A protein kinase was located in the cytosol of pea mesophyll cells. The protein kinase phosphorylates, in an ATP-dependent manner, chloroplast-destined precursor proteins but not precursor proteins, which are located to plant mitochondria or plant peroxisomes. The phosphorylation occurs on either serine or threonine residues, depending on the precursor protein used. We demonstrate the specific phosphorylation of the precursor forms of the chloroplast stroma proteins ferredoxin (preFd), small subunit of ribulose-bisphosphate-carboxylase (preSSU), the thylakoid localized light-harvesting chlorophyll a/b-binding protein (preLHCP), and the thylakoid lumen-localized proteins of the oxygen-evolving complex of 23 kDa (preOE23) and 33 kDa (preOE33). In the case of thylakoid lumen proteins which possess bipartite transit sequences, the phosphorylation occurs within the stroma-targeting domain. By using single amino acid substitution within the presequences of preSSU, preOE23, and preOE33, we were able to tentatively identify a consensus motif for the precursor protein kinase. This motif is (P/G)Xn(R/K)Xm(T/S) (n = 0–3 amino acids spacer and T*/S* represents the phosphate acceptor. The precursor protein kinase is present only in plant extracts, e.g. wheat germ and pea, but not in a reticulocyte lysate.

Protein import experiments into chloroplasts revealed that phosphorylated preSSU binds to the organelles, but dephosphorylation seems required to complete the translocation process and to obtain complete import. These results suggest that a precursor protein protein phosphatase is involved in chloroplast import and represents a so far unidentified component of the import machinery. In contrast to sucrose synthase, a cytosolic marker protein, the precursor protein protein kinase seems to adhere partially to the chloroplast surface. A phosphorylation-dephosphorylation cycle of chloroplast-destined precursor proteins might represent one step, which could lead to a specific sorting and productive translocation in plant cells.

Chloroplasts and mitochondria contain their own genome; however, the vast majority of their proteins is encoded in the nucleus and synthesized in the cytosol. In general these proteins carry NH2-terminal targeting domains which direct them to the proper organelle in a posttranslational event. Whereas some knowledge about the components and mechanisms which are involved in the recognition and translocation of the precursor on the organelar surface has accumulated (Hirsch et al., 1994; Schnell et al., 1994; Gray and Row, 1995; Soli, 1995), almost nothing is known about the events which take place in the cytosol, after the precursor emerges from the ribosome and before binding to the chloroplast. A loosely folded precursor, however, seems to be a prerequisite for membrane translocation. In all import systems aggregation of highly hydrophobic membrane proteins and premature folding has to be prevented. To attain or maintain such an import-competent, soluble conformation, some (Waegemann et al., 1990) but not all proteins need the help of cytosolic factors in vitro (Pilon et al., 1992). A 70-kDa heat shock protein (hsc70)1 was found to be involved in the transport of proteins to different destinations, e.g. mitochondria, endoplasmic reticulum, and chloroplasts (Deshaies et al., 1988; Zimmermann et al., 1988; Waegemann et al., 1990). It was also shown that precursor proteins readily interact with hsc70 during translation (Beckmann et al., 1990). Whereas this interaction seems a rather general phenomenon (Elli and van der Vies, 1991), two cytosolic factors have been described which act as specific chaperones for mitochondrial precursors. These factors, called presequence binding factor and mitochondrial import stimulation factor, have been purified from rabbit reticulocyte lysate and rat liver cytosol, respectively, and were shown to recognize mitochondrial presequences and stimulate their import into the organelle (Murakami et al., 1992; Hachiya et al., 1993, 1995). Such cytosolic factors which interact specifically with the organelar targeting domain could be even more important in a plant cell, because the plastids represent an additional compartment to which the cytosolic synthesized precursors have to be properly routed. Whereas the mitochondrial presequences share an overall similar framework, i.e. positively charged residues, amphiphilic α-helical structure in plants, fungi, and mammals (von Heijne, 1986; Hartl et al., 1989), the plastid-directing transit sequences are much more heterogeneous in length and secondary structure. The only common features of the chloroplast transit domains are an uncharged NH2-terminal region, a characteristic stromal processing site, and a particularly high content of serine and threonine residues (Karlin-Neumann and Tobin, 1986; von Heijne and Nishikawa, 1991; von Heijne et al., 1989). In order to understand the series of events which ultimately yield a specific and productive routing of precursor proteins to plastids, we have started an investigation on cytosolic components which could be involved in these processes.

1 The abbreviations used are hsc70, heat shock protein homologue of 70 kDa; SSU, small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase; Fd, ferredoxin; LHCP, chlorophyll a/b-binding protein of the light-harvesting complex; OE23, 23-kDa polypeptide of the oxygen evolving complex of photosystem II; OE33, 33-kDa polypeptide of the oxygen evolving complex of photosystem I; Fb, β-subunit of mitochondrial ATPase; MDH, malate dehydrogenase; pre, precursor form of; i-, intermediate processed precursor form of; wt, wild type; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ATP-γ-S, adenosine 5′-O-(thiotriphosphate).
Here we report the specific phosphorylation of several chloroplast precursor proteins within the stroma-targeting portion of the transit sequences, but not of their mature forms. Phosphorylation seems to inhibit import of precursor proteins into chloroplasts. Mitochondrial or peroxisomal precursors are not recognized by the protein kinase. The protein kinase, which belongs to the serine/threonine kinase family, is a plant-specific enzyme. It was shown to be located in the cytoplasm, but it also adheres to the chloroplast outer envelope.

