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Transfer of a chloroplast-bound precursor protein into the translocation apparatus is impaired after phospholipase C treatment

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We have studied the influence of phospholipase C treatment of intact purified chloroplast on the translocation of a plastid destined precursor protein. Under standard import conditions, i.e. in the light in the presence of 2 mM ATP translocation was completely abolished but binding was observed at slightly elevated levels. An experimental regime which allowed binding but not import of the precursor protein, i.e. in the dark in the presence of 10 μM ATP, demonstrated that translocation intermediates, normally detected at this stage, were missing in phospholipase treated chloroplasts. The precursor was completely sensitive to protease treatment, indicating that the transfer of the precursor from the receptor to the import apparatus was blocked by phospholipase treatment.

Protein import; Phospholipid; Phospholipase C; Chloroplast; Import receptor

1. INTRODUCTION

Most plastid proteins are coded for on nuclear genes, synthesized in the cytosol and subsequently transported in a post-translational event into the organelle [1,2]. To analyse and characterize the mechanism by which a precursor protein is translocated into the organelle it is necessary to define single steps in the import route [2,3]. By studying the nucleoside triphosphate dependency of protein translocation it has been possible to dissect the import process into two parts. Low amounts of ATP (5–100 μM) are necessary for binding [4,5], a condition which results simultaneously in the occurrence of protease-protected translocation intermediates [6,7], i.e. the precursor has moved partially into the outer envelope localized import apparatus. Higher concentrations of ATP (0.2–2 mM) are then necessary to chase the precursor protein localized in the import apparatus into the inside of the organelle [8–11].

The possible function of lipids in the import process has been implied in the case of the transport of apocytochrome *c* across the outer mitochondrial membrane [12,13]. Furthermore, it was demonstrated recently that fragments representing different parts of the transit peptide of pSSU interact specifically with lipids typically present in the outer chloroplast envelope [14], thus indicating that transit peptide–lipid interactions could also be involved in certain events in a productive chloroplast protein import pathway.

In this work we have evaluated the effect of phospholipase C treatment, which hydrolyses phosphatidylcholine into diacylglycerol and phosphocholine, of intact chloroplast on precursor protein import and binding. Hydrolysis of phosphatidylcholine by phospholipase C is shown to inhibit the transfer of the (most probably) receptor-bound precursor protein to the membrane-embedded import apparatus.

Abbreviations: SSU, small subunit of ribulose bisphosphate carboxylase; pSSU, precursor form of SSU; PLC, phospholipase C (EC 3.1.4.3).

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2. MATERIALS AND METHODS

Chloroplasts were purified from 2-week-old pea seedlings (*Pisum sativum*, var. Golf) grown in a greenhouse and purified through centrifugation on Percoll (Pharmacia) gradients [6,9]. Phospholipase C (PLC) (from *Bacillus cereus*, Boehringer-Mannheim, Germany) treatment was done at 4 U enzyme per mg chlorophyll for 20 min on ice in a buffer containing 50 mM HEPES (pH 7.6), 330 mM sorbitol and 0.5 mM $CaCl_2$ [15]. The PLC treatment was stopped by the addition of 10 mM EDTA. Intact organelles were separated from those damaged by the PLC treatment through centrifugation on Percoll gradients as above. Chloroplasts equivalent to 15 μg chlorophyll [16] were used in precursor protein import and binding assays [6]. Import experiments were done in the light in the presence of 2 mM ATP at 25°C for 10 min [6,9]. Chloroplasts were kept in the dark for 45 min prior to binding experiments to deplete internal ATP. The subsequent assay was done in the dark in the presence of 10 μM ATP at 25°C for 5 min. Protease treatment (thermolysin, Boehringer-Mannheim) was done depending on the experimental regime either before (750 μg protease mg^{-1} chlorophyll) or after precursor binding and import (100 μg protease mg^{-1} chlorophyll) [6,17]. Intact chloroplasts were repurified on Percoll gradients as above. In case chloroplasts were pretreated with both protease and PLC, the protease preceded the PLC treatment. Chloroplasts were repurified as above between the two manipulations. The precursor protein of the small subunit of ribulose bisphosphate carboxylase (pSSU) was synthesized by in vitro transcription–transla-

tion in the presence of [^{35}S]methionine in a reticulocyte lysate system as outlined in [18]. Experimental results were analysed by SDS-PAGE [19] followed by fluorography [20].

