Immunodetection of Rho-like plant proteins with Rac1 and Cdc42Hs antibodies

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Abstract

A few small GTP-binding proteins of the Ras superfamily have been identified in plants, including members of the Rho family: the proteins belonging to this group are known in mammalian and yeast cells to be involved in the control of polarity, cell morphogenesis and movement by regulating cytoskeleton organization. An investigation into where some Rho-like proteins are located in plant cells was made. The antibodies used, anti-Cdc42Hs and anti-Rac1, were raised against conserved characteristic sequences of Cdc42Hs and Rac1 mammalian proteins respectively. In fixed cells, Cdc42Hs antibody recognized epitopes generally colocalized with microtubules which may be implicated in the establishment of cell polarity, whereas the proteins recognized by Rac1 antibody seemed to be associated with organelle membranes. The same antibodies were used in Western blots of proteins from tobacco BY-2 and lucerne A2 suspension cells: Cdc42Hs antibody recognized three bands whereas Rac1 antibody revealed only one band of 18 kDa $M_r$. A [35S]GTP overlay revealed four bands of the same $M_r$ as those recognized in Western blots by Cdc42Hs and Rac1 antibodies.

Key words: Rho G-proteins, Cdc42, Rac1, Immunofluorescence, plant cells.

Introduction

The Ras superfamily of small GTP-binding genes code low molecular mass proteins (20–30 kDa) that are structurally and biochemically similar to the $\alpha$-subunit of the trimeric G proteins. These proteins which act as molecular switches are regulated by cycling between GTP- and GDP-bound forms, being activated upon binding of GTP and inactivated when the bound GTP is hydrolysed to GDP by the intrinsic GTPase activity (Nuofer and Balch, 1994). These conversions are modulated by interactions with cytosolic guanine dinucleotide-dissociation inhibitors (GDI) or stimulators (GDS), and GTPase activating proteins (GAP). However, these regulatory functions are dependent upon prior post-translational modifications of the carboxy terminus, with isoprenylation, cleavage and carboxymethylation (Hall and Zerial, 1995). Among these GTPases, four subsets of the Ras superfamily have been identified in plant cells: the Ran proteins which regulate the transition into M phase (Ach and Gruissem, 1994); the Arf proteins which may modulate vesicle budding and coating within the Golgi apparatus through a driver cycle (Regad et al., 1993); the Ypt/Rab proteins known to be part of the protein machinery involved in vesicular transport (Terryn et al., 1993; Hawes and Satiat-Jeunemaitre, 1996; Borg et al., 1997); and the Rho proteins (Yang and Watson, 1993; Delmer et al., 1995; Lin et al., 1996; Borg et al., 1997; Lin and Yang, 1997).

Most if not all of the studies on Rho in mammalian, Drosophila and yeast cells indicate that these proteins regulate actin organization, especially where the actin is required in cell motility and cell adhesion, although the exact effect on actin reorganization is highly cell-type dependent (Adams et al., 1990; Johnson and Pringle, 1990; Nobes and Hall, 1995; Murphy and Montell, 1996). However, they also seem to be involved in many other cellular processes such as signalling, growth control, endocytosis, and secretion (Allen et al., 1997). Many Rho and related proteins have now been identified in various eukaryotes and categorized into three major subfamilies:

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Abbreviations: ATP, adenosyl-triphosphate; BFA, brefeldin A; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GDP, guanosine 5’-diphosphate; GTP, guanosine 5’-triphosphate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PMSF, phenylmethylsulphonyl fluoride; PPB, preprophase band; SDS, sodium dodecyl sulphate; TBS, tris buffer saline.

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Cdc42, Rac and Rho, on the basis of the cellular functions and sequence homology (Chant, 1994; Chant and Stowers, 1995; Hall and Zerial, 1995).