MATERIALS AND METHODS

Construction of proSSU/SSU, preHLCP/LHCP, proOE32/I3OE23, proOE33, preFd, and preFb—Several plasmids for the overexpression of proteins in Escherichia coli were used. Pet 11c expression vector containing the cDNAs for preSSU and SSU, respectively, both from tobacco, were provided by Dr. R. Klein, University of Kentucky (Klein and Salvucci, 1992). cDNAs for preHLCP and LHCP from pea, both in pDS12 expression vectors are described in Paulsen et al. (1990). Dr. K. Cline, University of Florida, provided the cDNA clones for proOE23, I3OE23, and proOE33 from pea, all in pet 3c plasmids (Cline et al., 1993). The coding sequence for pea preFd was cloned in expression plasmid pet 21d by PCR using full-length preFd cDNA in BsdI as template. The sequence of the used forward primer which contains an in-frame Ncol site was 5′-CCCCCCCCATGGGACAACACCAACAGC-3′. The sequence of the reverse primer containing an in-frame Xhol site was 5′-GAAAAATTAAGCTTCCGATGTC-3′. The PCR product and vector pet 21d were digested with Ncol and Xhol and ligated. The preFd/pet 21d clone was obtained by sequencing and isolation of the overproduced proteins was done as described by Paulsen et al. (1990), with the exception that preFbβ was expressed at 30°C instead of 37°C. PreFβ and preFd were expressed with a C-terminal His-Tag.

Site-directed Mutagenesis of preSSU, preOE32, and preOE33—Excision of serine 31 and serine 34 of tobacco preSSU for alanine yielding preSSU-M31/S/A and preSSU-M34/S/A, respectively, or the exchange of both amino acids together for alanine resulting in preSSU-M31/34/S/A was done by PCR using preSSU cDNA in pet 11c as template. The primers for mutagenesis contain an AflII site, which is an endogenous restriction site in the preSSU cDNA near the mutated region. The sequence of the excision for serine was achieved by a single base exchange (T to G) for each serine. The forward primer sequences were 5′-CTGCTTCAAGCACGCTGCTCATCCGTTTAGCTTCGTTG-3′, 5′-CTGCTGCCGCTGCTCCTTTAGCACGTCCGTTAGCTTCGTTG-3′ and 5′-CTTGCTGCCGCTGCTCATCCGTTTAGCTTCGTTG-3′, respectively, or the reverse primer containing the mutation A into T (20–40
d long) in 125 mM Tris/HCl, pH 6.8, 0.1% SDS, 10% threitol, 100 mM sucrose, and 4 mM UDP with 70 mM MgCl₂, 2.5 μM ATP plus 2–5 μCi of [γ-32P]ATP (3000 C/mmole). For kinase activity, 10–20 μg of a cytoplasm-enriched protein fraction or 10 μg of wheat germ extract protein were added. The assays contained 1–3 μg of purified overexpressed proteins as substrates which were dissolved in 8 M urea prior to the experiment. The final urea concentration in the phosphorylation assay was 160 mM. The reaction was incubated for 5 min at room temperature and terminated with SDS sample buffer. The phosphorylation products were analyzed by SDS-PAGE (Laemmli, 1970) followed by autoradiography.

Analysis of Phosphorylated Amino Acid—For the determination of the phosphoacceptor amino acid, 10 phosphorylation assays with the respective protein substrate were performed and separated by SDS-PAGE. The gel was stained with Coomassie Blue, and the bands containing the phosphorylated proteins were excised from the gel. After equilibration (3 × 10 min) in 125 mM Tris/HCl, pH 6.8, 0.1% SDS, 10% glycerol, 1 mm EDTA, 45 mm β-mercaptoethanol, the respective protein was electroeluted from the gel slices according to the manufacturer’s instructions (Biometra, Göttingen, Germany). The eluted protein was precipitated with methanol and chloroform (Wessel and Flugge, 1984) and subsequently hydrolyzed in 6 N HCl for 2 h at 110°C. The hydrochloric acid was removed by evaporation, and the sample was dissolved in H₂O and analyzed by high voltage electrophoresis on Silica Gel 60 chromatography plates (Schleicher & Schuell) according to Hunter and Sefton (1980). The electrophoresis was performed in the presence of 200 g/L of phosphotungstic acid to reduce the electroosmosis at 500 V for 4 h, the plate was dried, sprayed with ninhydrin reagent to mark the phosphoamino acid standards, and exposed on x-ray film.

Sucrose SyntheseAssay—Sucrose synthase activity was measured by a coupled enzymatic reaction resulting in the conversion of NAD into NADH. The assay was performed with a commercial kit (Assay-As; Roche Diagnostics). The first step was performed in a final volume of 100 μl in 20 mM Hepes/KOH, pH 7.0, 5 mM dithiothreitol, 100 μM sucrose, and 4 mM UDP with 70 μg of protein of the chloroplast supernatant or cytoplasm. After 30 min at 25°C, the reaction was terminated by incubating for 4 min at 95°C. The produced UDP-glucose was measured in a second step which was performed in a final volume of 600 μl with 50 μl of the first enzyme reaction in 200 mM glycine, pH 8.7, 0.1 mM NAD, and 25 μg of UDP-glucose dehydrogenase. NADH production was determined at 343 nm.

Preparation of Wheat Germ Extract—Unroasted wheat-germ was
Proteins and were also adjusted to 8 M urea prior to the phosphorylation reaction. An autoradiogram is shown.

