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Detection of GTP-binding proteins in barley aleurone protoplasts

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We report the existence of several families of GTP-binding proteins in barley aleurone protoplasts. Partial purified plasma membrane proteins were separated by SDS-PAGE, transferred to a nitrocellulose filter and incubated with either antisera raised against a highly conserved animal G protein α subunit peptide/or *Ras* protein, or with [α-^{32}P]GTP. Two sets of proteins of M_r = 32–36 kDa and 22–24 kDa were strongly recognized by the antisera. Binding of [α-^{32}P]GTP was detected on Western blots with proteins of M_r = 22–24 kDa and 16 kDa. Binding was inhibited by 10^{-7}–10^{-6} M GTPγS, GTP or GDP; binding was not affected by 10^{-6}–10^{-5} M ATPγS or ADP. The kinetics, specificity and the effects of phytohormones in a [^{35}S]GTPγS binding assay were also studied in isolated plasma membranes of barley aleurone protoplasts.

GTP-binding; G protein; Phytohormone; Barley aleurone

1. INTRODUCTION

Plant cells respond to a large number of external and internal stimuli such as light, stress and hormones. These stimuli trigger plant signal transduction pathways in which cytosolic calcium levels, intracellular pH, inositol phosphates and ion-channels are involved [1–6]. However, little is known about the molecular mechanisms of the regulation of signal transduction. In animals and simple eukaryotes, it is evident that G proteins play an important role in signal transduction [7–8]. In plant cells, however, detection and cloning of G proteins have been carried out only in some species [9–12]. Functional evidence for G-proteins is still limited to a few observations such as regulation of K^+ current in *Vicia faba* L. guard cells [13] and blue light response in *Pisum sativum* L. cells [14].

Barley aleurone protoplasts have been used as a model system for studying plant hormones (e.g. abscisic acid (ABA) and gibberellins (GA)) induced responses. Both ABA and GA-mediated intracellular messengers, such as Ca^{2+} and pH changes are considered to be involved in these hormone signal transduction pathways [2,4,6,15,16]. We are interested in a possible role for G proteins in the signal transduction of these hormones in barley aleurone. As a first step, we investigated the presence of G proteins.

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Abbreviations. MES, 2-(*N*-morpholino)ethanesulfonic acid, EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PIPES, 1,4-piperazinediethanesulfonic acid.

2. MATERIALS AND METHODS

2.1 *Materials*

[α-^{32}P]GTP (3,000 Ci/mmol) and [^{35}S]GTPγS (1,355 Ci/mmol) were purchased from New England Nuclear (UK). MES, DTT, abscisic acid and gibberellic acid (GA_3) were obtained from Sigma Chemical Co. (St. Louis, USA), and cellulase Onozuka R-10 was from Yakult Honsha (Japan). Gamborg B5 was from Flow Laboratories (Irvine, Scotland) and PVP K25 was from Fluka Chemie (Switzerland).

2.2. *Isolation of protoplasts and partial purification of plasma membrane*

Barley (*Hordeum vulgare* L. cv Himalaya, harvested 1985; Department of Agronomy, Washington State University, Pullman, WA, USA) aleurone protoplasts were prepared essentially as described by Wang et al. [4,15]. The buffer we used for washing and incubation of protoplasts was 10 mM PIPES buffer consisting of 0.65 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM PIPES, pH 6.8.

Protoplasts at a density of 6×10^6/ml in PIPES buffer were diluted 10 times in receptor buffer containing Tris-HCl (20 mM, pH 7.5) and proteinase inhibitors [17], subsequently lysed by pressing them through 5 μm pores of a Nuclepore filter [17]. The homogenate was centrifuged at $10,000 \times g$ for 5 min at 4°C. After removal of supernatant, the pellet was washed once and resuspended in receptor buffer at a density equivalent to 5×10^6 protoplasts/ml.

2.3. *Western transfer analysis of membrane proteins*

The protein concentration of isolated membrane fractions was measured with a protein assay kit (Bio-Rad). The membrane fractions were solubilized in 5% SDS, and adjusted to loading buffer [18]. The mixture was incubated at 95°C 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 a nitrocellulose membrane as described by Klein et al. [19]. The nitrocellulose membrane was

blocked in phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST) plus 1% (w/v) BSA for 1 h and incubated overnight at room temperature with 1:500 diluted rabbit antiserum against the Gα-common peptide or monoclonal antibody against Ras, Y13-259 (Dupont, NEN) and for 2 h with 1:7,500 diluted goat anti-rabbit IgG or goat anti-mouse IgG conjugated to alkaline phosphatase (GARAP or GAMAP). The specific bands were visualized after incubation with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as substrate.