In plant tissues, low molecular weight GTP-binding proteins were identified using [α-32P]GTP overlays (Drøbak et al., 1995). Several such proteins have since been characterized biochemically, immunologically or by cDNA cloning (Terryn et al., 1993). An extensive screen of mRNA from 3-week-old nodules of Lotus japonicus has identified 33 different small GTP-binding proteins, which could be assigned to Ypt/Rab, Ran and two Rac subfamilies (Borg et al., 1997). These two Rac subfamilies contain all conserved residues or motifs unique to members of the Rho family, including the Asn-41 site for ADP-ribosylation by C3 toxin and the C-terminal CAAL (A, aliphatic amino acid; Just et al., 1994; Takai et al., 1995; Popoff et al., 1996). A Rho plant GTPase, Rop1Ps, cloned from garden pea (Yang and Watson, 1993), appears to be essentially expressed in pollen where the 21.3 kDa protein is associated with the apical membrane of the growing tube and the periphery of the generative cell, leading to speculation that in plants it modulates an actomyosin motor system involved in movement of the generative (male) cell. Like all plant Rho homologues identified so far, Rop1Ps is closer to the Rac subfamily (65% amino acid sequence identity) rather than the Rho subfamily (50% identity). Two Rac-like proteins have also been identified in cotton (Delmer et al., 1995).

As a prelude to the exploration of the function of Rho proteins and their potential involvement in the regulation of cytoskeleton organization, studies were undertaken to identify the Rho-like proteins in plant cells and their expression and localization. Plant cells are an attractive model for these studies for two main reasons.

(1) They are generally immobile without any of the Rho-mediated cellular processes associated with motility (formation of filopodia, lamellipodia, membrane ruffles, stress fibres, adhesion foci; Nobes and Hall, 1995). Plant cells may thus provide appropriate models to elucidate novel functions of these proteins.

(2) The relationship between different cytoskeletal components and their involvement in plant-specific functions is sometimes unique. For instance, contrary to the situation in mammalian cells, actin seems to be the cytoskeletal element involved in the maintenance of the Golgi apparatus (Satiat-Jeunemaître et al., 1996). Furthermore, microtubules are thought to be the major determinants regulating morphogenesis in plants (Lloyd et al., 1980). Therefore, the question to be addressed is the role for the Rho proteins in the regulation of the cytoskeletal network in plants: might they be associated with the reorganization of actin in plants as in yeast and animal cells? Might they be associated with the reorganization of microtubules in the context of morphogenesis in plants?

In order to study these hypotheses, the plant proteins recognized by antibodies directed against conserved sequences characteristic of mammalian Rho proteins were investigated.

In this paper, a [γ-35S]GTP overlay, immunoblotting and immunofluorescence studies using antibodies raised against conserved distinctive sequences of Rac1 and Cdc42Hs proteins were performed. Four physiological situations were examined in tobacco, lucerne or maize: proliferating suspension culture cells, non-proliferating suspension cells, root tissues, and plant protoplasts. Subsequently, the association of the Rac1- and Cdc42-like proteins with subcellular compartments or cytoplasmic elements was investigated. Pharmacological agents or physico-chemical stresses have been applied to the cells (cytochalasin D; oryzalin, cold; brefeldin A) to alter specifically the function of specific subcellular components (respectively actin filaments, microtubule arrays, the endomembrane system) so that the effects on the immunofluorescence patterns and the Rho proteins might be analysed. These results suggest that the proteins recognized by anti-Cdc42Hs antibody are in fact associated with microtubule arrays, and so this antibody may be a useful tool in cell morphogenesis studies. Further, the proteins recognized by anti-Rac1 antibody may be associated with the organelle membranes.

**Materials and methods**

**Biological material**

*Nicotiana tabacum* Bright Yellow-2 (BY-2) suspension-cultured cells were grown in a modified Murashige and Skoog medium (MSMO; Linsmaier and Skoog, 1965) in the dark at 26 °C with 100 rpm and subcultured every 7 d at 2/80 ml. For immunofluorescence studies and protein extracts, cells were sampled 3 d after subculturing (middle of their exponential phase of growth) or 11 d after subculturing (non-proliferating cells).

*Medicago sativa* ssp. *varia* clone A2 suspension-cultured cells were grown in the same conditions and subcultured every 7 d at 10/100 ml. Cells were sampled at similar times as for tobacco cells.

Maize caryopses (*Zea mays*, LG31, Limagrain, France) were immersed in tap water for 3 h and then allowed to germinate in Petri dishes on moist filter paper in the dark at 26 °C. Root apices were excised from 3-d-old shoots.

