM5Zf(+) (17). The SphI site was introduced at the original stop codon of the SSU-pGEM5Zf(+) using PCR with the primers 5′-CCCCCGGTAATCGTGGTC-3′ (reverse) and T7 (forward). A HindIII/PstI fragment obtained from preOE86 in pBSC5KII(+) (12) was ligated into the mutant SSU-pGEM5Zf(+) after restriction with HindIII and PstI. The resulting clone pre86SSUmOE86-pGEM5Zf(+) was sequenced. It contained the entire coding sequence of mature SSU (17) and amino acids 653–879 of preOE86 (12). Due to poor translation efficiency of SSU-pGEM5Zf(+), it was subcloned in one step into the vector pSELECT (Promega) after restriction with SphI/EcoRI.

preSSU-86A—A KpnI/BamHI fragment from SSU-86 in pGEM5Zf(+) was ligated into preSSU-pSP64 (17) after restriction with KpnI/BamHI, resulting in clone preSSU-pSP64 containing the entire coding region for preSSU (17) and amino acids 653–879 of preOE86 (12).

**OE86-C$_{c86-go}$**—For transcription-translation and expression in Escherichia coli a NcoI/XhoI fragment of mOE86-pET24c was cloned into pET21d (Novagen).

**COOH-terminal Deletions**—The COOH-terminal deleted polypeptides preOE86$_{c87}$, preOE86$_{c87-go}$, preOE86$_{c88-go}$, and preOE86$_{c87}$ were obtained by in vitro transcription-translation after restriction of preOE86$_{c87}$-pET17b using PstI, SpeI, HindIII, NcoI, and BamI, respectively.

**In Vitro Transcription-Translation and Expression of the Various Constructs**

mRNAs were obtained using either SP6 or T7 RNA-polymerase depending on the promoter present in the vector construct. The mRNAs were translated in a reticulocyte lysate system in the presence of $^{35}$S-labeled methionine and cysteine as described before (4).

**Isolation of Chloroplasts and Protein Import**

Chloroplasts were isolated from pea leaves as described before (13). A standard import assay contained chloroplasts equivalent to 30 µg of chlorophyll (19) and was carried out for 15–25 min at 25 or 4 °C in a final volume of 200 µl and 2–5% (v/v) translation product. Chloroplasts were treated with the protease thermolysin either before (750 µg of protease mg$^{-1}$ chlorophyll, 30 min, 4 °C) or after import (100 µg of protease mg$^{-1}$ chlorophyll, 15 min, 4 °C). Intact chloroplasts were recovered prior to further experimentation or analysis. The detailed procedure is as outlined in Ref. 13. ATP was removed from the translation mixture by the addition of hexokinase and glucose (20).

**RESULTS**

**Both Mature and preOE86 Interact with Intact Pea Chloroplasts**—To investigate the insertion of mOE86 and its precursor form preOE86, both were synthesized in a reticulocyte lysate and incubated with intact pea chloroplasts (Fig. 1A). PreOE86 binds to chloroplasts and is processed to the mature form, OE86 (Fig. 1A, lane 1). Upon protease treatment, processed mature OE86 is cleaved and a residual 52-kDa proteolytic fragment can be detected (Fig. 1A, lane 2). This 52-kDa fragment of OE86 represents a COOH-terminal portion of the protein (12) and can be used as a parameter for the correct folding of the protein in situ and in vitro (12, 23). These data indicate that preOE86 added to chloroplasts had inserted and folded correctly in the chloroplastic outer envelope. In contrast when translation product of mOE86 was offered to chloroplasts, binding was observed but much less 52 kDa breakdown product was seen (Fig. 1A, lanes 1–5). This indicates that the presequence of preOE86 was necessary for a productive translocation process. To characterize further the nature of the interaction between chloroplasts and preOE86 or mOE86, respectively, organelles were separated into a soluble and a membrane fraction. The membranes were then extracted at high pH (pH 11, 0.1 M Na$_2$CO$_3$). As shown (Fig. 1B) preOE86, processed mature OE86 or mOE86 were recovered exclusively in the soluble fraction (Fig. 1B, lanes 1 and 2 and lanes 5 and 6) and were resistant to extraction at high pH (Fig. 1B, lanes 3 and 4 and lanes 7 and 8), indicating that preOE86 as well as mOE86 interacted with chloroplasts by a protein-lipid interaction. The respective translation products were recovered in the soluble fraction (not shown and Ref. 12). These data indicate that an insertion or targeting signal exists in the mature form of OE86, which might help to anchor the protein into the membrane, whereas the presence of preOE86 seems to stimulate the productive translocation process.

