The Chloroplast Protein Import Receptors Toc34 and Toc159 Are Phosphorylated by Distinct Protein Kinases*

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The molecular composition of chloroplast outer and inner envelope translocons is fairly well established, but little is known about mechanisms and elements involved in import regulation. After synthesis in the cytosol, chloroplast targeted precursor proteins are recognized by outer envelope receptors Toc34 and Toc159. Phosphorylation plays an important role in regulation of Toc34 activity and preprotein binding. Using kinase renaturation assays, we have identified an ATP-dependent 98-kDa outer envelope kinase which is able to selectively phosphorylate Toc34 at a specific site. A 70-kDa outer envelope polypeptide phosphorylating Toc159 was identified by the same strategy. Antiserum against the 98-kDa kinase inhibits phosphorylation of Toc34, whereas labeling of Toc159 remains unaffected. Both kinases do not autophosphorylate in vitro and are unable to utilize myelin basic protein as substrate. We propose that distinct kinases are involved in regulation of chloroplast import via desensitization of preprotein receptors.

During evolution, chloroplasts have transferred the majority of their genes to the nucleus (1). This gene transfer required the genesis of novel regulatory and targeting mechanisms, which enabled the nuclear encoded proteins an efficient and faithful return to the organelle. In recent years, evidence has accumulated that protein phosphorylation and protein kinases play an important role in regulation and fine-tuning of protein translocation into various subcellular compartments. Upon translation in the cytosol, chloroplast, but not mitochondrial or peroxisomal, preprotein targeting sequences are phosphorylated by a specific protein kinase (2). Phosphorylated preproteins engage an oligomeric guidance complex consisting of a 14-3-3 protein dimer and a cytosolic Hsp70 (3). Enclosed within this complex, preproteins represent highly import-competent substrates. Translocation is initiated by tethering of the guidance complex to the chloroplast outer envelope (3, 4). Recognition and binding of preproteins is performed by two integral receptor subunits of the translocon at the outer envelope of chloroplasts (Toc complex) Toc159 and Toc34 (5). Both proteins have distinct nucleotide binding sites. Phosphorylation appears to play a role in the regulation of Toc34 receptor function (6). Direct interaction between unphosphorylated Toc34 in its GTP-bound state and the precursor form of ribulose-bisphosphate carboxylase/oxygenase small subunit could be demonstrated in vitro (6). In the phosphorylated state, Toc34 cannot bind GTP, with a concomitant strong decrease in its capacity to interact with precursor proteins (6). Little is known about the molecular mechanisms involved in regulation of Toc159. Recent evidence suggests, however, that the initial binding of at least some preproteins by Toc159 can be bypassed (7), pointing toward different specificities of the receptor subunits or indicating that Toc34 acts as a key receptor.

It has been demonstrated that phosphorylation specifically down-regulates the nuclear import pathways mediated by importin B and transportin (8). Target of this regulation is most probably a nucleoporin, rather than some other receptor or soluble component playing a role in nucleocytoplasmic trafficking. Further evidence for the involvement of protein phosphorylation in regulation of translocation into other cellular compartments exists (9), and several soluble protein kinases have been proposed to play a role in these processes (10, 11). Membrane-bound, or even peripherally associated, protein kinase activities that could account for phosphorylation of translocation machinery components remain enigmatic.

Although Toc34 and a 86-kDa proteolytic fragment of Toc159 are among the most prominently phosphorylated outer envelope proteins, several additional phosphoproteins have also been reported (12), and attempts have been made to purify and characterize enzymes involved in the phosphorylation (13, 14). Complex phosphorylation pattern of outer envelope polypeptides and requirements for both ATP and GTP implicated the existence of multiple protein kinases associated with this membrane. Indeed, a GTP-dependent phosphorylation was proposed to be mediated by an envelope kinase of the casein kinase II-type (14), whereas an ATP-dependent 70-kDa kinase was shown to operate independent of cyclic nucleotides, calcium, or calmodulin (13). The biological role and significance of various phosphoproteins as well as the substrate specificity of involved kinases remained, however, elusive.