**Obtaining protoplasts**

Protoplasts were prepared from 3-d-old BY-2 suspension cells. After a 10 min wash in their culture medium supplemented with 0.6 M sorbitol, cells were transferred to an enzymatic solution containing 0.1% cellulase RS (Onozuka), 0.1% macerozyme R10 (Onozuka), 0.03% pectolyase (Sigma) in culture medium with 0.6 M sorbitol. The cell suspension was agitated 20 rpm in darkness and at room temperature for 3 h. Then the cell suspension was filtered on 100 μm mesh nylon and centrifuged at 600 rpm for 3 min. The pellet of protoplasts was washed three times with the culture medium with 0.6 M sorbitol.

**Experimental treatments**

To assess the association of cytoskeletal components with epitopes recognized by Cdc42Hs and Rac1 antibodies, cells...
were treated for 1 h with a microtubule disrupting drug (oryzalin), a microtubule depolymerizing physical agent (cold), and an actin disrupting agent (cytochalasin D) as following:

Oryzalin (a gift of DowElanco, Letcombe Regis, UK) was added to the cell suspension at a final concentration of 10 μg ml⁻¹ from a stock solution of 50 mg ml⁻¹ in acetone.

Cytochalasin D (Sigma) was added to the cell suspension for a final concentration of 20 μg ml⁻¹ from a 1 mg ml⁻¹ stock solution in dimethylsulphoxide (DMSO).

For a cold treatment, the culture flasks were plunged into an ice container on a shaker for 1 h.

To assess the association of the endomembrane system with the epitopes recognized by Cdc42Hs and Rac1 antibodies, cells were treated for 1 h with a Golgi apparatus disrupting agent, brefeldin A (Alexis Corp.), for a final concentration of 100 μg ml⁻¹ from a stock solution of 20 mg ml⁻¹ in DMSO.

**Immunofluorescence**

The procedure were adapted from Satiat-Jeunemaitre et al. (1996). Biological material was fixed for 1 h at room temperature with paraformaldehyde 3% in a modified PBS (Phosphate Buffer Saline supplemented with 100 mM EGTA and 100 mM MgSO₄), pH 6.9. For protoplasts, 0.4 M sorbitol was added to this fixative. Cells, protoplasts or root apices were washed four times in PBS. Cell walls of BY-2 or A2 cells were partially digested by 20 min incubation in enzyme solution: 1% cellulase R10 (Onozuka), 1% pectinase (Sigma) in PBS, pH 6.9, and in the case of root apices by 12 min. After three washes in PBS, 10 μl of cells were allowed to dry on each well of a Vetcabond coated multiwll slide (Vector Laboratories); root apices were gently squashed on the slides in a drop of medium in order to release isolated root cells, and allowed to dry. Cell membranes were permeabilized with 0.5% Triton X-100 for 20 min and washed. Non-specific binding was blocked by 1% Bovine Serum Albumin (BSA). Primary antibodies were applied overnight at 4 °C. Slides were rinsed with a stream of PBS supplemented with 1% fish gelatine (Sigma), in PBS, pH 6.9, and in the case of root apices by 12 min. After three washes in PBS, 10 μl of cells were allowed to dry on each well of a Vetcabond coated multiwll slide (Vector Laboratories); root apices were gently squashed on the slides in a drop of medium in order to release isolated root cells, and allowed to dry. Cell membranes were permeabilized with 0.5% Triton X-100 for 20 min and washed. Non-specific binding was blocked by 1% Bovine Serum Albumin (BSA). Primary antibodies were applied overnight at 4 °C. Slides were rinsed with a stream of PBS supplemented with 1% fish gelatine (Sigma), and then secondary antibodies conjugated with fluorochrome were applied for 1 h at room temperature and in darkness. After a 1 h wash comprising five changes of PBS supplemented with 1% fish gelatine, slides were either mounted with Vetcashield (Vector Laboratories) or retained for a second immunostaining series.

The antibodies used were rabbit polyclonals anti-Cdc42Hs (directed to amino acids 166–182 near the carboxy terminus of human Cdc42; diluted 1:50; referenced as P1, Santa Cruz Biotechnology, Inc.) and anti-Rac1 (directed to amino acids 178–191 at the carboxy terminus of human Rac1; diluted 1:50; referenced as C-14, Santa Cruz Biotechnology, Inc.). A rat monoclonal antibody supernatant JIM84 was used neat as a Golgi marker (Horsley et al., 1993) and a rat monoclonal antibody YOL1/34 (Serotec) used at 1:25 dilution recognizes the α-tubulin.