2.4. *GTP-binding*

The transferred blots were first preincubated overnight at 4°C in blocking buffer (50 mM Tris-HCl, pH 7.5, 2 μM $MgCl_2$, 1 mM DTT, 0.3% Tween-20 and 5% milk powder) [20] and subsequently incubated for 0.5 h at 25°C in buffer B containing 10^{-9} M [α-^{32}P]GTP (3,000 Ci/mmol). The blots were washed three times with PBS for 5–15 min, air-dried, and autoradiographed for 12–72 h at −80°C with an intensifying screen. In competition experiments, the blots were incubated with buffer B containing 10^{-9} M [α-^{32}P]GTP and unlabelled competing NTPs or NDPs (GTPγS, GTP, GDPβS, GDP, ATPγS or ADP)

Binding of [^{35}S]GTPγS to membranes was measured at 0°C in an incubation volume of 100 μl containing 10 mM Tris, pH 8.0, 10 nM [^{35}S]GTPγS, 5 mM $MgCl_2$, and 80 μl membrane fractions isolated from 2×10^6 protoplasts/ml. The effects of ABA and GA on [^{35}S]GTPγS binding were measured at binding equilibrium (15 min). Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by centrifugation at 10,000 × *g* for 5 min at 4°C. The supernatant was aspirated and the pellet was dissolved in 80 μl 1% SDS, 1.2 ml Emulsifier (Packard) was added and radioactivity was determined. Nonspecific binding was determined in the presence of 0.1 mM unlabelled GTPγS [21].

2.5. *Immunological staining*

The blots were developed with alkaline phosphatase coupled to antibodies to mouse or rabbit IgG according to the ProtoBlot Western blot AP system technical manual (Promega). Antibodies utilized were an anti-*Ras* p21 monoclonal antibody (Y13-259) and a rabbit polyclonal antibody prepared against Gα-common peptide (Dupont, NEN).

3. RESULTS

3.1. *Immuno-detection of GTP-binding proteins and GTP binding on blots*

Antisera raised against a highly conserved amino acid sequence (Gα-common peptide) of animal G-protein recognized, in the membrane fraction of barley aleurone protoplasts, sets of proteins of M_r = 58, 50, 34–36 kDa and 22–24 kDa (Fig. 1B). However, proteins with a molecular weight of 34–36 kDa showed the strongest signal. Without addition of the first antibody, we did not detect any of the above mentioned bands by using the secondary antibody alone (Fig. 1A). At the bottom of the blot, one band (about 18 kDa) which gave a positive signal was considered to be a false positive, since it even appeared in the lane without addition of the first antibody (Fig. 1A,B). We have compared the electrophoretic mobilities of Gα immunoreactive bands with *Ras* immunoreactive bands. The 34–36 and 22–24 kDa bands were recognized by both anti-Gα and anti-*Ras* antibodies. Although *ras* antibody recognized some other bands (e.g. 72 kDa, 40 kDa etc.) as well, both antibodies commonly recognized the 34–36 and 22–24 kDa bands (Fig. 1C).

The same Western blot was used for radioactive [α-^{32}P]GTP binding as well. Autoradiography showed that there were GTP-binding proteins with an apparent molecular weight of about 22–24 kDa and 16 kDa in membrane fractions of barley aleurone protoplasts (Fig. 1D,E). Two strongly labelled bands of 22–24 kDa were found after short exposure time and one less strongly labelled band (16 kDa) was found after longer exposure time. These labelled bands could be chased by addition of unlabelled GTP but with different affinities (Fig. 1D,E). Proteins with a molecular weight of 22–24 kDa were also recognized by antibodies against both *Ras* and Gα subunit (Fig. 1B,C). However, the 16 kDa GTP binding band was not recognized by both antibodies (Fig. 1).

3.2. *Effects of temperature and plant hormones on GTP binding*

We tested the effects of temperature and the plant hormones abscisic acid and gibberellic acid on the binding of [^{35}S]GTPγS in the isolated plasma membrane fraction (Table I). Isolated membrane fraction was incubated with [^{35}S]GTPγS at 25°C and 0°C. The equilibrium binding was reached at about 15 min incubation for both temperatures (data not shown). At 25°C, a slightly higher amount of [^{35}S]GTPγS binding was found than at 0°C for the same input of isolated plasma membrane. When the [^{35}S]GTPγS and plant hormone were added to the isolated membranes at the same time for 15 min incubation (0°C), both abscisic acid (100 μM) and gibberellic acid (100 μM) had no significant effect on GTP binding (Table I).

