Note

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Involvement of GTP-binding Protein Immunologically Resembled Gαs and Gαolf in Phytoalexin Production of Cultured Carrot

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Abstract

A 38 kDa peptide in microsomal fraction prepared from oligogalacturonide elicitor-treated carrot cell culture showed an affinity to GTP after the separation by SDS-PAGE. Antibodies raised against the related alpha subunits of heterotrimer GTP-binding protein, anti-Gαs and anti-Gαolf, were found to crossreact with this peptide. In contrast, anti-G-proteins against other classes of α subunits of the trimeric complexes and low molecular weight monomers did not show the affinity to the peptide. These results suggest that a heterotrimer GTP-binding protein complex of which α subunit immunologically resembles Gαs and Gαolf may be involved in the early stage of oligouronide elicitor-induced phytoalexin biosynthesis in cultured carrot cells.

Key words: α subunit, cultured carrot cells, GTP-binding protein, heterotrimer complex, phytoalexin production, plant defense responses.

Abbreviation

Gα, GTP-binding protein α subunit.

Higher plants defend themselves by a variety of mechanisms when they are challenged by pathogenic microorganisms (Dixon *et al.*, 1994; Ebel and Mithoefer, 1998). Production of carrot phytoalexin, 6-methoxymellein, is triggered by the contact of the cells with oligogalacturonide elicitor which is liberated by partial hydrolysis of carrot cell walls by the action of extracellular pectinase and proteases secreted by invading fungi (Kurosaki, 2001). We have demonstrated (Kurosaki *et al.*, 1987, 2001) that oligouronide elicitor exhibits highly specific activity of inducing phytoalexin production in cultured carrot cells, and other defensive responses, such as chitinase induction, lignification and hypersensitive cell death, are essentially not observed in the cells stimulated by this elicitor. 6-Methoxymellein production in carrot cells is primarily controlled by the rate of transcription of genes encoding the biosynthetic enzyme proteins of the compound (Kurosaki, 2001), and increase in Ca^{2+} level in the cytosol is an essential early event in eliciting production of this phytoalexin (Kurosaki and Nishi, 1993). We demonstrated that the elevation of cytoplasmic Ca^{2+} concentration in cultured carrot is triggered by at least two independent mechanisms, a rapid breakdown of phosphatidylinositol in plasma membrane (Kurosaki *et al.*, 1987) and gating of cyclic AMP-dependent cation channel(s) (Kurosaki, 1997). This 'dual mechanism' for the second messenger liberation at the early stage of phytoalexin production in cultured carrot cells are analogous to that of the recognition of external stimuli in odor-sensitive animal cells (Liu *et al.*, 1994; Huque and Bruch, 1986). We have recently reported (Kurosaki *et al.*, 2001) that a certain GTP-binding protein(s) is involved in the transmembrane signaling pathway to liberate the second messengers in elicitor-treated carrot cells. In eukaryotic cells (Gilman, 1987; Bourne *et al.*, 1991), occurrence of two types of GTP-binding proteins has been demonstrated; heterotrimeric G-proteins composed by three subunits (α, β and γ) and monomer proteins with low molecular weight.

Although these two types of G-proteins have been also found in higher plant cells (Terryn *et al.*, 1993; Mulligan *et al.*, 1997), only very little is known about physiological functions of plant G-proteins. In the present study, we attempted to characterize the GTP-binding protein involved in the signal transduction mechanisms of oligogalacturonide elicitor-induced phytoalexin production in cultured carrot cells.

Cultured carrot cells (ft-2525 strain) in 80 ml of Murashige-Skoog's liquid medium (1962) were maintained on an Innova 2300 rotary shaker (New Brunswick Scientific Co., 150 rpm) in the presence of 3% (w/v) sucrose and 4.5 μM of 2,4-dichlorophenoxyacetic acid as described previously (Kurosaki *et al.*, 2001). Elicitor-active oligogalacturonides were prepared by partial hydrolysis of pectin fraction of cultured carrot cells as described previously in detail (Kurosaki *et al.*, 1987).