Partial Purification of the Precursor Protein Protein Kinase Activity—Precursor protein protein kinase activity was enriched from wheat germ extract. Immediately before each chromatography step the homogenate was spun at 250,000 × g for 20 min to remove membranes and insoluble material. The supernatant (50–60 mg of protein) was applied to a 18-ml Q-Sepharose FF column (Pharmacia Biotech Inc.) equilibrated in an ultrafiltration cell, and dialyzed overnight against 10 mM Hepes/KOH, pH 7.6, containing 100 mM potassium acetate, 1 mM magnesium acetate, and 0.5 mM CaCl₂ for 20 min on ice. The final chlorophyll concentration was 1 mg of chlorophyll/ml. The treatment was terminated by the addition of a 2-fold molar excess of α-macroglobulin for 15 min on ice. After resalination of the chloroplasts by centrifugation (1500 × g; 1 min). The organelles were resuspended in 330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6.

Analytical Methods—Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. SDS-PAGE was performed according to the method of Laemmli (1970). Silver staining of the gels was done as described in Blum et al. (1987).

RESULTS

Specific Phosphorylation of Chloroplast Precursor Proteins—Posttranslational modification of proteins by phosphorylation is an important mechanism in the regulation of cellular processes. The introduction of a phosphate group into a given polypeptide chain is likely to alter its three-dimensional conformation. The import-competent conformation or the exposure of certain motifs for recognition by chaperones, presquence binding factors, or receptors could be influenced by a phosphorylation event within the presquence. The high content of serine and threonine residues in the chloroplast transit sequences suggests a phosphorylation of precursor proteins. To address this question, several chloroplast precursor proteins and their mature proteins were overexpressed in E. coli, recovered in a partially purified form from inclusion bodies (Fig. 1A), and subjected to in vitro phosphorylation (Fig. 1B). As examples of typical soluble stromal proteins, we used the precursor and the mature forms of the small subunit of ribulose-bisphosphate carboxylase/oxygenase (preSSU/SSU) and of ferredoxin (pref/Fd). As a member of the thylakoid membrane the precursor and the mature form of the chlorophyll a/b-binding protein of the light-harvesting complex II (preLHCP/LHCP) were used. The transit sequence of preLHCP has typical stroma directing properties and is cleaved by the stromal proc-
Transit Sequence Phosphorylation

Phosphorylation in one step in the stroma. Two precursor proteins of the oxygen-evolving complex associated with photosystem II (preOE23/preOE33) were also analyzed. These proteins, which reside at the luminal side of the thylakoid membranes, possess, in contrast to preLHCP, bipartite presequences consisting of an amino-terminal stroma-targeting domain and an additional thylakoid transfer domain. The stroma-targeting domains of preOE23 and preOE33 are cleaved off in the stroma to yield intermediate sized precursors which are further translocated across the thylakoids and processed finally in the thylakoid lumen. For the OE23 protein this intermediate precursor lacking the stroma-targeting domain, but still possessing the thylakoid transfer domain (iOE23), was also available. Beside these chloroplast proteins we also used overexpressed precursors from the other organelles in the plant cell to which precursor proteins are directed by NH2-terminal-cleavable presequences in a posttranslational manner. As a constituent of plant mitochondria, the precursor of the β-subunit of the ATPase (preFβ) from tobacco was expressed, and as a member of the peroxisomes, we used the precursor and the mature form of malate dehydrogenase (preMDH/MDH) (gift of Ch. Gietl, München) (Gietl et al., 1994).

Each of the above described proteins was subjected to an in vitro phosphorylation assay with [γ-32P]ATP in the presence of cytoplasmic proteins isolated from pea mesophyll protoplasts (Fig. 1B). All chloroplast precursors examined, i.e. preSSU, preFd, preLHCP, preOE23, and preOE33 became phosphorylated (indicated by > in Fig. 1B). Their mature forms or the intermediate processed form of OE23 (iOE23), however, were not able to serve as a substrate for the cytosolic protein kinase activity, indicating that the phosphorylation occurred in the stromal targeting domain of the transit sequence. The cytoplasm used in the phosphorylation assays is likely to contain several protein kinases (Ranjeva and Boudet, 1987). We observed the phosphorylation of several endogenous proteins in the absence of added precursor proteins (Fig. 1B, lane 1). The pattern of endogenous phosphorylation varied with the different batches of cytoplasm used, while the precursor protein kinase activity seemed more stable. This could be due to the long isolation procedure of a cytosolic fraction from protoplasts (see “Materials and Methods”) and to different lengths of storage. The endogenous phosphoproteins were similar to those described by Hracky and Soll (1986). To ascertain that this phosphorylation is specific for chloroplast precursor proteins and not a general event, other precursor proteins carrying NH2-terminal presequences but which are destined for different locations inside the plant cell were also subjected to in vitro phosphorylation. As shown in Fig. 1B the plant mitochondrial preFβ could not be phosphorylated. The peroxisomal preMDH and also the mature MDH were phosphorylated only very weakly. We conclude that the radioactive label is probably incorporated into the mature part of MDH. Altogether these data demonstrate that a protein kinase is localized in cytoplasm enriched from pea mesophyll cells which is able to specifically phosphorylate precursor proteins destined for the chloroplast compartment. The phosphorylation seems to occur exclusively in the stroma-targeting domain of the presequence.

