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# ASSOCIATION OF MULTIPLE GTP-BINDING PROTEINS WITH THE PLANT CYTOSKELETON AND NUCLEAR MATRIX

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**Several types of GTP-binding proteins exist in plant cells. These include the *ras*-related low-molecular-weight monomeric GTP-binding proteins and the multi-subunit group which more closely resembles members of the mammalian heterotrimeric G-protein family. Proteins belonging to both of these families are known to be involved in cell signalling events and have until recently been assumed to be associated predominantly with membranes. We have investigated the possibility that GTP-binding proteins in plants also can be associated with membrane-free carrot (*Daucus carota* L.) cytoskeletons and nuclear matrices. Our results demonstrate that several low-molecular-weight GTP-binding proteins, and at least one G-protein α-subunit homologue, are associated with these cellular compartments.** 

The family of eukaryotic GTP-binding proteins is diverse with regard to both structure and function. Two classes of GTP-binding proteins have recently received particular attention due to their multiple roles in cellular signal transduction; these are the α/β/γ-subunit G-proteins and the low molecular weight GTPases of the Ras, Rho and Ypt/Rab groups. Plant genes encoding homologues of mammalian heterotrimeric G-protein α- and β-subunits have been identified (1,2) and immunological studies using antisera against conserved α-subunit domains have demonstrated the presence of several antigenic proteins in plant extracts (3). Low molecular weight GTP-binding proteins were first identified in plant tissues using [α-$^{32}$P]GTP-overlays (4) and since then, a considerable number of these proteins have been characterized biochemically, immunologically and by cDNA cloning (see ref. 5,6 for

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reviews). In this study we have investigated the possibility that GTP-binding proteins, in addition to their established association with cellular membranes, also may be associated with detergent-resistant components of the cytoskeleton and the nuclear matrix. Our data show that a number of low molecular weight GTP-binding proteins, and one homologue of a mammalian heterotrimeric G-protein $\alpha$-subunit, are associated with membrane-free cytoskeletal and nuclear fractions isolated from carrot cells.

## MATERIALS AND METHODS

**Cell culture and preparation of whole-cell extracts, microsomal membranes and plasma membranes.** Carrot (*Daucus carota* L.) cv. "Oxford" suspension cultures were maintained as described by McCann et al. (7). Whole-cell extracts were prepared by homogenization (polytron, Kinematika, UK) of cells in Minimum Protein Extraction Buffer (1 mM DTT, 1 mM EDTA, 30 mM Tris-HCl, pH: 7.2 containing 1 % (v/v) of a protease inhibitor cocktail: 1 mM benzamidine-HCl, 0.1 mg/ml phenanthroline, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mM PMSF) followed by removal of lipids and concentration of protein by the method of Wessel and Flugge (8). Microsomal membranes and plasma membranes were isolated and purified as described by Drøbak et al. (9).

**Preparation of detergent-resistant cytoskeletons, nuclei and nuclear matrices.** Protoplasts and cytoskeletons were prepared as described by Xu et al. (10) with the modifications outlined by Dove et al. (11). 0.05 % Triton-treated nuclei were isolated as described by Beven et al. (12). Intact nuclei and nuclear matrices were isolated from protoplasts prepared overnight using 0.01% (w/v) Y-23 (Seishin Pharmaceutical Co. Ltd., Tokyo, Japan) and 0.1% (w/v) RS-cellulase (Yakult Honsha Co. Ltd, Tokyo, Japan) in growth medium containing 0.5 M mannitol. Protoplasts were pelleted and resuspended in 0.5 M sucrose in buffer A: 10 mM NaCl, 10 mM MES, pH, 5.3. This suspension was transferred to volumetric flasks and centrifuged for 10 min at 150 xg. Intact protoplasts were collected from the neck of the flask and pipetted into 1.5 ml microcentrifuge tubes. Protoplasts were rapidly frozen in liquid nitrogen and thawed at 25°C in the presence of 1% (v/v) of the protease inhibitor cocktail. The protoplast suspension was passed through a 23 gauge needle 5 times and layered onto 100 $\mu$l of 54% sucrose in buffer A, underlaid by 50 $\mu$l Maxidens (Nycomed, Oslo, Norway). The tubes were centrifuged at 15,000 xg for 1 minute and the nuclei collected from the sucrose-Maxidens interface and resuspended in 0.4 M sucrose in buffer A. Triton X-100 treatment of intact nuclei was carried out in 0.4 M sucrose in buffer A containing 2 % (v/v) Triton X-100. After incubation for 15 minutes at room temperature the nuclear matrices were pelleted, washed 3 times with 0.4 M sucrose in buffer A, and resuspended.