Oligouronide elicitor (80 μg in 800 μl of 10 mM K-acetate buffer, pH 5.5) was added to 80 ml of 14-day-old cell culture (early stationary phase), and 10 ml aliquots of the cell suspension were removed at regular intervals. The cells were harvested by rapid suction, and were homogenized in 75 mM Mops-NaOH buffer (pH 7.6, containing 0.25 M sucrose) by sonic oscillation. Cell debris was removed by centrifugation (10,000 g, 15 min), and the microsomal proteins were precipitated by ultracentrifugation (100,000 g, 1 h). Microsome fraction thus prepared was subjected to SDS-PAGE (12% gel, 50 μg proteins per lane) according to the method of Laemmli (1970), and the separated proteins were transferred onto a nitrocellulose membrane filter (0.22 μm) on a semidry transfer cell (Bio-Rad, Trans-blot SD). After the blotting, the membrane was immersed and incubated in 40 ml of binding buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgSO_4$, 25 mM KCl, 1 mM EDTA, 5 mM dithiothreitol) containing 1% (w/v) bovine serum albumin at 25 ℃ for 1 h. After washing with the binding buffer without bovine serum albumin, the membrane was successively incubated with [^{35}S]GTP-γ-S (10 nM, 120 kBq) in 3 ml of the Tris buffer at 25 ℃ for 1 h (Kurosaki *et al.*, 2001). The nitrocellulose membrane was then washed with the binding buffer, air-dried, and the radioactivities associated with the separated proteins were analyzed on a bio-imaging analyzer (Fuji film, BAS-2000). As shown in **Fig. 1**, at least one of the microsomal proteins prepared from the elicitor-treated carrot cells appeared to show an affinity to GTP-γ-S, and a radioactive protein band was observed at the position corresponding to approximately 38 kDa. In contrast, no obvious signal was detected in the control culture which received the acetate buffer without the elicitor (data not shown). It is well known that GTP-binding proteins do not lose the affinity to GTP and GTP-γ-S even after SDS-PAGE under denatured condition (Sacchi *et al.*, 1996). This 38 kDa band could not be observed if the membrane was incubated with 1 μM of non-radiolabeled GTP for 10 min prior to the initiation of the binding reaction with [^{35}S]GTP-γ-S (data not shown). In contrast, we have reported (Kurosaki *et al.*, 2001) that the GTP-binding ability of the protein was not lost when it was preincubated with ATP instead of GTP. These results strongly suggest that the 38 kDa protein in the microsome fraction prepared from the elicitor-treated carrot cells is a GTP-binding protein. An attempt was made to detect G-protein(s) in elicitor-treated carrot cells employing highly purified plasma membranes prepared by aqueous two phase partitioning method (Graef and Weiler, 1989; Kurosaki, 1997). However, this did not succeed probably because the G-protein was unstable under this purification condition of the two phase system and lost the GTP-binding activity. This radiolabeled protein band of 38 kDa was observed only when the microsomes were prepared after 2 and 3 min of the elicitor treatment, and the samples obtained at time 0 and after 5 min did not showed the detectable binding activity to the GTP analogue (**Fig. 1**). In repeated experiments, this transient appearance of GTP-binding activity was somewhat varied in its timing, however, the radiolabeled 38

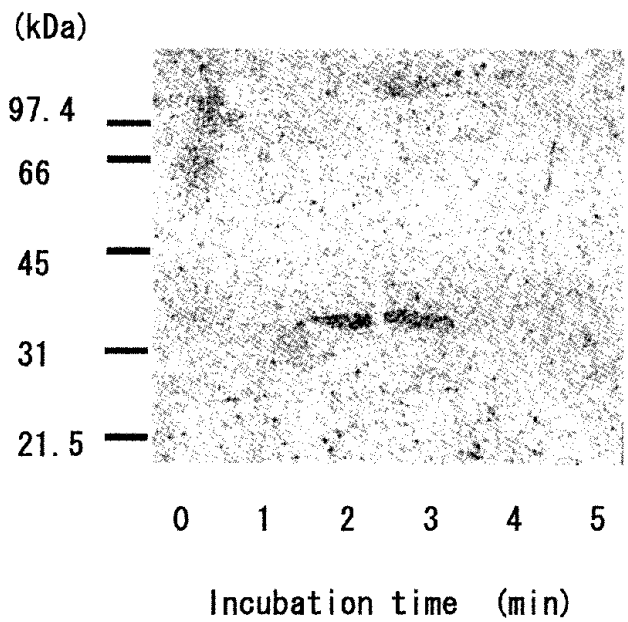

Fig. 1 Analyses of GTP-binding protein in cultured carrot cells treated with elicitor-active oligogalacturonides. Cultured carrot was treated with oligouronide elicitor, and the cells were harvested at regular intervals. Microsomal proteins prepared from the cells were separated by SDS-PAGE (12% gel), and transferred onto a nitrocellulose membrane. The membrane was then incubated with [^{35}S]GTP-γ-S, and after several washings, the radioactivities associated with the membrane were analyzed on a bio-imaging analyzer. Molecular mass of the radiolabeled protein was estimated with the standard markers (Bio-Rad, 97.4, 66, 45, 31 and 21.5 kDa).