To analyze the role of the presence of preOE86 in more detail, a hybrid protein was constructed, which consisted of the presence of preOE86, i.e. amino acids 1–149, fused in frame with the mature SSU of Rubisco (pre86SSU) to compare its targeting properties with that of preSSU. We were unable to detect any binding or interaction of pre86SSU translation product to chloroplasts either at 50 µM ATP or 2 mM ATP (Fig. 2A, lanes 1 and 2 and lanes 3 and 4) not even under very rapid recovery conditions by centrifugation through a silicon oil layer.
PreOEP86 binds to chloroplasts, whereas preOEP86542 and the presequence of preOEP86, namely preOEP86137 itself, nor with chloroplasts. Our results (Fig. 2B) between two stages in the interaction process of preOEP86 extraction and proteolytic fragmentation, we could distinguish and insertion into the membrane. Thus, by using alkaline struct was taken as a measure for a certain extent of folding detection of a protease-resistant fragment of a preOEP86 construct was as outlined in the legend to Fig. 1. TL, translation product. 10% of the total translation product. Integration of preOEP86 deletion proteins was also assessed by the appearance of a protease-resistant fragment (thermolysin (Th) fragment).

into HClO4 (24) (not shown). Although it cannot be excluded that targeting information present in the presequence of preOEP86 is hidden in the hybrid protein pre86SSU in vitro due to improper folding, we conclude that the presequence of preOEP86 does not contain enough information on its own to target a protein to chloroplasts, but additional internal OEP86 sequences are required for a productive targeting and insertion process.

We constructed a number of preOEP86 deletions to define further regions in the polypeptide that were responsible for a targeting and productive insertion (Fig. 2B). The mode of interaction between chloroplasts and preOEP86 deletions, for which a binding to chloroplasts was observed, was further analyzed by extraction at pH 11 (0.1 M Na2CO3) (25). The detection of a protease-resistant fragment of a preOEP86 construct was taken as a measure for a certain extent of folding and insertion into the membrane. Thus, by using alkaline extraction and proteolytic fragmentation, we could distinguish between two stages in the interaction process of preOEP86 with chloroplasts. Our results (Fig. 2B) indicate that neither the presequence of preOEP86, namely preOEP86137 itself, nor preOEP86542, bound to chloroplasts, whereas preOEP86697 and preOEP86775 bound with low efficiency (10–20% of full-length preOEP86) (Fig. 2B). Only preOEP86 and preOEP86775 bound to chloroplasts with similar yield, i.e., 5–10% of the total translation product added to a binding experiment. Both preproteins were completely recovered in the alkaline insoluble fraction, whereas preOEP86542 and preOEP86697 were recovered in the alkaline-soluble fraction (Fig. 2B). Thus, a short COOH-terminal stretch between amino acids 697–775 of preOEP86 seems to be responsible for the alkaline-resistant interaction with the chloroplastic outer envelope. To obtain further evidence for this notion a NH2-terminal deletion of OEP86 was constructed, namely OEP86-C699–879, which contained only the COOH-terminal 180 amino acids. OEP86-C699–879 inserted into the outer envelope membrane in a way that was resistant to alkaline extraction (Fig. 2B). When chloroplasts were treated with the protease thermolysin after an insertion experiment, no protease protected product could be detected (Fig. 2B) from any of the preOEP86 deletion constructs or from OEP86-C699–879 except for the full-length preOEP86 (Fig. 2B). From these data we conclude that a COOH-terminal portion of preOEP86 anchors the protein into the lipid bilayer. In addition the COOH-terminal portion of preOEP86 and its presequence might have to cooperate to insure not only targeting but a productive integration process of preOEP86 as evidenced by the appearance of the 52-kDa breakdown product from the full-length precursor only. Indeed this conclusion is consistent with studies presented below.

The COOH terminus of OEP86 might act as a sorting sequence and membrane anchor for the chloroplastic outer envelope membrane. To study this point we constructed hybrid proteins consisting of either the mature or the precursor form of SSU fused in frame to amino acids 653–879 of preOEP86. A chloroplasts were incubated with SSUc86 or SSU translation product in the presence of different concentrations of ATP. All other manipulations were as outlined on top of the figure and as described in the legend to Fig. 1. B, SSUc86 was recovered together with chloroplast membranes (P) and remained in the Na2CO3 insoluble fraction (P) and not in the soluble protein fractions (S) (methods as outlined in the legend to Fig. 1). TL, translation product; Th, thermolysin.