**GTP[$\gamma^{35}$S]-overlays.** Separation of polypeptides by SDS-PAGE, and electroblotting was carried out essentially as described by Drøbak et al. (4). For GTP[$\gamma^{35}$S] overlays the nitrocellulose sheets (0.45 $\mu$m membranes, Schleicher and Schuell, Dassel, Germany) with transferred proteins were washed thoroughly with three changes of buffer B: 50 mM Tris-HCl, pH: 7.5, 12 $\mu$M $MgCl_2$, 1 mM dithiothreitol, 0.3 % (v/v) Tween 20. For probing, GTP[$\gamma^{35}$]S (75 kBq/ml final concentration, sp.act. 51.5 TBq/mmol, N.E.N., U.K.) and 10 $\mu$M ATP were added to buffer B, and nitrocellulose strips were incubated with gentle agitation for 30 min at room temperature. After 3 washes with buffer B the strips were air dried and radioactive bands were visualized either by autoradiography or by phosphoimaging.

**Immunological methods.**

Peptides were transferred to nitrocellulose sheets as described above and briefly stained with Ponceau salt (Sigma, U.K.) in 0.3 % (v/v) trichloroacetic acid. After destaining in water the blots were washed 3 times in TBS containing 0.1 % (v/v) Tween 20 and incubated in: TBS containing 0.1 % (v/v) Tween 20, 5 % (w/v) Marvel (TBSTM) for 1.5 hr. Blots were then incubated with primary antibody overnight at 4°C in TBSTM. Following 3 washes in TBS + 0.1 % (v/v) Tween 20 the blots were incubated with horse radish peroxidase-conjugated anti-rabbit IgG immunoglobulins (DAKO, Denmark) in TBSTM for 1.5 hr at room temperature. After 3 washes with TBS, colour development was carried out by standard procedures. In control experiments, the diluted serum was preincubated with authentic $G_{Ara2}$ peptides (25 $\mu$g peptide/ml) at 37°C for 30 min and subsequent procedures carried out as above.

## RESULTS AND DISCUSSION

The distribution of low molecular weight GTP-binding proteins in whole-cell extracts, microsomal membranes, purified plasma membranes and isolated cytoskeletons from carrot cells is shown in **Figure 1**. A number of GTP-binding proteins with a $M_r$ ranging from approximately 20 kDa to 30 kDa are present in whole-cell extracts, microsomal membranes and in plasma membranes. In the cytoskeletal fraction, three major GTP-binding protein bands with the $M_r$ of 29.4 kDa, 26.9 kDa and 20.4 kDa were identified together with several minor bands. We have previously demonstrated that plant cytoskeleton preparations also contain nuclear proteins (10) so we further investigated the possibility of a nuclear localization of some of the GTP-binding proteins. **Figure 2** shows the distribution of low-molecular weight GTP-binding protein in cytoskeletons (lane 1) and in intact nuclei isolated