In this study, we present evidence for the existence of a chloroplast outer envelope ATP-dependent protein kinase, which can phosphorylate Toc34 receptor with high specificity. The 98-kDa kinase behaves as an integral membrane protein, and we propose it is a bona fide candidate for the regulator of this import receptor. Further, we identify Toc159 as a target of a distinct 70-kDa protein kinase activity, which does not phosphorylate Toc34. We conclude that protein import into chloroplasts could be regulated via two distinct protein kinases.

EXPERIMENTAL PROCEDURES
Standard Methods and Procedures—The bacterial strains TOP10 and BL21(DE3) were from Invitrogen (Groningen, The Netherlands). All chemicals used were from Roth (Karlsruhe, Germany) or Sigma
Expression and Purification of Recombinant Proteins—Toc34ATM5-His was overexpressed and affinity purified on Talon metal affinity column (CLONTECH, Heidelberg, Germany) as described in Ref. 6. Purified Toc34ATM5(S113A) was a generous gift from M. Jelic and E. Schleiff. Recombinant Toc75 was produced from pETT4–6His–Toc75 and purified according to Ref. 16.

Protein Purification and Microsequencing—Proteins were electrophoretically separated over the entire width of slab gels. After staining with Coomassie, individual protein bands were excised, rinsed with water, equilibrated in buffer (0.125 m Tris-HCl, pH 6.8, 1 mm EDTA, 0.1% SDS), and electroeluted. For internal sequencing, the protein was digested in the gel with protease LysC and peptides were separated by reverse-phase high performance liquid chromatography.

Peptide Synthesis, Coupling, and Antibody Production—A peptide was synthesized according to the obtained internal sequence of Oek98 with addition of a cysteine residue at the carboxyl terminus. Two hundred and fifty mg of thiol-activated Sepharose (Sigma) were washed three times in buffer A (20 mM NaPO4, pH 7.0, 150 mM NaCl, 1 mM EDTA). Four mg of peptide, dissolved in 500 mM of buffer A, were added to the purified resin and incubated overnight at 4 °C with agitation. This resin was used for four consecutive steps of rabbit immunization. The serum was purified with pulverized Escherichia coli proteins to reduce cross-reactivity.

Phosphorylation of Outer Envelope Proteins—Ten μg of outer envelope vesicles were resuspended in 50 μl of reaction buffer (10 μM ATP, 5 mM MgCl2, 0.5 MnCl2, 20 mM Tricine–KOH, pH 7.6), supplemented with 2 μCi of [γ-32P]ATP, and incubated at room temperature for 15 min. The reaction was stopped by addition of Laemmli loading buffer, and proteins were resolved on SDS-PAGE. Phosphorylation with [γ-32P]GTP was performed in the same manner. Radioactively labeled polypeptides were detected by autoradiography using x-ray films (Kodak X-Omat or a Fuji FLR-9000 phosphorimagener).

Immunoprecipitation of Radiolabeled Toc159—Fifty μg of outer envelope proteins were phosphorylated as described above. Membranes were solubilized with 2% SDS, boiled for 2 min, and diluted 10 times with IP buffer (50 mM Tris-HCl, pH 7.5, 80 mM NaCl, 2 mM EDTA, 1% Nonidet P-40). Antiserum against purified 86-kDa fragment of Toc159 was added in a ratio of 1:150 and incubated for 2 h at room temperature. One hundred μl of Protein A-Sepharose (Amersham Biosciences) was added and incubated for 3 h under constant agitation. The antibody-Sepharose conjugate was pelleted and washed three times with IP buffer and once again with IP buffer without Nonidet P-40. Proteins were released from the matrix by adding Laemmli loading buffer, boiling for 2 min, and finally analyzed by SDS-PAGE. Radioactively labeled proteins were detected by phosphorimager.