The Rho antibodies were labelled with anti-rabbit IgG conjugated either with FITC or Texas red. JIM84 and YOL1/34 were labelled with anti-rat IgG conjugated either with FITC or Texas red.

Slides were observed either with a Reichert Polysvar fluorescence microscope or a Sarastro 2000 Confocal microscope (Molecular Dynamics). Photographs were taken on Ilford 400 or 100 ASA film.

Protein extraction from BY-2 and A2 cells and protoplasts

Cells were collected, washed in PBS, centrifuged to a pellet at 1500 rpm and the 100 μl packed-cell volume suspended in 200 μl of ice-cold lysis buffer, pH 7.0 (20 mM Bis-Tris, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 5 mM glycerol, 1.5% polyvinyl pyrrolidone) in the presence of 4 μM sodium cacodylate and protease inhibitors (1 mM PMSF, 1 μM peptatin, 10 μM leupeptin, 10 μM E-64), and lysed on ice by vortexing with an homogenizer. Cell lysates were spun at 10000 g for 3 min. The supernatant containing the soluble and membranous proteins was kept for analysis.

**Immunoblot analysis**

Protein samples were treated by SDS-lysis buffer (Laemmli, 1970) containing 5% β-mercaptoethanol, heated at 100 °C for 3 min, and separated on a 12% SDS-polyacrylamide gel at 25 mA in a Bio-Rad system.

The molecular weight markers (14 to 97.4 kDa; Bio-Rad) were run in parallel with the protein extracts. Gels were either stained with Coomassie Brilliant blue or proteins on gels were transferred to nitrocellulose sheets (0.45 μm; Schleicher and Schuell) by the method of Towbin et al. (1979), and stained with Ponceau S (Sigma) in 1% acetic acid to verify equal loading in each lane. After destaining in water, the sheets were blocked with a solution containing 5% milk powder, 10 mM TBS 0.1 M, pH 7.4 and 0.05% Tween 20 for 90 min prior to incubation with the primary antibody overnight at 4 °C in the milk buffer (Rho antibodies, 1:250; YOL1/34, 1:250). Primary antibodies were detected using alkaline phosphatase conjugated anti-rabbit or anti-rat IgG antiserum (Promega) at 1:5000 dilution. Colour development was carried out by standard Nitro-blue tetrazolium/BCIP procedures.

**[γ³²P]GTP overlays**

After separation of proteins by SDS-PAGE and electrotransfer, the nitrocellulose sheets were washed three times in 50 mM TRIS-HCl, pH 7.5, 1 mM DTT and 0.1% Tween 20. For probing, [γ³²P]GTP (5 μCi ml⁻¹ final concentration) and 10 μM ATP were added to this buffer, and nitrocellulose strips were incubated at 37 °C for 30 min and at 24 °C for 30 min. After three washes with buffer, the strips were air-dried and radioactive bands were visualized by autoradiography with HyperFilm βmax at −80 °C for 5–10 d.

**Results**

The patterns obtained after immunofluorescence staining of tobacco cells, lucerne cells or maize root cells with Cdc42Hs and Rac1 antibodies were the same in the three materials. Therefore results presented here will focus mainly on the tobacco cells.

**Cdc42Hs antibodies often follow a microtubule-like pattern**

Cdc42Hs antibody revealed various fluorescent patterns according to the physiological state of the cells. Interphase cells showed transverse tubular structures organized in an helical manner around the cell (Fig. 1A). This network is not comparable to that described for actin in tobacco cells (data not shown), but mimics the pattern described in interphasic microtubule staining (Fig. 1B); the serial sections made by confocal microscopy never revealed some endoplasmic microtubule staining. However, one constant difference between the Cdc42Hs antibody and
Fig. 1. Localization of the epitopes recognized by anti-Cdc42Hs and anti-tubulin antibodies in interphase BY-2 cells by immunofluorescence. (A) Staining by Cdc42Hs antibodies reveals tubular structures organized in an helical manner around the cell, mimicking the pattern described for interphasic microtubules. In all interphase cells, the nucleoli are also stained. (B) Staining by tubulin antibodies: characteristic cortical microtubular arrays arranged in helical manner are stained. Note that the nucleoli are not stained. In neither case were structures decorated if the first antibody was omitted (photos not presented) (A, ×750; B, ×720).