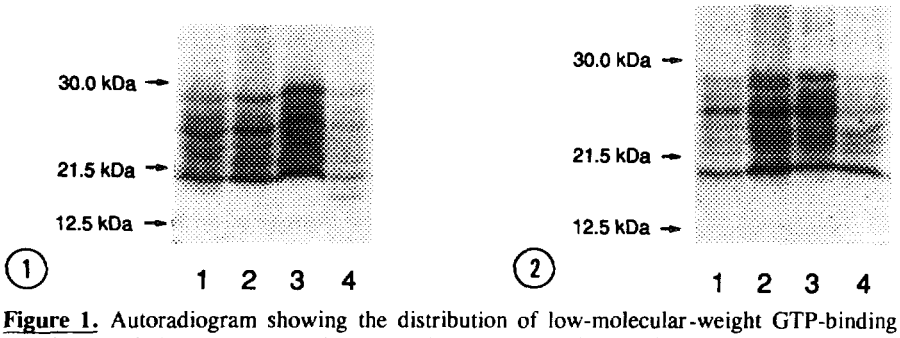


**Figure 1.** Autoradiogram showing the distribution of low-molecular-weight GTP-binding proteins in whole cell extracts (lane 1), microsomal membranes (lane 2), purified plasma membranes (lane 3) and cytoskeletons (lane 4). Each lane contains approximately 30 $\mu$g of protein.

**Figure 2.** Autoradiogram showing the distribution of low-molecular-weight GTP-binding proteins in cytoskeletons (lane 1), intact nuclei (lane 2), nuclei treated with 0.05 % Triton X-100 (lane 3) and nuclei treated with 2 % Triton X-100 (lane 4). Each lane contains approximately 30 $\mu$g of protein.

in the absence of detergent (lane 2) and in nuclei treated with 0.05 % (v/v) and 2.0 % (v/v) Triton X-100 respectively (lane 3-4). The 29.4 kDa, 26.9 kDa and 20.4 kDa proteins associated with isolated cytoskeletons also appear to be present in the nuclear preparations but there are also obvious differences in the protein pattern (note in particular the presence of the prominent band of approximately 28.6 kDa in intact nuclei which is absent in all other fractions). The Triton X-100-sensitive nuclear proteins are likely to be associated with the nuclear membranes whereas the detergent-resistant proteins in lane 4 most probably are associated with nuclear matrix structures. Although these data do not allow us to unequivocally determine whether any of the low-molecular weight GTP-binding proteins have an exclusively nuclear or cytoskeletal localization they nevertheless show that these proteins can be associated both with nuclear membranes and with the detergent-resistant components of the cytoskeleton/nuclear matrix. Several low molecular weight GTP-binding proteins are known to interact with the cytoskeleton and nucleus of mammalian cells. The **rac** and **rho** proteins in particular have received attention since it was shown that **rho** links plasma membrane receptors to the assembly of focal adhesions and actin stress fibers and **rac** links receptors to the polymerization of actin at the plasma membrane (13). A cDNA encoding a plant **rho**-homologue, named **Rho1Ps,** has been cloned from pea (14). In the present study we investigated the possibility that one, or more, of the low-molecular weight GTP-binding proteins associated with the cytoskeleton could be relatives of the mammalian **rho**- and **rac**-proteins. Immunoblot analysis (ECL detection) using polyclonal antisera against mammalian **rhoA**, **rac1** and **G25K (cdc42)** however proved inconclusive (data not shown). A diagnostic feature of the mammalian **rho** proteins is their susceptibility to ADP-ribosylation by *Clostridium botulinum* exotoxin C3 (15). In spite of using a range of incubation procedures and varying concentrations of *C. botulinum* exotoxin C3, we did not find any evidence for ADP-ribosylation of any of the low-molecular weight GTP-binding proteins associated with the cytoskeletal fraction. This suggests either, that these protein are only distant relatives of the mammalian Rho family or, that special conditions are needed to ADP-ribosylate **rho**-related plant GTP-binding proteins *in vitro* (positive controls using authentic **rho** proteins all showed high levels of ADP-ribosylation under all conditions employed). In addition to **rac** and **rho** two other low-molecular weight GTP-binding proteins, **rap2B** and **ran**, have been found to interact specifically with the cytoskeleton and/or nucleus. Torti et al. (16) thus showed that **rap2B** in resting platelets is completely detergent soluble, whereas in platelets aggregated with thrombin, or the $Ca^{2+}$-ATPase inhibitor thapsigargin, a significant amount of **rap2B** becomes associated with the cytoskeleton. The only known low molecular weight GTP-binding protein which is specifically associated with the nucleus is **ran** (**ra**s-related **n**uclear protein, 17). A **ran** homologue was recently identified in plant cells (18) but whether

it, like in mammalian cells, plays a role in nuclear protein import (17) remains to be elucidated.