Kinase Renaturation Assays—Renaturation of kinase activity was carried out according to a modified protocol from Ref. 17. Outer envelope vesicles were separated in 12.5% SDS-PAGE and electroblotted onto a PVDF membrane (Hybond-P, Amersham Biosciences). The membrane was then incubated in denaturation buffer (7 mM guanidine HCl, 50 mM Tris-HCl, pH 8.3, 50 mM dithiothreitol, 2 mM EDTA). After rinsing in washing solution 1 (30 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 140 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA, 5 mM MgCl2, 40 μM CaCl2, 1% (w/v) BSA, 0.1% (v/v) Nonidet P-40). Buffer was supplemented with either 0.05% (v/v) casein (5% dephosphorylated solution, Sigma) or phosphovit (w/v). Specific substrates and synthetic peptides were supplemented in the amount of 20 ng/cm2 of membrane. During this step the kinase substrate bound to the membrane. The membrane was then incubated with blocking solution (5% (w/v) BSA, 30 mM Tris-HCl, pH 7.5) in which 1.2% unspecific or 50 ng/cm2 specific substrates were supplemented. Phosphorylation was performed for 1 h at room temperature in reaction buffer (30 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 80 mM CaCl2, 100 μCi of [γ-32P]ATP). Upon completion, the membrane was washed twice with washing solution 1, once with washing solution 2 (30 mM Tris-HCl, pH 7.5, 0.05% (v/v) Nonidet P-40), and again in washing solution 1. Loosely bound (<2 μCi) of 32P]ATP was removed by incubation of the membrane in 1 mM KOH for 10 min. The membrane was neutralized with washing solution 1, air-dried, and exposed to x-ray film or a phosphorimaginer plate.

For in-gel renaturation, outer envelope extracts containing 10 μg of protein were fractionated in an 12.5% SDS-PAGE containing 0.25 mg/ml myelin basic protein (MBP). Protein denaturing, renaturing, and kinase activity assays in the gel were performed as described (18).

Precursor Binding to Phosphorylated Toc34—Twenty μg of outer envelope proteins were separated in 10% SDS-PAGE and electrophoresed onto PVDF membrane. Membrane strips containing Oek98 were excised, and the kinase protein activity was renatured as described above. Stripes were then saturated with blocking solution containing either Toc34ATM or Toc34ATM(S113A) at concentration of 17 μg/ml, and phosphorylation was performed as described using 1.25 μCi of ATP. Blocking solution was removed by a short wash with washing solution 1, and the controls were incubated with in vitro translated [35S]methionine and [35S]cysteine (1.17 Ci/mmol) labeled precursor of ribulose-biphosphate carboxylase/oxygenase small subunit (preSSU) in binding buffer (20 mM Tris, pH 7.5, 120 mM NaCl, 1.25 μM ATP, 5 mM MgCl2, 1 mM GTP, 0.5 mM EDTA). After 20 min of incubation at 22 °C, unbound preSSU was removed by two rounds of extensive washing with the binding buffer. The amount of bound preSSU was quantified by scintillation counting.

Control experiments were performed in the same manner, except the kinase activity of Oek98 has not been restored by the renaturation step.

Quantification of the Phosphorylation—Phosphorylation was quantified from phosphorimagering data using Aida Image Analyser version 3.10 analysis software (Raytest, Straubenhardt, Germany).

RESULTS

Preprotein Receptor Toc159 Is a Phosphoprotein—Certain proteins contained within chloroplast outer envelope membranes can be phosphorylated by endogenous kinase(s) in vitro. At least a dozen different phosphoproteins can be phosphorylated on a 12.5% SDS-polyacrylamide gel and visualized by autoradiography following a 15-min incubation with [γ-32P]ATP (Fig. 1A; Ref. 12). The most conspicuously phosphorylated proteins are in the molecular mass range of 86, 34, and 16 kDa. The 34-kDa phosphoprotein has recently been identified as the preprotein import receptor Toc34. Labeling in the presence of [γ-32P]GTP