Fig. 2. Localization of the epitopes recognized by anti-Cdc42Hs (A, B, C) and anti-tubulin (D, E, F) antibodies in dividing BY-2 cells by immunofluorescence. In prophase (A, D), the Cdc42Hs epitopes (A) appear associated with the microtubular preprophase-band (D). In metaphase (B, E), the Cdc42Hs staining (B) is associated with the mitotic spindle (E). In late anaphase/early telophase (C, F), the Cdc42Hs staining (C) is associated with the microtubular phragmoplast (F) (A, D, ×400; B, E, ×1000; C, F ×1100).
tubulin staining patterns was that in the former case the nucleoli were stained too.

In dividing cells, the Cdc42Hs antibody followed reorganization of the network typical of microtubules.

In early prophase, the fluorescent structures aggregated in a girdle in the middle of the cells (Fig. 2A), apparently marking the future site of division. This organization recalls the preprophase band (PPB) of microtubules as outlined by a tubulin staining (Fig. 2D). However, the periphery of the nucleus was clearly outlined in the case of tubulin staining, whereas the fluorescence was more diffuse around the nuclear zone in the case of Cdc42Hs antibody staining.

In metaphase cells, the condensed chromatin was negative, and the labelling was concentrated in a spindle shape (Fig. 2B), resembling metaphasic microtubules (Fig. 2E). At cytokinesis, Cdc42Hs antibody decorated the phragmoplast (Fig. 2C) in a pattern similar to that described by microtubules in the mid-zone of the cell (Fig. 2F), where short tubular structures ran perpendicular to the newly forming cell plate. Moreover with Cdc42Hs antibody, diffuse fluorescence was observed at the location of the reforming nuclei on each side of the phragmoplast.

To analyse the juxtaposition of the epitopes recognized by Cdc42Hs and YOL1/34 antibodies further, double immunostaining was performed (Fig. 3). This confirmed the co-localization of the two staining patterns for the various microtubular arrays: PPB (Fig. 3A, E), mitotic spindle (Fig. 3B, F, C, G), phragmoplast (Fig. 3D, H). However, in addition, Cdc42Hs antibody lit up the nucleus in preprophase (Fig. 3A), and the reforming nuclei in telophase (Fig. 3D), notably the nucleoli.

The finding that Cdc42Hs antibody staining co-localized with microtubules in plant cells (when, in the first place, a structural correlation with actin was expected) led to the further characterization of the effects of cytoskeleton disrupting drugs on the cellular distribution of the epitopes recognized by Cdc42Hs antibody. In order to test an association between proteins recognized by Cdc42Hs antibody and actin cytoskeleton, cells were treated with cytochalasin D then processed for Cdc42Hs antibody and actin cytoskeleton, cells were treated with cytochalasin D then processed for Cdc42Hs antibody and actin staining. The cytochalasin treatment effectively disorganized the actin network (data not shown). However, the pattern of proteins recognized by Cdc42Hs antibody was not altered by actin disruption (Fig. 4A).

The effects of various agents known for disrupting microtubule structures were then tested. Oryzalin fragmented the microtubule network in a typical manner as already described in the literature (data not shown). Interestingly, the Cdc42Hs antibody pattern was also shatttered and, in a similar way (Fig. 4B), the transverse helical arrays in interphasic cells becoming fragmented. However, the nucleolar staining was maintained. The depolymerization of microtubules by cold treatment (data not shown) was associated with a disruption of the proteins recognized by Cdc42Hs antibody in the same way as with oryzalin (Fig. 4C).

These observations suggested that indeed the proteins recognized by Cdc42Hs antibody might be in close association with the microtubule cytoskeleton rather than the actin one.