Determination of the Phosphorylation Sites—For most protein kinases the amino acids serine, threonine, or tyrosine serve as phosphoacceptor amino acids. A common feature of all chloroplast transit sequences is their high content (20–30%) of serine and threonine. To determine the identity of the phosphoacceptor group the 32P-labeled precursor proteins were separated by SDS-PAGE, excised from the gel, and electroeluted. The eluted proteins were precipitated by CHCl3/MeOH followed by acid hydrolysis at 110 °C. The hydrolysate was analyzed by high voltage electrophoresis on silica gel thin layer chromatography plates. We found that serine serves as the phosphate acceptor in preSSU (Fig. 2A), preFd (Fig. 2B), and preOE23 (Fig. 2C), whereas preOE33 was phosphorylated exclusively on a threonine residue (Fig. 2D). In the case of preLHCP, both amino acids incorporated labeled phosphate in almost equal amounts (Fig. 2E). An endogenous phosphorylation site with threonine as phosphate acceptor is present very close to the NH2 terminus of mature LHCP. This site is involved in the regulation of light harvesting in the thylakoids in vivo (Bennett, 1991). Thus this internal phosphorylation site might also be recognized in our in vitro phosphorylation experiments when the precursor form and cytosol are used in the phosphorylation assay. Taken together these results suggest that the functional precursor protein kinase belongs to the serine/threonine kinase family.

To determine the phosphorylation sites exactly and to find a putative consensus motif in the chloroplast transit peptides phosphorylated preSSU was treated with the protease trypsin. This resulted in one radioactive labeled fragment (not shown). Sequencing of this peptide revealed that this fragment represents indeed a part of the preSSU transit sequence from tobacco. The fragment comprises amino acid 31–39 of preSSU. Three serines are present in this fragment which could serve as potential phosphate acceptors (Fig. 3). Two of these serines were exchanged for alanine by in vivo mutagenesis. The mutations affected serine 31 and serine 34 which were either separately resulting in the clones cpreSSU-M31/S/A and cpreSSU-M34/S/A or simultaneously yielding cpreSSU-M31/34/S/A (Fig. 3). The three mutated cDNAs were subsequently expressed in E. coli (Fig. 4A, lanes 1–4, C) and subjected to in vitro phosphorylation as before (Fig. 4A, lanes 1–4, 32P). The phosphorylation experiments of the mutated precursor proteins resulted in a reduced 32P incorporation into preSSU-M31/34/S/A (Fig. 4A, lane 2, 32P) and no incorporation into preSSU-M34/S/A and preSSU-M31/34/S/A (Fig. 4A, lanes 3 and 4, 32P). From these data we conclude that serine 34 is the actual phosphoacceptor in tobacco preSSU, whereas serine 31 might function in the recognition or binding of the kinase.

With the knowledge of this phosphorylation site we analyzed other transit sequences for homologies. A similar motif was found in preOE23. The respective serine 22 was exchanged for alanine resulting in cpreOE23-M22/S/A (Figs. 3 and 4B, lane 2, C). The phosphorylation experiment using the mutated protein clearly shows that preOE23-M22/S/A is not phosphory-
**Fig. 3. Amino acid sequence of chloroplast precursor proteins and the introduced mutations.** Amino acids are listed in the single-letter code. A, the marked box in preSSU-WT indicates the phosphorylated tryptic fragment which was sequenced. The amino acids which are involved in the formation of the putative consensus motif are underlined. TTD refers to the thylakoid transfer domain, mature refers to the mature part of the respective protein. B, a putative consensus motif for chloroplast precursor protein phosphorylation is shown. The asterisk marks the phosphorylation site. n represents a variable amino acid spacer.

**Fig. 4. Determination of the phosphorylation site in several chloroplast precursor proteins.** A, wild-type preSSU (lane 1) and preSSU-M3134-SA (lane 2), preSSU-M34-SA (lane 3), or the double mutant preSSU-M3134-SA (lane 4) were either subjected to SDS-PAGE and Coomassie Blue staining as indicated by the letter C at the bottom of the figure or to in vitro phosphorylation in the presence of cytoplasm as indicated by a 32P (autoradiogram) at the bottom of the figure. B, Coomassie Blue staining of wild-type preOE33 (lane 1) or the mutant preOE33-M22-SA (lane 2) in part C and the phosphorylation of the respective protein in part 32P. C, Coomassie Blue staining of expressed wild-type preOE33 (lane 1) and mutant preOE33-M21-T/A (lane 2) in part C and the phosphorylation of the respective protein in lane 1 and 2 of part 32P. D, phosphorylated preOE33-M21-T/A was excised from the gel and subjected to phosphoamino acid analysis. The positions of the phosphoamino acid standards phosphoserine (P-Ser) and phosphothreonine (P-Thr) are indicated beside the autoradiogram.

Determined any more indicating that serine 22 is indeed the phosphoacceptor amino acid in the pea preOE23 transit peptide (Fig. 4B, lane 2, 32P). A similar sequence homology was found in preOE33 (Fig. 3). From the phosphoamino acid analysis we knew that this protein is labeled on a threonine residue. Therefore we exchanged threonine 21 for alanine resulting in cpreOE33-M21-T/A (Fig. 3). The overexpressed protein (Fig. 4C, lane 2, C) was subjected to the kinase assay (Fig. 4C, lane 2, 32P). To our surprise the mutated protein was only slightly less phosphorylated than the wild-type precursor (Fig. 4C, compare lane 1, 32P and 2, 32P). This observation could be due to three reasons. First, the mutation did not affect the phosphoacceptor amino acid. Second, threonine 21 acts as phosphoacceptor group, but the kinase can also use another residue with a lower efficiency if threonine 21 is not available. And third, with the exchange of threonine 21 into alanine a new phosphorylation site was created which could also be used by the kinase with reduced efficiency. In order to address this question we analyzed the phosphoacceptor amino acid in preOE33-M21-T/A by acid hydrolysis and thin layer chromatography. As shown in Fig. 4D the mutated preOE33-M21-T/A was now exclusively phosphorylated on a serine in contrast to the wild type (Fig. 2D). From these data we conclude that threonine 21 is the phosphoacceptor group in pea preOE33. We cannot decide now whether the kinase is able to switch to serine phosphorylation or whether a new phosphorylation motif was created.