![Image](https://via.placeholder.com/150)

FIG. 1. Phosphorylation of outer envelope proteins. A, ATP is a more efficient phosphorydor for phosphorylation of Toc34 and a 86-kDa polypeptide then GTP. The phosphorylation of 10 μg of outer envelope proteins was determined as described under “Experimental Procedures,” followed by autoradiography. Left, positions of molecular mass standards in kDa. B, immunoprecipitation (IP) of phosphorylated Toc159 and its major 86-kDa proteolytic fragment using an antiserum against purified 86-kDa polypeptide. Toc34 is also precipitated as a result of sequence similarity. C, phosphopabeling of the 86-kDa fragment of Toc159 is resistant to alkali and acidic treatments. Phosphoproteins were transferred onto PVDF membrane and treated for 60 min in 0.5 M Tris-HCl, pH 8.8, 1 M NaOH, or 0.5 M HCl at 65 °C respectively, and subsequently autoradiographed.
resulted only in one prominent phosphoprotein above 100 kDa, whereas the 86-, 34-, and 16-kDa proteins are much more weakly labeled (Fig. 1A).

The possibility that the 86-kDa polypeptide could be a proteolytic fragment of the preprotein import receptor Toc159 prompted further investigations. We have previously established (19) that antibodies raised against a purified 86-kDa protein, formerly designated Toc86 (20), efficiently immunoprecipitate Toc159 from the outer envelope preparations. The serum recognizes proteolytic fragments of Toc159 as well as Toc34 (19). The latter is caused by a stretch of high sequence similarity around the putative nucleotide binding domain (15, 20, 21). Immunoprecipitation was performed after phosphorylation of 50 μg of outer envelope proteins with [γ-32P]ATP. The immunopellet contained mostly the phosphorylated 86-kDa proteolytic fragment of Toc159 and little intact receptor (Fig. 1B). Phosphorylated Toc34 could also be detected. Toc159 is most probably labeled on serine or threonine residues, because phosphorylation proved to be resistant to both alkali or acid treatments (Fig. 1C). This is consistent with previous results, which demonstrated that the prevailing labeling of outer envelope proteins is on serine or threonine residues (13).

Functional Renaturation of Outer Envelope Kinases—To identify the envelope-localized Toc159 and Toc34 kinase(s), a renaturation assay was performed using SDS-PAGE separated proteins. Twenty μg of outer envelope proteins were resolved on 12.5% SDS-PAGE and transferred onto PVDF membrane. First we performed renaturation assays in the presence of the unspecific kinase substrates casein and phosvitin, using both [γ-32P]ATP and [γ-32P]GTP as phosphodonors. After extensive washing of unbound or loosely bound nucleoside triphosphate and denaturation with KOH, only those components that are able to transfer the phosphate group to itself or the surrounding substrate can be detected. No kinase activity could be detected in the outer envelopes under these conditions (Fig. 2A). This result indicated either that the renaturation assay failed or that the outer envelope kinases have a high substrate specificity. A positive control renaturation (Fig. 2A), performed with commercially available catalytic subunit of bovine heart protein kinase, favors the latter idea.

Therefore, heterologously expressed and purified Toc34 (Toc34ΔTM) was used as a substrate. This protein lacks the transmembrane domain at its COOH terminus, but contains the cytosolic nucleoside triphosphate binding domain as well as the proposed phosphorylation site. After overexpression, the protein was purified to near homogeneity by affinity chromatography on a Talon affinity matrix (Fig. 2B) and used as a substrate in kinase renaturation assays.