A third way to disrupt the microtubule pattern was to make protoplasts. The cortical microtubular network was still recognizable even though it had lost its regular helical pattern and was randomly organized (Fig. 5B). In contrast, no cortical nor organized structures could be observed with Cdc42Hs antibody in protoplasts (Fig. 5A). A diffuse staining had replaced the tubular network and the nuclei were no longer stained. By sequential sampling, the collapse of the anti-Cdc42Hs staining pattern could be assigned to the first 20 min of protoplast formation. This observation suggests that the loss of cell polarity during protoplast formation leads to redistribution in the cytoplasm of the epitopes recognized by Cdc42Hs antibody. Indeed, at this stage, double immunostaining with YOL1/34 and Cdc42Hs antibodies clearly shows the dissociation of the two staining patterns in protoplasts. These features were especially noticeable in dividing protoplasts (Fig. 5C, D). These results indicate that the epitopes recognized by Cdc42Hs antibody are not always co-localized with microtubules, and that they are not necessarily related to tubulin structures.

**Cdc42Hs antibody recognizes several bands on Western blots**

On immunoblots of protein extracts from tobacco cells, Cdc42Hs antibody recognized one major band of approximately 55 kDa $M_r$. In some blotting experiments, lighter bands of 35 kDa and, quite exceptionally, of 18 kDa were sometimes observed too. This 55 kDa protein band is higher than the tubulin band (50 kDa) recognized by YOL1/34 (Fig. 6A). In proteins extracted from lucerne cells, Cdc42Hs antibody recognized three bands, namely a major band of approximately 54 kDa $M_r$ and two weaker bands of approximately 35 and 19 kDa $M_r$ (Fig. 6B). Biochemical treatments such as incubation of extracts with 8 M urea never modified the relative intensities of these bands, therefore there was no evidence for dimerization of a smaller protein.

**[$\gamma^{35}S$]GTP overlays**

The bands revealed on blots were evident on autoradiography too. The distribution of [$\gamma^{35}S$]GTP-binding proteins in extracts from BY-2 cells is shown in Fig. 7. Three major GTP-binding protein bands were detected at approximately 19 kDa, 35 kDa and 54 kDa $M_r$, together with several minor bands. The band of approximately
54 kDa $M_r$ may correspond to the band of approximately 55 kDa recognized on Western blots by anti-Cdc42Hs.

**Rac1 antibody decorates several subcellular structures**

Rac1 antibody gave a punctuate pattern throughout the whole cell (Fig. 8A). Two classes of forms were decorated: a population of small units looking like typical immunostaining of plant Golgi stacks (compare Fig. 8A with Fig. 8C), while the others were of larger diameter in a droplet shape. At first sight, this pattern did not change throughout the cell cycle, and dividing cells displayed the same staining as interphase cells. However, in some cases the Rac1 antibody outlined the new cell plate in late anaphase/ telophase in a similar way to that which the JIM84 antibody recognizes Golgi membranes (compare Fig. 8B with Fig. 8D).

These observations suggest that the epitopes recognized by Rac1 antibody are associated with various cytosolic structures, some of which move to the cell plate during cytokinesis.

In double immunostaining of tobacco cells with JIM84 (a Golgi marker) and Rac1 antibodies, the population of small units stained with Rac1 antibody co-localized with Golgi stacks. However, the staining of the 'droplet shape' population did not co-localize with the Golgi marker (data not shown). Rac1 antibody apparently recognizes some other cytosolic structures beyond Golgi stacks.

The effects of a Golgi-disrupting agent, brelefdin A (BFA), upon the patterns of Rac1 and JIM84 antibodies were compared. After a 1 h BFA treatment, the small Golgi stacks coalesced in the cell to form larger fluorescent domains (Fig. 9B). When BFA treated cells were stained with Rac1 antibody, similar broadening of some fluorescent spots was observed (quite distinct from the pre-existing 'droplet-shape' population), but not throughout the cell (Fig. 9A). This reinforces the interpretation that while a protein recognized by Rac1 antibody may be associated with Golgi membranes, it appears also to be associated with some other BFA-insensitive subcellular structures.

The results with anti-Rac1 staining are more clear-cut on immunoblots: proteins extracted from tobacco cells show a unique band with an $M_r$ of approximately 18 kDa (Fig. 6C). A similar band has been observed in extracts from maize root extracts and lucerne extracts (data not shown). These 18 kDa proteins may well be homologous to Rac1 proteins in mammalian cells (21 kDa).

Moreover, in the [$\gamma^{35}$S]GTP overlay experiment it was found that one band, $M_r$ approximately 19 kDa, bound [$\gamma^{35}$S]GTP (Fig. 7).