Altogether these results suggest a consensus motif in the chloroplast transit peptides which could serve as a putative recognition site for the cytosolic precursor protein kinase. We postulate that the motif consists of a turn-promoting residue (P/G) followed by a basic amino acid (R/K), an hydroxylated group (S/T) and finally by the actual phosphoacceptor amino acid (S/T) (Fig. 3). The spacing between this residue seems to be variable (see Fig. 3 and “Discussion”).

The Precursor Protein Kinase Is Present in Different Plant Sources—In order to find a suitable source for the enrichment of the cytosolic protein kinase and for other studies, a soluble extract from wheat germ was tested for its capability in chloroplast precursor phosphorylation. The wheat germ extract showed specific phosphorylation of all chloroplast precursor proteins tested (Fig. 2), but not of their mutated forms (not shown) and was therefore used as a starting material for purification (see “Materials and Methods”). Interestingly, rabbit reticulocyte lysate, which was also tested as a source, was not able to use chloroplast precursors as substrate for protein phosphorylation, suggesting that the kinase is a plant-specific enzyme (not shown). Irrespective of the source of the precursor protein kinase, i.e. wheat germ (Fig. 2), partially purified preparation (Fig. 5), or pea cytosol (Fig. 1), its specificity was established using various precursors and their mutated forms.

**Significance of Precursor Phosphorylation for Protein Import—**In a first approach import studies were conducted using the mutated preSSU-M34-SA which can not longer serve as a substrate for the precursor protein kinase. These experiments revealed that the mutated protein is imported into chloroplasts as efficient as the wild type preSSU indicating that the phosphorylation of the transit sequence is not prerequisite for the translocation event in vitro (Fig. 5B and not shown). Earlier results had, however, demonstrated that the phosphatase inhibitors NaF and NaMoO4 inhibit protein import into plastids in a reversible manner (Függe and Hinz, 1986; Schindler et al. 1987) raising the possibility that dephosphorylation of the precursor protein during the import process might indeed be a regulatory step.

Different experimental approaches were used to test this idea (Fig. 5, A and B). First, we wanted to know at what stage during import dephosphorylation of phosphorylated preSSU (P-preSSU) occurred, i.e. early during translocation at the envelope membranes or in the stroma before or during processing. To elucidate this a preSSU processing mutant was constructed and synthesized from the tobacco cDNA clone in analogy to a preSSU mutant described for pea (Archer and Keegstra, 1993). Due to an amino acid exchange (M54-R/D) close to the stromal processing site, this precursor is impaired in normal processing but yields an intermediate form (iSSU), which is about 2 kDa smaller than the precursor form. Import efficiency of the mutant preSSU is similar to wt-preSSU (Fig. 5A, lanes 5–8). The intermediate processing site in preSSU-M54-R/D is most likely NH2-terminal of the phosphorylation site in preSSU, which is

2 K. Waegemann, unpublished results.
at amino acid position 34. We reasoned that, if removal of the phosphate from preSSU would occur via or after processing, iSSU-M54-R/D should still be phosphorylated. To test this we used ([γ-32P]ATP)γS to label both wt-preSSU and preSSU-M54-R/D. ATP-γS is accepted as a phosphate donor by protein kinases but thiophosphorylated proteins are not or much more slowly dephosphorylated by protein phosphatases (McGowan and Cohen, 1988; MacKintosh, 1993). This seems also true in plants as thiophosphorylated preSSU is not dephosphorylated by a soluble protein extract from pea chloroplasts over a 60-min period (not shown).3

32P-Labeled wt-preSSU is imported into intact chloroplasts and processed, and the mature form appears protease-protected inside the organelles (Fig. 5A, lanes 5 and 6). The mutant 32P-labeled preSSU-M54-R/D is imported and processed with similar efficiency as the wt-preSSU, only processing yields an intermediate form (iSSU) (Fig. 5A, lanes 5 and 7 and 8). This intermediate form is also detected in the experiment using wt-preSSU but to a much lesser extent. In parallel assays nonradioactive preSSU and preSSU-M54-R/D, both labeled by phosphorylation with a partially purified kinase fraction from a wheat germ lysate and [γ-32P]ATP-γS prior to import into chloroplasts, were used. The thiophosphorylated forms of preSSU and preSSU-M54-R/D accumulated at the chloroplast surface in comparison to preSSU or preSSU-M54-R/D phosphorylated by ATP, presumably because complete translocation was inhibited or slowed down by the thiophosphate group in the precursor proteins. No labeled iSSU or iSSU-M54-R/D was detectable (Fig. 5A, lanes 1-4) inside chloroplasts, although the thiolabeled precursor cannot be dephosphorylated in the time course (5 min) of the import experiment (see above) (not shown). This indicates that the thiophosphorylated precursor had not yet reached the stroma for processing. In contrast, the chloroplast-bound preSSU and preSSU-M54-R/D yield two new distinct thiophosphorylated translocation intermediates (P-TimA and P-TimB) upon protease treatment (Fig. 5A, lanes 2 and 4). In addition the thiophosphorylated precursor forms of both proteins seem to be more protease-protected than the controls (Fig. 5A, lanes 6 and 8). P-TimA and P-TimB are recovered in the envelope membrane fraction and not in the soluble chloroplast protein (not shown). These data indicate that dephosphorylation of preSSU might be necessary for complete translocation into the stroma, i.e. if dephosphorylation is hindered by introduction of a thiophosphate group, the import process comes to an early stop shortly after binding.

