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Detection of small GTP-binding proteins in the outer envelope membrane of pea chloroplasts

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We found small GTP-binding proteins in the outer envelope membrane of pea chloroplasts. The proteins in this membrane were separated by SDS-PAGE, transferred to a nitrocellulose filter, and incubated with [α-^{32}P]GTP. Three GTP-binding proteins with the molecular weight of 24 000 were found. Binding was prevented by 10^{-8}–10^{-7} M GTP or by 10^{-7} M guanosine 5′-[γ-thio]triphosphate or GDP; binding was unaffected by 10^{-8}–10^{-6} M ATP. Thermolysin treatment of intact chloroplasts resulted in the loss of GTP-binding activity, suggesting that these proteins were in the cytosolic side of the outer envelope membrane.

Chloroplast outer membrane; Small GTP-binding protein; Pea

1. INTRODUCTION

Chloroplasts respond to external and internal stimuli such as light and hormones. Chloroplasts send signals to nuclei and cytoplasm for intracellular communication [1]. The molecular mechanisms of such signal perception and transduction are largely unknown. One approach to understanding these phenomena in chloroplasts is to study chloroplast envelope proteins, which may be involved. GTP-binding proteins are conserved molecular switches found in various cells. They respond to input signals and affect intracellular signalling systems [2–4]. Here we looked for small GTP-binding proteins in pea chloroplasts and detected some in the outer envelope membrane.

2. MATERIALS AND METHODS

2.1. *Isolation of leaf components*

Pea seedlings (*Pisum sativum* cv. Alaska) were grown under cycles of 12 h of light and 12 h of dark for 12 days at about 20°C. Their leaves were homogenized with buffer A (50 mM tricine-KOH, pH 8/0.5 mM dithiothreitol/0.1 mM EDTA/1 mM benzamidine/5 mM ε-amino caproic acid/1 mM phenylmethylsulfonyl fluoride) and the extract was centrifuged at 16 000 × *g* for 30 min. The total soluble and insoluble fractions were obtained. The insoluble fraction was washed with 80% acetone and dissolved in 5% SDS. Intact chloroplasts were isolated from the 12-day-old leaves as described elsewhere [5] except that the homogenization buffer and Percoll gradient did not contain Mg^{2+} or Mn^{2+}. Envelope membranes were obtained by hypertonic lysis of chloroplasts and by sucrose density gradient centrifugation as reported before [6]. The stroma and thylakoid membrane were recovered from this sucrose density gradient [7]. The outer and inner envelope membranes were separated on a sucrose density gradient as described previously [8].

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2.2. *Assay of acyl-CoA synthetase and ATPase activity*

Acyl-CoA synthetase was assayed as reported elsewhere [9]. The reaction was started by the addition of 1.0 μg of membrane protein and incubation was for 3 min at 25°C. The free acids were extracted and the radioactivity in the aqueous phase was counted. The initial reaction rates were used to calculate enzyme activity. ATPase activity was assayed as described before [10].

2.3. *Protein separation and transfer to nitrocellulose blots*

The protein concentration of the isolated leaf components was measured with a protein assay kit (Bio-Rad). The membrane fractions were made soluble in 5% SDS, and the solution was adjusted to Laemmli loading buffer [11]. The mixture was heated in boiling water for 2 min, and 5–30 μg of each fraction was separated by SDS-PAGE (12.5% gel). The proteins in the gels were transferred electrophoretically to nitrocellulose filters as reported previously [12].

2.4. *Thermolysin treatment*

Isolated chloroplasts were treated with 100 μg/ml thermolysin and the treated chloroplasts were repurified with a Percoll gradient as described elsewhere [13].

2.5. *GTP-binding assay*

The transferred blots were first preincubated overnight at 6°C in buffer B (50 mM Tris-HCl, pH 7.4/5 mM $MgCl_2$/0.3% Tween 20/0.5 mM EDTA) [14] and next incubated for 1 h at 37°C in buffer B containing 10^{-9} M [α-^{32}P]GTP (3000 Ci/mmol). The blots were washed three times with 50–100 ml buffer for 5–15 min, air-dried, and autoradiographed for 12–72 h at −70°C with an intensifying screen. In competition experiments, the blots were first incubated with buffer B containing an unlabelled competing substance (GTP, guanosine 5′-[γ-thio]triphosphate (GTP[γS]), GDP, GMP, ATP, UTP, or CTP) and next incubated with buffer B containing an unlabelled competing substance and 10^{-9} M [α-^{32}P]GTP.