3.3. *Specific GTP binding in the plasma membrane*

The specificity of the binding sites was tested by competition experiments (Fig. 2). Incubation of the blots with 10^{-7} M GTP or GTPγS or GDP or GDPβS strongly blocked the binding of [α-^{32}P]GTP. ATPγS and ADP (10^{-5} M) did not affect the binding of [α-^{32}P]GTP. This specificity was also studied in insoluble membrane fractions of the protoplasts by using [^{35}S]GTPγS (Fig. 3). Fig. 3 shows that the amount of [^{35}S]GTPγS binding was strongly reduced when the unlabelled GTP, GTPγS

Table I

Effects of temperature and hormones on GTP binding*

Conditions	% of [^{35}S]GTPγS binding
Binding at 0°C	100
Binding at 25°C	115 ± 8
+ 100 μM ABA (at 0°C)	101 ± 5
+ 100 μM GA (at 0°C)	105 ± 3

*Results of at least 3 independent triplicated experiments for each condition are shown. The amount of binding at 0°C is calculated as 100% binding. 100% means the specific binding (4,094 cpm/1.5 × 10^5 protoplasts equivalent) and 50,000 cpm input of [^{35}S]GTPγS. The data are presented as means ± S.D.

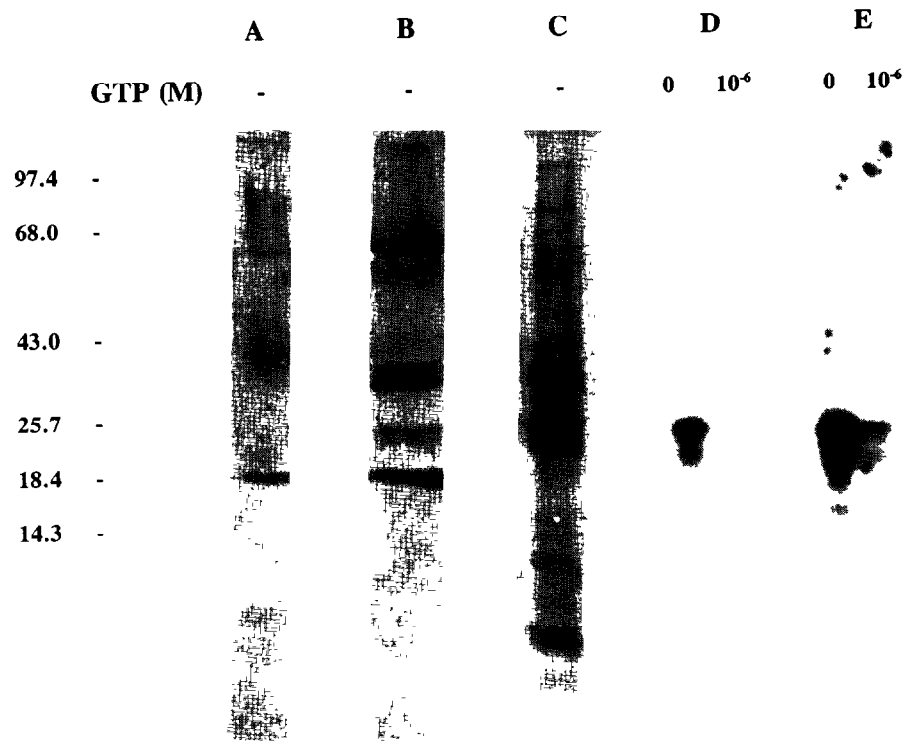

Fig. 1. Immuno-detection of GTP-binding proteins and [α-^{32}P]GTP binding in barley aleurone protoplasts. 25 μg of isolated plasma membrane was electrophoresed on a 12.5% SDS-PAGE and transferred to nitrocellulose membrane One part of the membrane was incubated with the first antibody (rabbit antiserum against Gα-common peptide lane B or *Ras* Y13-259 lane C) and secondary antibody (GARAP). Lane A is without the incubation of the first antibody. Another part of the membrane was used for [α-^{32}P]GTP binding (D,E) Autoradiographs demonstrated that a short exposure time (12 h) gave a set of bands M_r = 22–24 kDa (see D) and a long exposure time (72 h) provided an additional band M_r = 16 kDa (see E). Addition of unlabelled GTP (10^{-6} M) was able to compete the radioactive binding. Molecular mass markers are indicated on the left

and GDP were added but not with the addition of ATP and ADP. For guanine nucleotides, the half maximal inhibition of binding was reached at a concentration of about 10^{-8} M. At a concentration of 10^{-4} M ATP, only slight inhibition of [^{35}S]GTPγS could be observed (Fig. 3).