To obtain further experimental evidence for this notion, we conducted import experiments in the presence of NaF, a broad range protein phosphatase inhibitor (Fig. 5B). Unlabeled wt-preSSU was phosphorylated by [γ-32P]ATP and a partially purified kinase fraction from wheat germ extracts (see "Material and Methods"). 32P-Labeled preSSU bound to chloroplasts in the absence or presence of NaF. Protease treatment of organelar bound precursors yielded 32P-labeled P-TimA and P-TimB. In the absence of NaF, 32P-labeled translocation inter-

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3 J. Soll, unpublished results.
mediates accumulate to about 10–20% of the NaF level (compare Fig. 5B, lanes 2 and 4). When we used thio phosphorylated preSSU in a standard import assay in the presence of NaF, binding of preSSU to chloroplasts increased, compared to that in the absence of NaF (Fig. 5B, lanes 5 and 7). Upon protease treatment P-TimA and P-TimB and protease protected precursor protein were detected in the absence (Fig. 5B, lanes 5 and 6) of NaF. In the presence of NaF the amount of protease protected preSSU and P-TimA and P-TimB increased similar to the increased amount of binding (Fig. 5B, lanes 7 and 8). The detection of P-TimA and P-TimB in the absence (Fig. 5, A and B) or presence (Fig. 5B, lanes 1–9) of NaF indicates that NaF per se does not deviate the precursor from its normal import to a bypass pathway. This is corroborated by earlier findings (Flüge and Hinz, 1986; Schindler et al., 1987). In a parallel experiment we used [35S]labeled wt-preSSU which had been incubated prior to the import with unlabelled ATP and partially purified kinase. In the absence of NaF, import occurred normally (Fig. 5B, lanes 9 and 10), while in the presence of NaF almost no import was detectable, and bound preSSU accumulated again (Fig. 5B, lane 11). Subsequent protease treatment resulted in the occurrence of P-TimA and P-TimB (Fig. 5B, lane 12, indicated by an arrowhead). The difference in labeling intensities of P-TimA and P-TimB in Fig. 5B, lanes 8 and 12, is due to (i) differences in the specific activity of the labeled educts, i.e. [γ-32P]ATP, γS and 35S-labeled preSSU and (ii) stoichiometric phosphorylation of preSSU in vitro, i.e. the import reactions presented in Fig. 5B, lanes 9–12, contain two different precursor proteins, one which is phosphorylated and one which is not. The nonphosphorylated wt-preSSU subpopulation probably gives rise to the additional translocation intermediates (Tim1–4) seen in Fig. 5B, lane 12. The yield of Tim1–4 in these experiments varied, probably due to the different ratio of phosphorylated to nonphosphorylated preSSU (a typical result out of five repeats is shown in lanes 9–12). Tim1–4 are most likely identical to deg 1–4, which have been described as translocation intermediates in the preSSU import pathway into chloroplasts for the reticulocyte lysate-synthesi

sized precursor protein (Waegemann and Soll, 1991). To test this we used the nonphosphorylatable mutant preSSU-M34S/A subjected it to a mock phosphorylation treatment and assayed its import characteristics in the absence or presence of NaF. In the absence of NaF, preSSU-M34S/A imports normally and is processed to the mature form (Fig. 5B, lanes 13 and 14). In the presence of NaF, preSSU accumulates at the chloroplast surface, and almost no import occurs. After a protease treatment, translocation intermediates appear that are not related to phosphorylation, namely Tim1–4 (Fig. 5B, lanes 15 and 16). Translocation intermediate homologues to P-TimA and P-TimB are not generated to a significant amount from preSSU-M34S/A. From these data we conclude that a phosphorylated chloroplast precursor protein cannot be imported into the organelle, but that dephosphorylation is required sometime after binding but before translocation.

The Cytosolic Precursor Protein Kinase Is Associated with the Chloroplast Outer Surface—Our initial results indicated that the chloroplast precursor specific protein kinase is a soluble enzyme in the cytoplasm (Fig. 6, lanes 1 and 2). Proteins exist, however, which have a dual localization, i.e. soluble and membrane-associated. We thus wanted to test whether a portion of the kinase is also bound to the chloroplast surface. For this purpose isolated intact pea chloroplasts were incubated with preSSU in the presence of [γ-32P]ATP. This experiment shows that chloroplasts are indeed able to phosphorylate preSSU (Fig. 6, lanes 3–6). However, only a small portion of the labeled precursor could be isolated together with the organelles after centrifugation (Fig. 6, lanes 3 and 4), whereas the main portion of 32P-labeled preSSU was recovered in the supernatant (Fig. 6, lanes 5 and 6). These results are consistent with the observation that the initial interaction of precursor proteins with the chloroplast surface is a reversible process (Olsen et al., 1989; Perry and Keegstra, 1994). Tight and irreversible binding of preSSU to the chloroplasts requires between 20 and 50 µM ATP (Olsen et al., 1989), concentrations which are about 10-fold higher than those used for the phosphorylation of preSSU, which was 2.5 µM. When the organelles were pre-

![Fig. 6. Localization of the protein kinase activity. A cytoplasm-enriched fraction from pea mesophyll cells (20 µg of protein) was incubated in the absence (lane 1) or presence (lane 2) of overexpressed preSSU with [γ-32P]ATP. A protein kinase assay was performed with isolated intact pea chloroplasts (equivalent to 15 µg of chlorophyll) in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of preSSU. After completion of phosphorylation, the organelles were resolated by centrifugation. Either the pellet, containing the intact chloroplasts (P) or the supernatant (S) was subjected to SDS-PAGE and autoradiography. Intact chloroplasts were pretreated without (lanes 7 and 8) or with (lanes 9 and 10) thermolysin (Th) (10 µg of Th/100 µg of chlorophyll) as indicated in the figure. After termination of the digestion with a 2-fold molar excess of α-macroglobulin, the organelles were resolated by centrifugation and subjected to in vitro phosphorylation in the presence (lanes 8 and 10) or absence (lanes 7 and 9) of preSSU. After completion of the reaction, the assays were divided into pellet and supernatant by centrifugation. The autoradiogram of the resulting supernatants (S) is shown. In vitro phosphorylation with chloroplast supernatant, which was prepared as described under “Materials and Methods,” was performed in the absence (lane 11) or in the presence (lane 12) of preSSU. The position of preSSU is indicated by an arrowhead in each autoradiogram.