3. RESULTS

All members of the plastid family are surrounded by a pair of unit membranes called the inner and outer envelopes [21]. The outer envelope, which forms the boundary of the organelle to the cytoplasm, is unique in its protein and lipid composition [21,22]. The role of proteinaceous components of the outer envelope in the protein import process into chloroplasts has been established [1,2]. Here we treated chloroplasts with PLC, which hydrolyses phosphatidylcholine to diacylglycerol (membrane bound) and phosphocholine (water soluble) to assess the possible involvement of phosphatidylcholine in protein translocation. As shown in Fig. 1 import of pSSU into intact chloroplasts is completely abolished by PLC treatment as demonstrated by the failure of mature SSU to accumulate inside the organelle (lanes 5 and 6). In contrast binding of pSSU to the organelle surface was not inhibited but seemed to increase slightly (Fig. 1, lane 5). PLC, which was inactivated by boiling for 5 min, did not influence the import efficiency of pSSU import (Fig. 1, lanes 3 and 4). This indicated that the enzymatic hydrolysis of phosphatidylcholine by PLC was responsible for the inhibition of protein import. Increasing the concentrations of PLC, from 1 to 4 U resulted in increased import inhibition (not shown). Higher concentrations of PLC resulted in an almost complete loss of chloroplast intactness. When the temperature was raised from 4 to 8°C, respectively, during the PLC treatment of intact organelles, residual import decreased further (8°C) or was not detectable anymore at 12°C (not shown). Again the amount of intact chloroplasts was greatly reduced under these conditions (4 U PLC, 12°C). Together these data demonstrate that import into chloroplasts is inhibited due to the enzymatic properties of PLC.

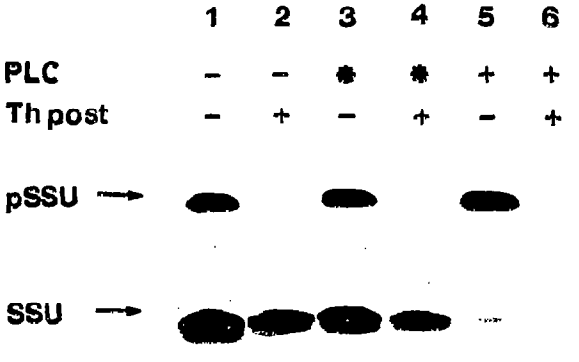

Fig. 1. Influence of phospholipase C treatment of intact pea chloroplasts on the import of pSSU. Translocation of pSSU was studied with chloroplasts in the light with 2 mM ATP either not treated (lanes 1, 2) or treated with inactivated PLC (*, lanes 3, 4) or active PLC (+, lanes 5, 6). PLC (*) was heat denatured (100°C) prior to the assay. After completion of the import experiment chloroplasts were treated with thermolysin (Th post, even numbers). A fluorogram is shown.

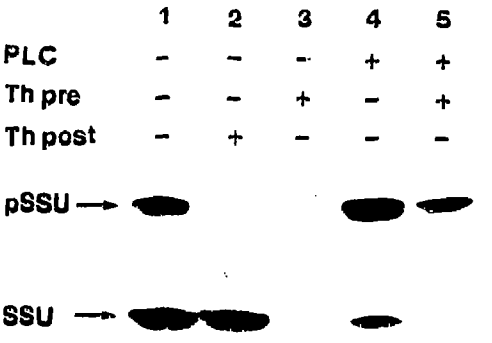

Fig. 2. Binding of pSSU to intact chloroplasts after phospholipase C treatment is specific. Chloroplasts were treated with thermolysin (lane 3) or PLC (lane 4) or thermolysin plus PLC prior to binding and import of pSSU in intact chloroplasts (Th pre). Import with non-treated organelles is shown in lanes 1 and 2. All other conditions are as in Fig. 1.

Binding and import of precursor polypeptides are most likely initiated by proteinaceous receptor proteins exposed on the chloroplast surface [1,2]. In order to differentiate between specific and non-specific binding and import, organelles were treated with the non-penetrating protease thermolysin. As demonstrated in Fig. 2 thermolysin treatment largely abolishes binding and import of pSSU (compare lanes 1 and 3). Chloroplasts treated with PLC show levels of pSSU import higher to that of protease-treated ones (Fig. 2, compare lanes 3 and 4), but again the import rate is drastically reduced

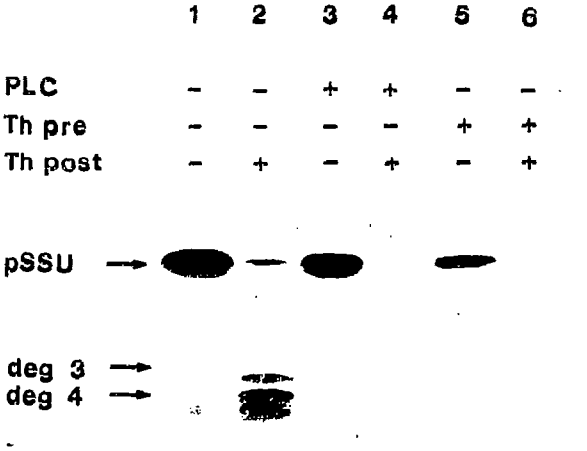

Fig. 3. The transfer of pSSU on the chloroplast surface into the import apparatus is blocked by phospholipase C treatment. Chloroplasts were pretreated with PLC or protease as outlined in the figure. Incubation of intact organelles with pSSU was done in the dark at 10 μM ATP. After completion of pSSU binding, chloroplasts were re-isolated and treated with thermolysin (even numbers). The translocation intermediates obtained in control experiments (lane 2) are numbered deg 3 and deg 4 (the nomenclature of translocation intermediates is as in [6]).