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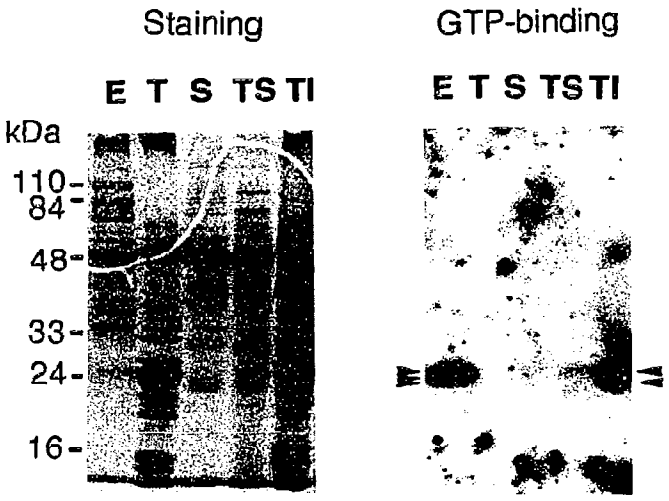

Fig. 1. GTP-binding activity in pea chloroplast fraction. 30 μg of envelope (E), thylakoid (T), stromal (S), total soluble (TS) and total insoluble (TI) proteins was separated by 12.5% SDS-PAGE, and GTP-binding was assayed as in section 2. 15 μg of protein was stained with Coomassie brilliant blue.

3. RESULTS

Autoradiography showed that there were GTP-binding proteins of the molecular weight of about 24 000 in the envelope but not in the stromal or thylakoid fractions (Fig. 1). One strongly labelled band was found and two less strongly labelled bands were also found in the vicinity by shorter exposure time (data not shown). In the total leaf proteins, GTP-binding activity was found in insoluble fraction (TI in Fig. 1) and at least two bands were found under the conditions tested. These two bands probably do not arise from envelope, because insoluble fraction contained a very small amount of envelope proteins.

To find where GTP-binding proteins were in the envelope, the envelope fraction was further fractionated into outer and inner membranes. Contamination of each fraction was examined by an assay of marker enzyme activity (Fig. 2A). Acyl-CoA synthetase, a marker enzyme in the outer envelope [9], was found in both

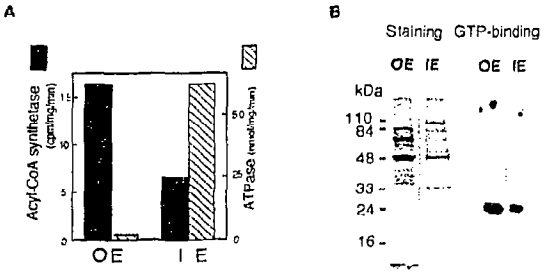

Fig. 2. Location of GTP-binding activity in the envelope. The envelope fraction was separated into the outer envelope (OE) membrane and inner envelope (IE) membrane. (A) Acyl-CoA synthetase activity and ATPase activity in each fraction were assayed as described in section 2. (B) 30 μg of protein of each fraction was separated and assayed as in the legend of Fig. 1. 20 μg of protein was stained with Coomassie brilliant blue.

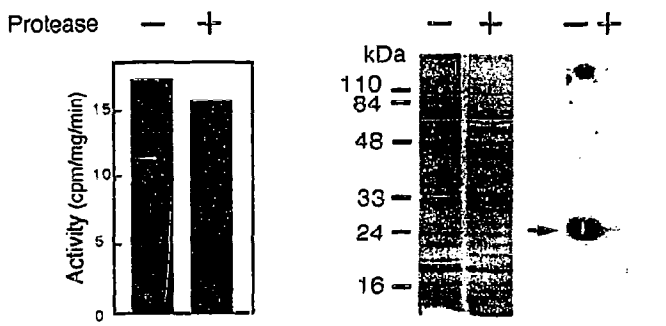

Fig. 3. Effects of thermolysin on GTP-binding activity in outer envelope membrane proteins. From the chloroplasts treated with thermolysin, envelope fraction was isolated and the outer membrane fraction was prepared as described in section 2. Acyl-CoA synthetase activity and GTP-binding activity were assayed as described in the text. 10 μg of protein was used for silver staining and 30 μg of protein for GTP-binding assay.

fractions, but ATPase, a marker enzyme in the inner envelope [10] was found in only the inner envelope fraction. So, the outer membrane fraction was not contaminated by the inner membrane fraction, but the inner membrane fraction was contaminated by the outer membrane fraction. Re-fractionation of the inner membrane with a sucrose gradient confirmed that the outer membrane fraction had contaminated this fraction (data not shown). The 24-kDa bands of the outer membrane fraction were intensely labelled and those of the inner membrane fraction were less intensely labelled (Fig. 2B). Because of the contamination we found, we concluded that the 24-kDa proteins were in the outer membrane.