 Autoradiography revealed a single protein band with an apparent molecular mass of 98 kDa (Fig. 2C) in the presence of [γ-32P]ATP but not [γ-32P]GTP as the phosphodonor. This correlates with the results of the in vitro phosphorylation assays (Fig. 1A). We conclude that Toc34 is phosphorylated in an ATP-dependent fashion by a putative kinase of 98 kDa molecular mass. To further address the substrate specificity of the 98-kDa kinase, we then asked if this polypeptide is able to phosphorylate Toc159, specifically the 86-kDa proteolytic fragment, which itself is an excellent phosphorylation substrate (Fig. 1A). The 86-kDa peptide was prepared from high resolution SDS-PAGE of separated outer envelope proteins (Fig. 2D). The polypeptide was electroeluted from gel slices, concentrated, and used as a substrate in renaturation assays. The 98-kDa kinase failed to phosphorylate the substrate and was accordingly not detected. Instead, an additional 70-kDa protein was elicited (Fig. 2C). Again, only ATP but not GTP can serve as a phosphodonor. Toc75, the preprotein translocation channel and most abundant translocon subunit, however, failed to serve as a substrate for both renatured protein kinases (Fig. 2C). This further indicates the specificity for the preprotein receptors Toc34 and Toc159. We designated the two identified protein kinase activities OEK98 and OEK70, for outer envelope kinase of 98 and 70 kDa, respectively.

OEK98 Can Efficiently Recognize the Toc34 Phosphorylation Site—The phosphorylation site of pea Toc34 was determined and shown to be positioned at serine 113. The amino acid sequence around Ser-113 does not resemble any other canonical phosphorylation motif found in the PROSITE data base. This indicates that the Toc34 kinase may not belong to a conventional protein kinase class. To further test OEK98 selectivity and to establish the minimum motif requirements for phosphorylation, we used a synthetic peptide matching the sequence of the phosphorylation site. The peptide (DMALNIIKSFLDLKT) contains Ser-113 at position 9, surrounded by a hydrophobic pocket. Kinase renaturation assays supplemented with the peptide revealed that OEK98 can efficiently phosphorylate this substrate, whereas OEK70 activity remained undetected (Fig. 3). To exclude the possibility that phosphorylation occurred unspecifically, we offered an arbitrary peptide (TSKYTSVLPGORVLC), containing two serine residues at

2 E. Schleiff and J. Soll, unpublished data.
OEK98 and OEK70 Are Probably Not Mitogen-activated Protein (MAP) Kinases—Regulation of protein translocation into chloroplasts can be envisioned as a part of a signal transduction cascade, linking the organelle with cytoplasmic and nuclear events. Certain members of the MAP kinase family have been implicated in regulation of protein trafficking between the nucleus and the cytoplasm (10). The intriguing possibility of OEK98 and OEK70 being related to MAP kinases prompted us to investigate whether they can phosphorylate MBP, a specific substrate for this protein kinase subclass. For this purpose, in-gel renaturation assays were carried out. Both OEK98 and OEK70 failed, however, to phosphorylate MBP under given conditions, suggesting that they are probably not related to the members of MAP kinase cascade (Fig. 3).

OEK98 Is an Intrinsic Membrane Protein—To test if OEK98 is an integral or peripheral membrane protein, outer envelope membranes were extracted using different salt and urea treatments. Soluble and insoluble proteins were separated by centrifugation, and kinase activity was assayed by renaturation experiments using Toc34ATM as a substrate. In every case we observed the OEK98 activity exclusively in the membrane fractions (Fig. 4A). This strongly suggests that OEK98 is an integral membrane protein. The membrane association properties of OEK98 are comparable with those of Toc64 (Fig. 4A), an integral component of the outer envelope translocon (4). The Toc34 phosphorylation site is located within a large hydrophilic, cytoplasmic-exposed domain. It is therefore reasonable to assume that the catalytic domain of OEK98 is also oriented in the similar manner. To test this assumption, outer envelope membranes, which are isolated as right-side-out vesicles (22) were incubated with increasing concentrations of trypsin and membrane fractions were separated from the supernatant by centrifugation. Activity of OEK98 was subsequently assayed by kinase renaturation in the presence of Toc34ATM. Low concentrations of trypsin resulted in the accumulation of a major 55-kDa proteolytic fragment, which still exhibited kinase activity (Fig. 4B, lane 2). An additional minor band at ~60 kDa could also be detected. Treatments with higher protease concentrations diminished the kinase activity present in the membrane fraction with simultaneous occurrence of a soluble 14-kDa fragment still possessing kinase activity (Fig. 4B, indicated by an arrowhead). After proteolysis with 10 ng of trypsin, the phosphorylation activity was completely abolished in both the membrane and the soluble fractions. Together, our data indicate that the kinase domain of OEK98 is exposed to the cytosol and can be released by protease treatment. Both membrane extraction and protease digestion experiments were performed for OEK70 membrane association properties; however, kinase activity could not be restored after any of these treatments (data not shown).