to control levels (Fig. 2, lanes 2 and 4). Binding of pSSU to the chloroplast surface is slightly increased in PLC-treated organelles under import conditions; this is probably due to the block of pSSU translocation and subsequent enrichment of pSSU on receptor sites. The sum of organelle-bound pSSU and imported, mature SSU inside the chloroplasts in control experiments is, however, equivalent or higher in comparison to PLC-treated organelles (see also Figs. 1 and 2). To assess whether pSSU binding to chloroplasts after PLC treatment was still mediated by proteinaceous receptors, chloroplasts were treated successively with protease and lipase. This regime resulted in very low quantities of precursor polypeptide bound to the organelle surface (Fig. 2, lane 5). Import was not detected in these experiments. The difference in binding between thermolysin-treated chloroplasts (Fig. 2, lane 3) and that which is detected after PLC and thermolysin double treatment (Fig. 2, lane 5) could be due to non-specific adhesion to the organelle surface. In summary the data strongly indicate that most of the binding of pSSU to the chloroplast surface after PLC treatment is still mediated by receptor polypeptides.

It is possible to stop the translocation process of pSSU into chloroplasts at different stages, e.g. binding or partial translocation into the import apparatus by manipulating the temperature and or ATP concentrations during the import assay [4,6,10]. To analyse at which step PLC blocked the translocation process we carried out import reactions of pSSU into chloroplasts in the dark in the presence of 10 μM ATP. These conditions resulted in partial precursor translocation into the import apparatus which could be detected as protease-protected translocation intermediates after thermolysin treatment [6,7] (Fig. 3, lanes 1 and 2). When chloroplasts, which had been treated with PLC, were incubated with pSSU under the conditions described above and subsequently subjected to proteolytic digestion by thermolysin no translocation intermediates could be detected (Fig. 3, lanes 3 and 4). Binding to the chloroplast surface at 10 μM ATP was nearly as efficient as in control experiments (Fig. 3, lanes 1 and 3), but less than at 2 mM ATP (compare Figs. 1 and 2). Chloroplasts which had been protease treated prior to the incubation with pSSU showed residual binding in the dark in the presence of low ATP. Together these data strongly indicate that the PLC treatment blocks a transfer step of the precursor polypeptide from a surface-exposed localization, e.g. bound to the receptor, to the import machinery, i.e. insertion into the translocation complex.

4. DISCUSSION

Monogalactosyldiglyceride, digalactosyldiglyceride and phosphatidylcholine are the three major lipid constituents of the outer chloroplast envelope membrane [21]. Phospholipase C hydrolyses specifically phosphatidylcholine exposed on the cytosolic leaflet of the outer envelope in intact chloroplasts [15]. Under proper conditions chloroplasts remain largely intact [15] and can be used for further studies. As demonstrated in this work destruction of phosphatidylcholine inhibits precursor protein translocation at a step after binding but before insertion into the import machinery. In PLC-treated chloroplasts the precursor protein remains fully sensitive to externally added protease under various conditions (compare Figs. 1 and 3). It is therefore tempting to assume that the precursor protein is blocked at the receptor stage.

Hydrolysis of phosphatidylcholine by PLC drastically changes the lipid composition of the outer chloroplast envelope and could thus lead to a destabilization of the import machinery. The mechanisms by which such an inhibition is working are not clear but could be the following:

(i) Receptor molecules are randomly distributed in the outer envelope membrane. Upon binding of a precursor protein they move laterally in the plane of the membrane to interact and transfer the precursor protein into the import machinery. Hydrolysis of phosphatidylcholine hinders this diffusion process and thus inhibits the docking and transfer process.

(ii) It is possible to isolate a protein complex from envelope membranes which seems to house at least some of the receptors for precursor recognition and large parts of the translocation machinery as one unit [7]. It is possible that phosphatidylcholine is an essential constituent of this translocation unit. After phosphatidylcholine hydrolysis the components of this unit can no longer interact properly or lose their biological activity.

(iii) Finally a direct interaction of the precursor proteins with phosphatidylcholine might also be necessary for a productive translocation process.

Even though the mechanism through which PLC is acting is speculative at the moment, it is clear that our results should facilitate the identification of a pSSU receptor protein in the outer chloroplast envelope [23]. The bound precursor protein remains fully protease sensitive, indicating that it is interacting only with a limited number of components on the outer chloroplast envelope surface, e.g. the receptor polypeptides. Chemical crosslinking reagents have been succesfully used to identify constituents of the import machinery in other systems [24]. A precursor protein which is halted at the receptor stage or at very early steps of the translocation process should give rise to a very limited number of crosslinked products. This approach is now being applied to aid in the characterization of single constituents of the chloroplast envelope translocation apparatus.

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