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**Transit Sequence Phosphorylation**
have to be imported into the chloroplasts. The import is medi-
ated, while internal chloroplast protein kinases were not
affected, (Fig. 6, 12). The respective chloroplast
pellet had an residual activity of about 10% (not shown). These
data suggest that a portion of the precursor protein protein
kinase is in a loose association with the organelar surface.

To test the specificity of the association of this largely cyto-
solic enzyme with the chloroplasts, we analyzed the distribu-
tion of sucrose synthase, a cytosolic marker enzyme (Stitt and
Steup, 1985), with that of the protein kinase activity in iso-
lated cytoplasm and in chloroplasts. Quantification of the kinase
activity and sucrose synthase activity in the chloroplast super-
natant and in isolated cytoplasm shows that both fractions were
able to phosphorylate preSSU, but sucrose synthase activ-
ity could be detected exclusively in the cytoplasm (Fig. 7).
Therefore we conclude that the protein kinase is a cytosolic
enzyme which might specifically interact and adhere to the
chloroplast surface. The nature of this interaction remains to
be elucidated.

**DISCUSSION**

Most chloroplast proteins are synthesized in the cytosol and
have to be imported into the chloroplasts. The import is medi-
ated by an NH$_2$-terminal transit sequence which contains all of
the information necessary for translocation in vitro. However,
the transit sequences of the different chloroplast proteins are
very heterogenous in length and also in secondary structure.
Attempts to dissect the presequences into functional domains,
which are important for certain steps in protein import, have
been successfully conducted for some precursor proteins, i.e.
preSSU (Reiss et al., 1989) and preFd (Pilon et al., 1995). But

![Transit Sequence Phosphorylation](Image)

Fig. 7. **Protein kinase activity is associated with chloroplasts.** Protein kinase activity of cytoplasm or chloroplast supernatant (35 µg of protein, respectively) was determined by performing an in vitro phosphorylation experiment in the presence of preSSU and [γ-32P]ATP. Radiola
tilated preSSU was quantified in both reactions by basic densitometry of the autoradiograms. Sucrose synthase activity was measured in identical amounts (70 µg of protein) of both fractions as described under "Materials and Methods." Maximal enzymatic activity
of the protein kinase and sucrose synthase in the cytoplasm was set to
100%.

Unfortunately these motifs do not fit to the presequences of all
or most chloroplast precursors. On the way to find such a
common motif, we addressed the question whether a specific
phosphorylation in the transit sequence could be such a motif.
Here we have reported on the phosphorylation of five different
overexpressed chloroplast precursor proteins. Each of the pro-
cursors, i.e. preSSU, preFd, preLHCP, preOE23, and preOE33,
was labeled in the transit sequence. Precursors containing a
bipartite presequence, i.e. preOE23 and preOE33, are phos-
phorylated in the stromal targeting domain of the transit peptide
as indicated by the fact that iOE23 could not serve as a sub-
strate for the precursor protein kinase. Other precursor pro-
cursors with NH$_2$-terminal cleavable presequences, i.e. mito-
ochondrial preF$_1$β or peroxysomal preMDH, were not phos-
phorylated or like the MDH in the mature part of the protein. We
are currently trying to overexpress more mitochondrial preci-
sor proteins from plants to broaden the basis for this notion.

Phosphoamino acid analysis showed that the phosphoryla-
tion of the chloroplast transit sequences occurs on serine or
threonine residues. For preSSU, preOE23, and preOE33, the
phosphorylation site was determined exactly. Comparison of these
sequences revealed a loose motif for a consensus se-
quence which is defined by a turn-promoting residue (P/G)
followed by a spacer, a basic amino acid (R/K), a hydroxylated
residue (S/T), a spacer, and the actual phosphoacceptor amino
acids (S*/T*). The length of the spacer can vary from protein to
protein. The kinase belongs to the serine/threonine protein
kinase family and was shown to be a cytoplasmic enzyme which
might associate loosely with chloroplasts. The precursor pro-
cursor protein kinase was detected in phylogenetically different
plants, the dicotyledonous pea versus the monocotyledonous
wheat and in developmentally very different stages, i.e. pea
leaf mesophyll cells and wheat germ embryos. To date it is not
clear whether the chloroplast precursors are phosphorylated in
the cytoplasm during translation or before binding to the or-
ganelle, or if they are phosphorylated at the chloroplast surface
during initial phases of chloroplast-precursor interaction
(Keegstra et al., 1989).