To identify the orientation of GTP-binding proteins in the outer envelope, we did protease protection studies using intact chloroplasts. Thermolysin partially degrades the surface proteins of chloroplasts [13] but does not degrade acyl-CoA synthetase, which seems not to be on the outside of the outer envelope [15]. We compared the protected GTP-binding activity with that of acyl-CoA synthetase. The results showed that GTP-binding activity was almost completely degraded but that acyl-CoA synthetase activity was not (Fig. 3), indicating that GTP-binding proteins were susceptible to thermolysin and therefore on the cytoplasmic side of the outer envelope.

The specificity of the binding site was tested by competition experiments (Fig. 4). Incubation of the blots with 10^{-8}–10^{-6} M GTP or 10^{-7}–10^{-6} M GTP[γS] or GDP totally blocked the binding of [α-^{32}P]GTP. ATP (10^{-8}–10^{-6} M) and GMP (10^{-8}–10^{-6} M) did not affect the binding of [α-^{32}P]GTP (Fig. 4). UTP and CTP did not affect the binding at 10^{-8}–10^{-7} M but slightly blocked the bindings at 10^{-6} M. Thus, binding was specific for the di- and triphosphate forms of guanine nucleotides.

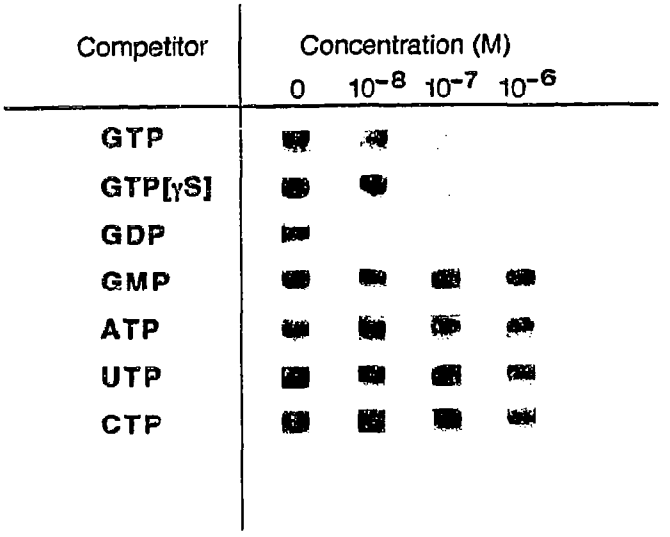

Fig. 4. Competition by various nucleotides for the binding of [α-^{32}P]GTP to envelope membrane proteins. The GTP-binding activity of envelope membrane (20 μg of protein) was assayed as in section 2 in the presence of the indicated concentration of an unlabeled nucleotide. A 0 M concentration indicates incubation with 10^{-9} M [α-^{32}P]GTP only.

4. DISCUSSION

Here, we found GTP-binding proteins of the molecular weight of 24 000 in the chloroplast outer envelope by a GTP-binding assay [16] that measures small GTP-binding proteins of low molecular weight but not large G proteins with an $\alpha\beta\gamma$ subunit structure [17]. Under the conditions tested, the binding specificity was similar to that of the small GTP-binding proteins in the *ras* superfamily of such proteins [18]. That chloroplast GTP-binding proteins were on the cytoplasmic side of the outer envelope suggests that these proteins may be involved in communication between the cytoplasm and the chloroplast, probably from the chloroplast to the cytoplasm.

Several GTP-binding proteins are present in the plasma membrane of higher plants [19–23] and in spinach thylakoid membranes [24], although we did not detect them in the thylakoid fraction under the conditions tested (Fig. 1). These proteins resemble the α subunit of animal G protein in some ways [20,21,23]. A few small GTP-binding proteins with molecular weights of 23 400–28 500 have been found in hypocotyl microsomal fractions by an assay method that ours resembles [22]. The proteins detected in the insoluble fraction from total leaf (Fig. 1) may be those of microsomal fractions. The physiological role of these proteins is unknown except for their possible involvement in the regulation of protein kinase activity [24] and in phytochrome-mediated signal transduction in *Avena* seedlings [23]. A putative *Arabidopsis thaliana* gene homologous to the *ras*-related gene [25] and G protein α gene has been cloned [26]. Experiments are now in progress to characterize chloroplast GTP-binding proteins.

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