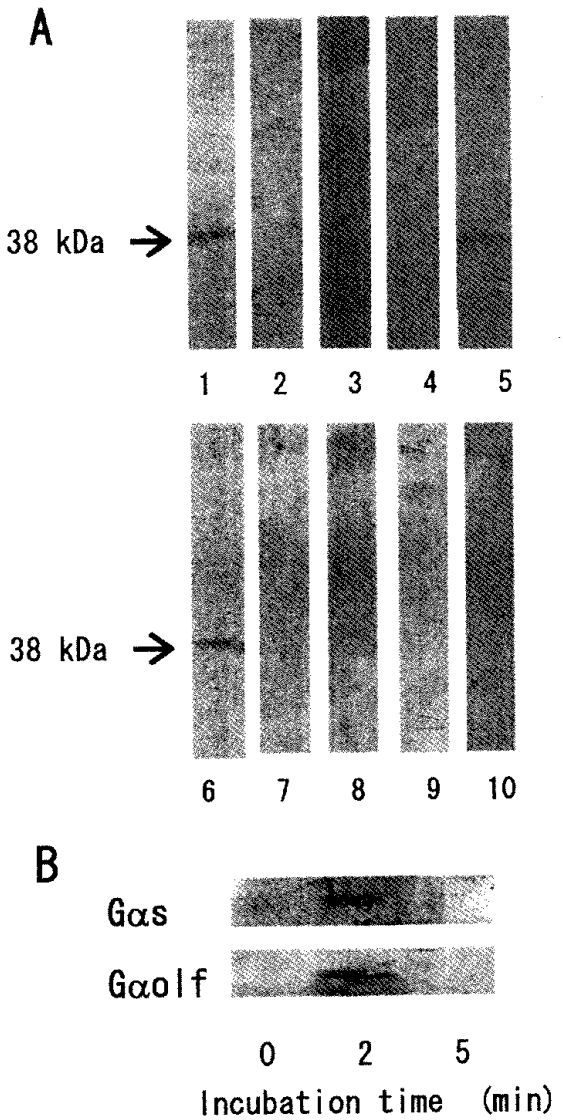

Fig. 2 Immunoblot analyses of GTP-binding protein in elicitor-treated carrot cell culture. (A), The 38 kDa protein specifically observed in elicitor-treated carrot cells was probed with several poly- and monoclonal (shown in parentheses below) antibodies raised against various G-proteins. 1, Treated with an antibody raised against common structure of the α subunit of heterotrimeric GTP-binding protein (poly); 2, anti-Rac (poly); 3, anti-Ras (mono); 4, anti-Rho (mono); 5, anti-Gαs (poly); 6, anti-Gαolf (poly); 7, anti-Gαi (mono); 8, anti-Gαi3 (mono); 9, anti-Gαo (mono); 10, anti-Gαq (poly). Antibodies against Rac, Ras, Rho, Gαolf, αi, Gαi3, and Gαq were obtained from Santa Cruz Biotechnology while anti-Gαcommon, Gαs, Gαo were from BIOMOL Research Laboratories. (B), Change in the intensities of 38 kDa peptide immunologically detected with anti-Gαs and Gαolf. Microsome proteins were prepared from cultured carrot cells immediately before (time 0), 2 and 5 min after the elicitor-treatment. They were separated by SDS-PAGE, and were probed with anti-Gαs and Gαolf after being blotted onto a nitrocellulose membrane.

kDa band was reproducibly observed within 2 to 4 min after the elicitor-treatment. At present, it is not clear whether the transient appearance of the 38 kDa GTP-binding protein in microsomal fraction of oligouronide elicitor-treated carrot cells is due to the gene expression or the translocation of the protein from cytoplasmic space to the membrane (Kieffer *et al.*, 1997). We reported previously (Kurosaki *et al.*, 2001) that low but significant levels of GTP-hydrolytic and GTP-binding activities were observed even in control carrot culture which did not receive the elicitor. Although no information is available to explain this apparent discrepancy, it is possible that the constitutive GTP-binding activity is too low to detect on the bio-imaging analyzer and only the induced activity is sufficient to be monitored in the present experimental system.

In order to characterize the biochemical properties of the GTP-binging protein transiently appeared in elicitor-treated carrot cells, antibodies raised against various G-proteins were obtained from commercial sources and their reactivities toward the 38 kDa protein were examined (**Fig. 2A**). These antibodies, either mono- or polyclonal, were prepared employing synthetic peptides of the conserved or diverse regions of several GTP-binding proteins obtained from animal cells as the antigens. Microsomal proteins prepared from elicitor-treated carrot cells (after 2 min of the treatment) were separated by SDS-PAGE, and were blotted onto the nitrocellulose membrane. After blocking with 1% (w/v) bovine serum albumin, the membrane was incubated with antibodies raised against various GTP-binding proteins (appropriately diluted according to the instruction manual) in 5 ml Tris-buffered saline at 4 ℃ overnight, respectively. The membrane was then incubated with Protein A conjugated with alkaline phosphatase (Bio-Rad) at 25 ℃ for 2 h, and were successively washed with the buffered saline. Primary antibodies bound to the 38 kDa peptide were visualized by color development reaction with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazorium chloride as the substrates. Although antibody raised against the common structure of GTP-binding site in the α-subunit of heterotrimeric G-proteins appeared to crossreact with the 38 kDa peptide, treatment of the peptide with several antibodies toward monomeric G-proteins, Ras, Rac and Rho, showed