An Antiserum against OEK98 Can Inhibit Toc34 Phosphorylation—To obtain further information about the OEK98 identity, we purified the protein from outer envelope preparations and subjected it to microsequencing. The position of OEK98 protein was identified in high resolution SDS-PAGE, the protein was excised and enriched. To assess the purity and homogeneity of the sample, extracted protein was further resolved onto high resolution acrylamide gels. The kinase activity of isolated protein was checked by renaturation. The resulting polypeptide band was digested with the protease LysC, and
Immunodetection was performed using ECL reaction. Inner envelopes (IE) were separated on a 12.5% acrylamide running gel. Molecular mass standards in kDa.

The serum (anti-OEK98) was used for immunoblot analyses of outer envelope preparations. The serum was used for production of a synthetic peptide. The peptide, containing an additional cysteine at the carboxyl terminus, was then coupled with thiol-Sepharose resin and used as an antigen. The serum (anti-OEK98) was directly added to the reaction in a ratio of 1:100 (data not shown). These data further substantiate the concept of two kinase receptors.

Because conventional phosphorylation assays cannot distinguish between substrate phosphorylation and possible kinase functional properties of Toc159 are regulated in a similar manner.

**DISCUSSION**

Phosphorylation of Toc34 at Ser-113 Decreases Preprotein Binding—To establish the functional link between phosphorylation of Toc34 at Ser-113 and the preprotein recognition, we used renatured OEk98 activity to phosphorylate Toc34 and then analyzed the binding of radiolabeled preSSU. Nonphosphorylatable Toc34ΔTM(S113A) was used in a control experiment. When the OEk98 activity is not re-established by renaturation, both Toc34ΔTM and Toc34ΔTM(S113A) have comparable affinity for the preSSU (Fig. 7). This demonstrates that the exchange of serine 113 to alanine does not change Toc34 structure in a way that would influence its ability to recognize and bind precursor protein. Upon phosphorylation of Toc34ΔTM by OEk98, the binding of preSSU is reduced by ~50% as compared with Toc34ΔTM(S113A) (Fig. 7). This is a strong indication that phosphorylation of Toc34 at Ser-113 has a specific effect on Toc34 function, namely the preprotein binding.
autophosphorylation, they are not suitable in the search for kinases. They can also create a considerable amount of confusion and ambiguity as to the real identity of kinase substrates (25). Therefore, we have employed functional renaturation assays in a search for outer envelope kinases. This approach has so far been successfully used in several investigations of other chloroplast protein kinases (25, 26). Renaturations in the presence of unspecific substrates failed to show kinase activity in outer envelopes, but simultaneously demonstrated that, at least in vitro, no protein from this chloroplast subfraction is able to autophosphorylate. Previous studies (13) also demonstrated that protein kinases of outer envelopes are selective in substrate specificity. Casein, phosvitin, and histones II-S were poorly accepted, or not at all. This observations and our findings prompted us to use more specific substrates. Subsequently, the activity of a 98-kDa kinase could be restored to detectable levels only in the presence of Toc34 as the substrate and ATP as the phosphodonor. Similarly, the Toc159 kinase is elicited only when we offer the 86-kDa peptide as a substrate and ATP as a phosphodonor.