The presence of low molecular weight GTP-binding proteins in both the cytoskeletal fraction and in nuclei and nuclear matrices prompted us to investigate whether members of the heterotrimeric G-protein $\alpha$-subunit family also shared this cellular localization. **Figure 3** (top lane) shows an immunoblot of whole-cell extracts, microsomal and plasma membrane proteins as well as cytoskeletal and nuclear proteins probed with $G_{Ara2}$ (W5) antiserum raised against the 15 C-terminal amino acids of a $G_{\alpha}$-subunit homologue (GPA1) from *Arabidopsis thaliana* (1). Several proteins are recognized by the $G_{Ara2}$ antiserum, but after preincubation of the antiserum with the appropriate control peptide a major band with an approximate $M_r$ of 47.5 kDa was shown to be specifically recognized by the antiserum (**Figure 3,** bottom lane). Whole-cell extracts and microsomal fractions contain the $G_{Ara2}$ antigen but the data in **figure 3** demonstrate that the primary localization is the plasma membrane. It is significant, however, that both the cytoskeletal and nuclear fractions contain the $G_{Ara2}$ antigen. In both of these fractions the $G_{Ara2}$ antigen levels are significant, but low. There are several lines of evidence which indicate that a similar situation exists in activated mammalian cells. Sarndahl et al. (19) showed that a substantial part of the $\alpha$-subunit of the G*n* protein (G*n*$\alpha$) in unstimulated neutrophils is associated with a cytoskeletal fraction, but that rapid release from the cytoskeleton is induced by cellular stimulation with the chemotactic factor fMet-Leu-Phe or by direct G-protein activation by GTP$\gamma$S or $AlF_4^-$. It is worth noting that the preparation of both the cytoskeletal and nuclear fraction used in these studies involves conversion of cells into protoplasts - a procedure which recently was shown to lead to cellular activation (20),

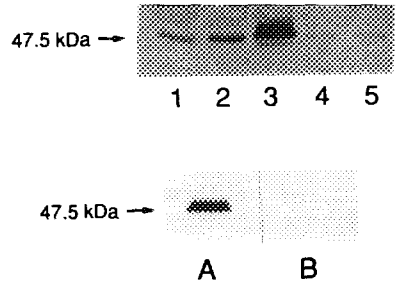


**Figure 3.** Anti-$G_{Ara2}$-immunoblots of subcellular fractions isolated from carrot cells. Upper panel: lane 1: whole-cell extract, lane 2: microsomal membranes, lane 3: purified plasma membranes, lane 4: cytoskeletons, lane 5: intact nuclei. Each lane contains approximately 30 $\mu$g protein. Lower panel: anti-$G_{Ara2}$-immunoblot of purified plasma membranes without (left lane), or with (right lane), preincubation of the primary antibody with authentic $G_{Ara2}$ peptide. Each lane contains approximately 30 $\mu$g protein.

and we have preliminary data which suggest that the procedure employed in the preparation of subcellular fractions indeed affects the distribution of the $G_{Ara2}$ antigen. The physiological role(s) of the cytoskeletal and nuclear GTP-binding proteins identified in this study remains to be determined but the demonstration that members of two signal transducing GTP-binding protein families can be associated with these cellular compartments in plants raises several important questions. We are currently investigating the nature of the cytoskeletal and nuclear GTP-binding proteins in more detail and are testing the hypothesis that their association with the cytoskeleton and nuclear matrix can be influenced by specific plant cell agonists.

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