no obvious band after the visualization reaction. Among the several antibodies against α-subunits of various classes of heterotrimer G-proteins, only anti-Gαs and anti-Gαolf appeared to crossreact with the peptide and showed a band at the position corresponding to 38 kDa while antibodies raised against other α-subunits of heterotrimeric GTP-binding proteins, Gαo, Gαq and several Gαi isoforms, did not show the visual band under the present conditions. This finding appeared to agree with our previous report (Kurosaki *et al.*, 2001) that mastoparan, an activator of trimer-type G-proteins, stimulates phytoalexin production in cultured carrot cells even in the absence of elicitor. In the repeated experiments, as well as in the GTP-γ-S-binding assay shown in **Fig. 1**, this 38 kDa band could be immunologically detected only in the microsomes prepared after 2-4 min of the elicitor-treatment, and the samples obtained at time 0 and after 5 min did not show the band by the incubation with either anti-Gαs or anti-Gαolf (**Fig. 2B**). These series of the results suggest the possibility that the heterotrimeric GTP-binding protein complex of which α subunit immunologically resembles Gαs and Gαolf would participate in the early stage of signal transduction mechanisms in phytoalexin induction in oligogalacturonide elicitor-treated carrot cell culture.

It has been shown (Gilman, 1987; Bourne *et al.*, 1991) that *Mrs* of α subunits of heterotrimeric GTP-binding proteins in animal and yeast cells are varied in some extent (39-52 kDa). In contrast, the sizes of the subunits from plant sources reported so far have been estimated to be approximately 45 kDa (Terryn *et al.*, 1993; Mulligan *et al.*, 1997). At present, it is not clear whether or not the 38 kDa peptide observed in the present experiments is a novel class of α-subunit of plant heterotrimer G-proteins specifically activated in infected cells. However, the fact that the peptide crossreacts with the antibodies raised against the specific α-subunits of animal cells, Gαs and Gαolf, might suggest the possibility that the peptide is a product of a certain ortholog gene of these unique subunits, and its properties and behavior are somewhat different from those of other α-subunits of plant G-proteins.

It has been recently shown that G-proteins participating into hypersensitive response (Rajasekhar *et al.*, 1999; Kawasaki *et al.*, 1999) and oxidative burst (Kieffer *et al.*, 1997) in infected plant cells are low molecular weight monomers but not heterotrimeric complexes. It is possible therefore that phytoalexin production and some of other defense responses in higher plants are mediated by different classes of GTP-binding proteins. It has been shown (Gilman, 1987; Bourne *et al.*, 1991) that adenylyl cyclase is a common effecter of the heterotrimer G-protein complexes composed by either Gαs or Gαolf. Present results appear to be consistent with our previous finding that direct activation of cyclic nucleotide-sensitive cation channels is an essential event to activate Ca^{2+} cascade that leads to the expression of genes encoding biosynthetic enzymes of carrot phytoalexin (Kurosaki, 1997). In higher plants, only very limited information is available for cyclic AMP-generating systems, therefore, characterization of the GTP-binding protcin(s) which would activate cyclic nucleotide synthesis might provide important evidence to understand this second messenger in plant cells.

We showed previously (Kurosaki *et al.*, 1987) that the rapid breakdown of phosphatidylinositol takes place upon the contact with oligogalacturonide elicitor as another half of the 'dual mechanism' for the second messenger liberation. However, antibody against Gαq, which is considered to play an important role in the activation of phosphatidylinositol cycle in animal cells (Gilman, 1987; Bourne *et al.*, 1991), was shown not to crossreact with the peptides in elicitor-treated carrot microsome. It would be possible that the activation of G-protein involved in phosphatidylinositol responses does not occur within the experimental period of this study, alternatively, the activity of Gαq is, if any, too low to detect under the present conditions. In addition, the possibility cannot be excluded that the structure of Gαq-like protein in carrot is somewhat different from animals, and the antibody against animal Gαq was not able to recognize the protein of plant source.

Further characterization and molecular cloning of this GTP-binding protein involved in transmembrane signaling of phytoalexin biosynthesis in carrot cells is in progress in our laboratory.

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