4. DISCUSSION

We found GTP-binding proteins of a molecular weight of 34–36 kDa and 22–24 kDa in the membrane fraction of barley aleurone protoplasts by immuno-detection and of a molecular weight of 22–24 kDa and 16 kDa by a GTP-binding assay on Western blots. The GTP-binding assay on Western blots measures small GTP-binding proteins of low molecular weight but not the α subunit of large G proteins with a heterotrimeric αβγ subunit structure [22]. Under our conditions tested, the binding specificity was similar to that of the small GTP-binding proteins in the *Ras* superfamily of such proteins [23]. Under the experimental conditions used, plant hormones (abscisic acid and gibberellic acid) had no significant effect on GTP binding. This might be due to the experimental conditions or that the G-proteins we detected are not directly coupled to hormone receptors.

Several GTP-binding proteins have been detected in the plasma membrane of higher plants. Some of them in some way resemble the α subunit of animal G protein [10–12]. A few small GTP-binding proteins with molecular weights of 22–28 kDa have been found in hypocotyl microsomal fractions and chloroplast membranes by similar methods [10]. The physiological role

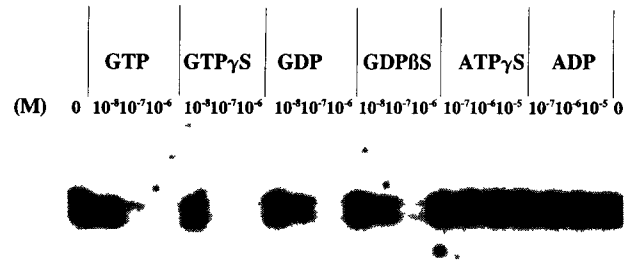

Fig. 2. Competition by various nucleotides for the binding of [α-^{32}P]GTP to barley aleurone protoplast plasma membrane protein on the Western blot. The GTP-binding activity of membrane (20 μg protein) was assayed as described in section 2 in the presence of the indicated concentration of an unlabelled nucleotide A zero M concentration indicates incubation with 10^{-9} M [α-^{32}P]GTP only.

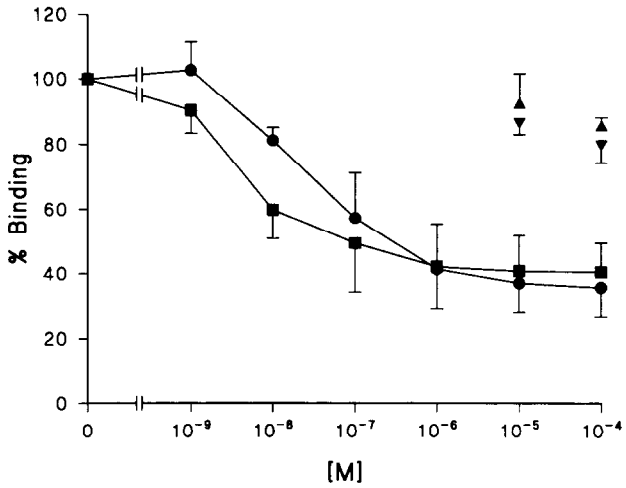

Fig. 3. Competition by various nucleotides for the binding of [^{35}S]GTPγS to the insoluble plasma membrane fraction. The GTP-binding activity of membrane (from 1.6×10^5 protoplasts) was assayed as described in section 2 in the presence of the indicated concentration of an unlabelled nucleotide. At 0 concentration, incubation was without unlabelled compounds and with [^{35}S]GTPγS (7×10^{-10} M) only. ●, Addition of unlabelled GTP; ■, addition of unlabelled GDP; ▲, addition of unlabelled ATP; ▼, addition of unlabelled ADP. The error bars are standard deviations. The means of three independent experiments in triplicate are presented.

of these small G proteins is not clear though some data indicate that these proteins may be involved in protein kinase activity [24] and phytochrome-mediated signal transduction [25]. Recently, Thompson reported that stress causes a translocation of the 28 kDa GTP-binding protein from the Golgi and endoplasmic reticulum to the plasma membrane in *Dunaliella salina* [26]. The possible role of this small GTP binding protein in the regulation of α-amylase secretion, intracellular pH and calcium in barley aleurone protoplasts is under current investigation.

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