**Discussion**

The Rho-like proteins identified so far in plant cells have mainly been found by cDNA cloning: Rho1Ps in pea seedlings (Yang and Watson, 1993), Rac9 and Rac13 in cotton fibres (Delmer et al., 1995), Rop1 in pollen tubes (Lin et al., 1996) and two other small GTP-binding proteins belonging to the family Rho/Rac proteins in root nodule from *Lotus japonicus* (Lj-Rac1 and Lj-Rac2; Borg et al., 1997). Rare are the experimental approaches assessing the biochemistry, the localization or the cellular function of these proteins in plant cells. The elegant studies of Lin et al. (1996) and Lin and Yang (1997) on pollen tubes have localized Rop1 to the cortical region of the tube apex and in the periphery of the generative cell and implicated it in tip growth and movement of the generative cell: they demonstrate for the first time the potential role of a Rho protein in plants.

The complementary approach reported in this paper was to exploit similarity between potential plant Rho proteins and mammalian Rho proteins by using antibodies raised against distinctive conserved sequences of mammalian Rho proteins and so to immunostain plant cells. Among the various commercial antibodies tested, Cdc42Hs and Rac1 antibodies appeared especially interesting as they revealed organized staining patterns in all the plant tissues observed.

**Proteins recognized by anti-Cdc42Hs antibody may play a role in cell morphogenesis**

Cdc42Hs is the mammalian homologue of the *Saccharomyces cerevisiae* cell division cycle protein, Cdc42Sc, previously shown to function in the process of polarized growth in yeast (Johnson and Pringle, 1990; Ziman et al., 1993). Cdc42Sc has been shown to localize to the site of bud emergence at the plasma membrane. In mammalian cells, Cdc42Hs also appears critical for normal cell growth and differentiation (Nobes and Hall, 1995); Cdc42 has been reported to influence other cellular activities such as formation and targeted delivery of Golgi derived vesicles, or cell cycle check-point control (Erickson et al., 1996). Little is known regarding the specific biochemical mechanisms by which Cdc42Hs is regulated or how its various effector activities are manifested in vivo.

In plant cells, Cdc42Hs antibody reveals a highly organized pattern, lighting up nucleoli and mimicking microtubule staining in walled cells, but becoming diffuse in the cytoplasm when cells have lost their polarity (i.e. in protoplasts).
The immunoblotting experiments on tobacco cell protein extracts reported here and on tobacco protoplast protein extract (data not shown) do not allow us to identify a unique protein involved in such decoration. In the two cases, one band was recognized by the Cdc42Hs antibody in protein extracts from tobacco cells or from protoplasts, at approximately 55 kDa. This is not compatible with the molecular weight of small GTP-binding proteins (between 20 and 30 kDa) and therefore this protein cannot be a strict equivalent of a Rho GTPase. However, surprisingly, this 55 kDa protein still binds GTP in overlays which are expected to favour monomeric G-proteins: therefore, the band merits further investigation.

In some blotting experiments on tobacco or lucerne protein extracts, 18 kDa bands were sometimes observed too. This 18 kDa protein may be a candidate for a Cdc42-like protein in plant cells. Note that for all these bands observed on Western blots, correlative bands were observed with GTP overlay experiments as well.

The question remains as to which protein is associated with the fluorescent pattern described by the anti-Cdc42Hs antibody. Because the immunofluorescence pattern of Cdc42Hs antibody is the same in the two species, and because the major band of 54–55 kDa is common between the two, it is suspected that this protein is responsible for the fluorescent pattern.

Hypothesizing that the proteins recognized by immunofluorescence may indeed be part of the Cdc42 subfamily of the Rho proteins, it may be surprising at first sight that they appeared linked to the microtubule network, when most of the Rho protein regulatory functions reported in mammalian cells appear to be linked with actin dynamics. The plant cytoskeleton has functions distinctive to those in animal cells: plant cells are not motile, and therefore do not have an actin biology linked for instance to membrane ruffling, membrane protrusion, or substrate adhesion. To assure a subcellular function, plant cells may also use a cytoskeleton partner different from that observed in animal cells: for instance, plant cells appear to maintain the 3-D organization of the Golgi apparatus via actin, and not via microtubules as in mammalian cells (Satiat-Jeunemaitre et al., 1996). Moreover, the microtubules (rather than actin) appear pre-eminent in plant morphogenesis, even if their exact