Import studies using the nonphosphorylatable preSSU-M34
S/A revealed that the mutated proteins are imported into chlo-
roplasts as efficient as the wt-preSSU, indicating that phos-
phorylation of the transit sequence is not prerequisite for the translo-
cation in vivo. However, if phosphorylation of plastid-
destined precursor proteins would be complete during or after
translation in vivo, then dephosphorylation by a protein phos-
phatase could be a regulatory step in the import process, i.e. a
phosphorylated precursor would not be translocated into chlo-
roplasts. This is not without precedent in posttranslational
protein translocation. It has been reported that the nuclear
import of several proteins, e.g. lamin B$_2$, SV40 T-antigen, and
yeast transcription factor SW15 (Hennekens et al., 1993; Jans
et al., 1991; Moll et al., 1991; Rihs et al., 1991), is inhibited by
phosphorylation of a serine or threonine residue near the nu-
clear localization signal. Only upon dephosphorylation are
these proteins imported into the nucleus. To evaluate such a
possibility, we used two approaches. First, thiophosphorylated
precursor proteins were used for import. The thiosphosphate
group is not or only very slowly released from the phosphopro-
tein by all known protein phosphatases (McGowan and Cohen,
1988; MacKintosh, 1993). In addition we used preSSU-M54
R/D, a precursor form that imports normally but is aberrantly
processed. The processing site of preSSU-M54-R/D is most
likely NH$_2$-terminal to the phosphorylation site; however, no
thiosphosphorylated imported ISSU-M54-R/D was detectable in
standard import assays. The thiosphosphorylated preSSU-M54
R/D bound normally to intact chloroplasts and became partially
protease-protected, indicating that it had moved into the translocation machinery. The phosphorylated translocation intermediates P-TimA and P-TimB are different in size to translocation intermediates (deg 1–4) described before for wt-preSSU, which was synthesized in a reticulocyte lysate system (Waegemann and Soli, 1991). The translocation intermediates deg 1–4, which are identical to Tim1–4, thus seems to originate from the nonphosphorylated precursor form, e.g., after dephosphorylation (this study, Fig. 5B, lanes 12 and 16) (Waegemann and Soli, 1991). Tim1–4 are most prominent in import experiments in the presence of preSSU-M34-S/A, the nonphosphorylatable mutant, corroborating this idea.

Furthermore, P-TimA and P-TimB are detected from a wt-preSSU phosphorylated either by \([\gamma-32P]ATP\) or by phosphorylation with \([\gamma-32P]ATP\). The chloroplast-bound \([32P]\)preSSU yields P-TimA and P-TimB upon protease treatment, demonstrating that the thio-phosphate group did not result in an artificial translocation intermediate. P-TimA and P-TimB are also detected from organelar bound \(^{35}S\)-labeled preSSU phosphorylated with unlabeled ATP in the presence of NaF upon protease treatment. NaF is a protein phosphatase inhibitor, which had been shown before to reversibly inhibit preSSU import into chloroplasts (Flügge and Hinz, 1986; Schindler et al., 1987). Since in vitro phosphorylation of precursor proteins is substoichiometric, P-TimA and P-TimB appear in combination with the nonphosphorylated Tim. Hence it is not possible to experimentally follow the dephosphorylation of preSSU and the concurrent appearance of SSU. It remains to be established that a cycle of precursor phosphorylation-dephosphorylation is a general and essential part of the import process in vivo. Other possibilities have also to be considered, e.g., that this process could represent a regulatory phenomenon, which is activated only under certain developmental or environmental conditions to control precursor protein import into plastids.

The precursor protein protein phosphatase is most likely localized in the outer envelope membranes since P-TimA and P-TimB are membrane-bound early translocation intermediates. The precursor protein protein phosphatase would represent a new yet to be identified component of the chloroplast import machinery. Preliminary experiments to classify the protein phosphatase using specific inhibitors failed. Calyculin and microcystin, potent inhibitors of PP1 and PP2A (Mackintosh and MacIntosh, 1994), were without influence on preSSU import. NaMoO₄ (40 mM) had the same inhibitory effect as NaF, while vanadate (1 mM) (Hunter, 1995) was without influence. To test for PP2B- or PP2C-specific activities is more complex in a crude system (Shendikar, 1994) and was not tried in this study.

NaF seems to inhibit an additional step in the import pathway downstream to dephosphorylation of the precursor protein since preSSU-M34-S/A is not completely imported in the presence of NaF. Precursor import into chloroplasts requires nucleoside triphosphates at various steps in the translocation pathway, e.g., for binding (Olsen et al., 1989) or for pulling the precursor across the membrane by the ATP-dependent action of hsc70 (Stuart et al., 1994). Furthermore, key components of the chloroplast outer envelope import machinery, i.e. OEP86 (Hirsch et al., 1994) and OEP34 (Seedorf et al., 1995; Schnell et al., 1994), are prominent phosphoproteins of this membrane. Their activity might be regulated by a phosphorylation-dephosphorylation cycle (Soll, 1995), which could also be influenced by NaF and result in the inhibition of complete preSSU-M34-S/A translocation into chloroplasts. One of these events or one yet to be identified process seems also be inhibited by NaF and impairs import.

**Protein phosphatases do not merely reverse the effect, which is provoked by phosphorylation, but are highly regulatory enzymes themselves (Mumby and Walter, 1993; Hunter, 1995). Therefore, the function(s) of a precursor protein protein phosphatase might be to regulate or modulate precursor uptake in dependence of the chloroplast import competency, e.g., energy or reduct state. Another possibility might be the developmental regulation of import into chloroplasts during chloroplast differentiation and maturation. It has been reported that mature chloroplasts from fully expanded pea leaves lose their competency to import precursor proteins, while chloroplasts from young, rapidly expanding leaves showed highest import rates (Dahlin and Cline, 1991). Another possibility would be that the precursor protein protein kinase is not always in an activated state in vivo, i.e. a nonphosphorylated precursor could short-circuit the protein phosphatase. It is clear, that much remains to be learned on the exact purpose of this regulatory circuit in protein import into chloroplasts.**

In conclusion phosphorylation of chloroplast destined precursors in the cytosol in combination with their dephosphorylation at the chloroplast envelopes might represent one step in a cascade of events which ultimately lead to specific sorting and translocation to plastids in plant cells.

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Transit Sequence Phosphorylation

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