FIG. 2. The presequence of preOEP86 cannot target a passenger protein to chloroplasts but is necessary together with a COOH-terminal portion of OEP86 for a productive translocation process. A, pre86SSU does neither bind nor import into chloroplasts independent of the ATP concentration. Experimental conditions were as outlined in the legend to Fig. 1. TL, translation product, 10% of which was added to an experiment. Th, thermolysin. B, truncated preOEP86 polypeptides were synthesized as described in the legend to Fig. 1. Extraction and proteolytic fragmentation after treatment of preOEP86-pET17b with different restriction enzymes (see “Materials and Methods”). OEP86-C699–879 was constructed and synthesized as described under “Materials and Methods.” Binding and interaction with chloroplasts was done and analyzed as above. The yield of binding was quantified by laser densitometry of the exposed x-ray films. +++, >5% binding; +, 2–5% binding; +, <2% binding of added translation product. Integration of preOEP86 deletion proteins was also assessed by the appearance of a protease-resistant fragment (thermolysin (Th) fragment).
protein in which preSSU was fused to amino acids 653–879 of preOEP86 (see above) resulting in preSSUc86. The fusion protein preSSUc86 bound to chloroplasts in the presence of 50 μM ATP (Fig. 4A, lane 1), conditions that also favor binding of preSSU but that do not allow maximal translocation (Fig. 4A, lane 5) (26, 27). When chloroplasts were treated with the protease thermolysin after such a binding experiment, translocation intermediates of preSSU were detected, namely Tim 3 and Tim 4 (Fig. 4A, lane 6), which have been described before (13, 28). Chloroplast-bound preSSUc86 gave rise to identical translocation intermediates as preSSU (Fig. 4A, lanes 2 and 6) although with low yield, indicating that some preSSUc86 had entered the general import route across the envelope membranes. Under conditions that allow complete translocation of a precursor protein, i.e. 2 mM ATP, processed mature SSU was detected protease protected inside chloroplasts from preSSU, whereas very little precursor remained at the organellar surface (Fig. 4A, lanes 7 and 8). In contrast most of preSSUc86 remained bound to the chloroplast surface in a protease-accessible way in the presence of 2 mM ATP (Fig. 4A, lanes 3 and 4). Between 10–20% of preSSUc86 was found in the processed form, SSUc86, protease protected inside chloroplasts (Fig. 4A, lanes 3 and 4) corroborating our earlier notion that preSSU can indeed function, although with low efficiency, as a carrier for c86. Differences in the interaction of preSSU and preSSUc86 with intact chloroplasts are indicated by differences in localization and mode of interaction. At 50 μM ATP preSSU is recovered together with the membranes, but the majority (between 70 and 80%) is extractable by pH 11 (Fig. 4B, lower panel, lanes 1–4). This is in agreement with data reported before (13) under these conditions. Only very little of preSSUc86 is extractable by 0.1 M Na2CO3 (Fig. 4B, upper panel, lanes 3 and 4). The alkaline soluble form of preSSUc86 is most likely the fraction that is bound to the general protein import machinary of chloroplasts via the preSSU portion of the hybrid protein, whereas the insoluble form of preSSUc86 represents the fraction that interacts with the chloroplastic outer envelope via the COOH terminus of OEP86. Processed mature SSU and SSUc86 are recovered in the soluble stroma, and the remaining protein in the membrane fraction is completely alkaline extractable (Fig. 4B, lanes 5–8).

The results obtained so far indicated that firstly the presequence of preOEP86 was necessary for an efficient and productive translocation pathway, although it did not contain chloroplast targeting information. Secondly, the COOH terminus of OEP86 is necessary to anchor the protein into the chloroplastic outer envelope. Thirdly, the COOH terminus could also play a role as a sorting signal. As demonstrated in Fig. 2, OEP6-C<sub>699–879</sub> was able to insert in an alkaline-resistant way into the chloroplastic outer membrane. Therefore, we wanted to know if overexpressed OEP6-C<sub>699–879</sub> was able to influence binding and insertion of preOEP86 into chloroplasts. In the presence of overexpressed OEP6-C<sub>699–879</sub> proper insertion and folding of preOEP86 translation product was reduced by about 70%, as demonstrated by the reduced yield of mature OEP6 and the 52-KDa fragment (Fig. 5, lanes 2 and 4). To detect competition at the level of preOEP86 binding to chloroplasts is much more difficult because a significant amount of binding in vitro seems to be nonproductive (see above). Furthermore we do not expect that competition occurs at the level of nonspecific and nonproductive interaction with the bulk lipid phase. However, the results show that OEP6-C<sub>699–879</sub> associates with partially inhibits most likely proteinaceous components involved in the productive targeting and insertion pathway (Fig. 5). In an attempt to differentiate further between the role of lipids...
and proteinaceous components in the preOEP86 insertion pathway, chloroplasts were treated with the protease thermolysin prior to an insertion experiment. PreOEP86 bound to chloroplasts either not treated or treated with protease in the presence of ATP. This binding was largely resistant to alkaline extraction (not shown). Only untreated chloroplasts, however, were able to properly insert and fold the protein as deduced from the 52-kDa fragment (not shown). Binding of mOEP86 or OEP86-C<sub>699–879</sub> to protease-treated chloroplasts occurred with similar yields as to nontreated chloroplasts. These data indicate that proteinaceous components are involved in the productive binding and insertion process of preOEP86 (12). When these components are removed a nonproductive interaction occurs in vitro between the outer envelope lipids and preOEP86 most likely via the COOH terminus.