Several lines of evidence indicate the substrate specificity of OEK98 and OEK70. First, none of the tested unspecified substrates could be utilized by the kinases. Also, specific substrates could not be interchanged between the kinases and therefore, each renaturation assay possessed an build-in control experiment. Second, recombinant Toc34 protein harboring a modified phosphorylation site was not accepted as a substrate. Furthermore, a synthetic peptide corresponding to the determined phosphorylation site in Toc34 was efficiently utilized. On the other hand, an arbitrary peptide or Toc75, containing several serine residues, could not be used as an alternative substrate. Third, antibodies generated against a peptide derived from an internal peptide sequence of OEK98 efficiently blocked phosphorylation of Toc34, whereas labeling of Toc159 remained unaffected. However, the latter experiment must be interpreted with caution because we have not used Fab fragments and therefore cannot rule out unspecific cross-linking. Additionally, it is possible that OEK98 is in closer proximity to Toc34 than to Toc159, so the inhibitory effect could be more pronounced for Toc34.

The active kinase domain is located in the cytoplasmically exposed region of OEK98 and can be released as a 14-kDa soluble peptide. As an intermediate in this proteolytic degradation pattern, we observed a 55-kDa fragment, which was still membrane-bound but seemed to have increased enzymatic activity. It is tempting to speculate that this fragment contains the catalytic domain that is rendered into a more active state because of the lack of a regulatory domain, which has been digested away. Similar examples of increased catalytic activity upon proteolysis exist in other systems and usually lead to a constitutive activation of the kinase (27–29). Already, the size of OEK98 could be an indicator of its receptor-kinase nature (30). Receptor-protein kinases in this molecular size range have been reported previously in plant cells (31); however, this is the first report of high molecular weight protein kinases being associated with photosynthetic organelles. However, one argument that speaks against a possible receptor-protein kinase nature of both OEK70 and OEK98 is an apparent lack of their autophosphorylation activity. This has also been noticed in previous studies describing the 70-kDa outer envelope kinase (13).

Although both kinases failed to phosphorylate myelin basic protein, and are therefore unlikely members of the mitogen-activated protein kinase cascade, they could still be a part of a different signal transduction circuit, linking chloroplasts with the cytosol and the nucleus. Located at the outer surface of chloroplasts, OEK98 and OEK70 could lie upstream of various signal transduction pathways, synchronizing protein import into organelles with translation and, eventually, transcription of nuclear encoded plastid proteins.

The inactivation of import receptors by phosphorylation suggests that cells have evolved mechanisms to regulate almost the entire chloroplast protein transport pathway. The preprotein recognition of both Toc159 and Toc34 could be differentially regulated via OEK70 and OEK98. Recently, it has been shown that Toc34 is most probably a major preprotein receptor, because the recognition capacity of Toc159 could be bypassed in vitro (7). Phosphorylation can have a profound effect on the biochemical function, selectivity, and sensitivity of the import receptors. It is possible that the functional state of the kinases can be adopted by reception of regulatory signals coming from either the organelle or the cytosol, depending on the overall requirements of the cell. The nuclear encoded plastid proteins may require different import rates, depending on the stage of plastid development, physiological states of the cell, or conditions under which photosynthesis is performed. The process of desensitization, which requires phosphorylation of a receptor rendering it insensitive for a given agonist (32), may be responsible for granting certain proteins a higher order of import priority. Polypeptides that are less important for the proper chloroplast function under, for example, rapidly changing environmental conditions, would therefore not interfere with the transport of essential components. Such a mechanism could be concerted with short term adaptation mechanisms (33), maintaining a steady state of multisubunit photosynthetic complexes before transcription and translation processes are re-modulated to meet a new demand. The kinases could themselves be perceptive for particular protein requirements or could be regulated by a still unknown mechanism.

The identification of auxiliary components involved in import regulation and, in particular, elucidation of the signal transduction mechanisms connecting import with physiological responses are important emerging topics of current research in the protein translocation field.

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