To analyze the role of ATP in the translocation pathway, the translocation products of preOEP86, preOEP86<sub>775</sub>, mOEP86, OEP86-C<sub>699–879</sub> and preSSU were depleted of ATP by the addition of hexokinase and glucose (20). Binding to chloroplasts was then assayed either in the absence of exogenously added ATP or in the presence of 20 μM ATP. Binding was quantified by laser densitometry of the exposed x-ray films. The bars represent the x-fold stimulation of binding in the presence of ATP in comparison with the absence of ATP. A typical result out of three repeats is shown.

![Image](http://www.jbc.org/)

**DISCUSSION**

To date only two proteins have been identified, namely OEP86 and OEP75, that require an NH<sub>2</sub>-terminal cleavable presequence for productive targeting to an organellar outer membrane. The common denominator of these proteins is that both are localized in the chloroplastic outer envelopes and both are constituents of the protein import machinery (2, 8). Although preOEP75 follows the general import pathway (15) it seemed evident from preliminary studies (12) that preOEP86 uses a unique translocation pathway. In this study we demonstrate that the presequence has to cooperate with a COOH-terminal portion of preOEP86 to guarantee the efficient and productive translocation into the outer envelope. The function of the presequence of preOEP86 seems at least in part different from that of other presequences, because it has no ability to target preOEP86<sub>137</sub> and preOEP86<sub>647</sub> or a passenger protein, namely pre66SSU to chloroplasts. Our results demonstrate, however, that the presequence of preOEP86 is required for a productive translocation pathway. Firstly, the yield of OEP86 insertion is stimulated 3–4-fold when preOEP86 instead of mOEP86 is offered to chloroplasts. Secondly, only preOEP86 and preOEP86<sub>775</sub> show a significant dependence on ATP for binding to chloroplasts, whereas mOEP86 and OEP86-C<sub>699–879</sub> do not. The presequence of preOEP86 is negatively charged in contrast to normal chloroplast directing presequences, which carry an overall positive charge. The negative charges might therefore inhibit the interaction with receptor components of the general protein import machinery of chloroplasts and lead to a specific and novel translocation pathway for preOEP86.
Although the presequence of OEP86 is necessary for productive insertion of the preprotein, a specialized receptor for it was taken as a measure for productive translocation. A typical result out of three repeats is shown.

The productive translocation of preOEP86 into the chloroplastic outer envelope is not a spontaneous process but requires yet to be identified proteinaceous components. This conclusion is drawn from the evidence that (i) protease-treated organelles are unable to integrate preOEP86; (ii) hydrolysis of ATP is required for productive binding of preOEP86 but not for the final folding reactions; (iii) preOEP86 translocation can be competed with the overexpressed OEP86-C<sub>699</sub>–<sub>879</sub> exc and (iv) the translocation is strictly temperature-dependent. In general the translocation of other proteins into the chloroplastic outer envelope does not require ATP or protease-sensitive chloroplast surface components (3, 4, 6).

The carboxyl terminus of OEP86 is necessary to anchor the protein into the envelope membrane. However, it can also function as a membrane anchor for a soluble protein as demonstrated for SSUc86. SSUc86 is oriented in the outer envelope in a way that exposes SSU to the cytosol. This orientation is like that in the authentic OEP86, the NH<sub>2</sub> terminus of which protrudes also into the cytosol (12). We were unable to detect a protease-protected fragment from inserted SSUc86 indicating that only a short transmembrane region of SSUc86 was protease-resistant, too short to warrant detection. The other possibility would be that SSUc86 represents a monotopic protein (29), a possibility that has been considered before for OEP86 (12).

Envelope transfer stroma targeting domains can be used as carriers to import passenger proteins that contain several hydrophobic transmembrane α-helices into the organelle. The COOH terminus of OEP86, i.e. amino acids 653–879, however, represents a strong detour signal, which directs preSSUc86 to the chloroplastic outer envelope. About 10–20% of the hybrid protein enters the general import pathway as demonstrated by the appearance of processed SSUc86 inside the organelle at 2 mM ATP. At 50 mM ATP translocation intermediates were detected that were identical for preSSU and preSSUc86 as expected if translocation proceeds from the NH<sub>2</sub> terminus to the COOH terminus and comes to a stop at ATP concentrations that do not allow complete translocation, i.e. 50–100 mM ATP (13, 28).

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A Protein Import Receptor of Chloroplasts Is Inserted into the Outer Envelope Membrane by a Novel Pathway
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