![Fig. 4. Modification of the Cdc42Hs-like protein pattern in BY-2 cells after 1 h incubation in cytoskeleton disrupting agents. (A) After a 40 μg ml⁻¹ cytochalasin D treatment, the Cdc42Hs antibody staining characteristic of interphase cells is not modified, transverse helical arrays and nucleoli being stained. (B) After a 10 μg ml⁻¹ oryzalin treatment, the Cdc42Hs antibody pattern is modified: organized arrays are fragmented and helical structures are no longer recognizable. The nucleoli remain positive. (C) After a cold treatment (2 °C), the Cdc42Hs staining pattern is similar to that obtained after an oryzalin treatment (A, ×900; B, ×570; C, ×880).](http://jxb.oxfordjournals.org/)

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Fig. 5. Modification of the Cdc42Hs-like protein pattern in BY-2 cells after protoplasting. Immunofluorescence of Cdc42Hs (A, C) and tubulin (B, D) antibodies on BY-2 protoplasts. (A) Cdc42Hs antibody staining shows a diffuse fluorescence in the cytoplasm. Neither the nucleoli nor tubular structures initially outlined in the whole cells are recognizable. (B) Tubulin staining still reveals microtubular arrays randomly organized in the protoplast. (C, D) Double-labelling showing that in dividing protoplasts the anti-Cdc42 staining is diffuse, although tubulin staining is still well localized in a characteristic pattern (mitotic spindle associated with a metaphase) (A, ×1350; B, ×1260, C, D, ×1120).

involvement in the control of transport vesicles and cell wall organization is still not clear (Roberts et al., 1985; Satiat-Jeunemaitre, 1992). Accordingly, proteins recognized by anti-Cdc42Hs may indeed play a role in morphogenesis in plant cells, as they do in yeast or mammalian cells, but via microtubules.

At present, it has not been possible to characterize the nucleolar decoration by Cdc42Hs antibody. Immunoblots were performed on protein extracted from nuclei, revealing similar blotting patterns to those for whole cells, but the purity of these nuclear fractions was not quantified (data not shown). It has been proposed that in activated mammalian cells, Cdc42Hs may provoke sequentially the activation of two nuclear protein kinases and the stimulation of DNA synthesis. By flow cytometry, a multiparametric analysis of Cdc42Hs staining and DNA staining was made on tobacco protoplasts, but the shift of the staining pattern from ‘microtubular-like’ to ‘diffuse cytosolic’ when preparing protoplasts for such analyses render interpretation of the experiment unfeasible.

Nuclear or nucleolar proteins participating for instance in the organization of microtubule arrays during cytokinesis have been identified in eukaryotic cells (Baluska et al., 1997). Similarly, the Cdc42Hs epitopes recognized in nucleoli of interphase cells may be released and relocated to mitotic spindles in the course of mitosis.

Whatever the contribution of the protein recognized by anti-Cdc42Hs on nuclear activities may be, it is
apparently co-ordinated with the cytoplasmic events as the decoration of the cytoplasm and nucleoli were always concomitant.

Whatever the proteins recognized by this anti-Cdc42Hs, these results indicate that this antibody is a good tool to work on cytoskeleton/polarity processes in plant cells.

**Anti-Rac1 antibody recognizes proteins associated with intracellular membranes**

Homologues of Rac proteins have been previously described in plant cells (Delmer et al., 1995; Borg et al., 1997). In maize roots, lucerne roots, lucerne cell suspension, tobacco cell suspension or tobacco protoplasts, anti-Rac1 reveals similar immunofluorescence staining, describing two populations of small bodies. These results indicate that some components of these units are Golgi stacks. The other bodies recognized by Rac1 antibody may well be representative of other intracellular organelles such as peroxisomes.

Anti-Rac1 antibody recognized only one major band of approximately 18 kDa both in tobacco cell protein extracts and in lucerne and maize root protein extracts (data not shown). The immunofluorescent pattern is probably due to this 18 kDa protein. The GTP overlay experiments have shown that some 18 kDa proteins bound GTP. Therefore, it is postulated that in plant cells anti-Rac1 indeed recognizes a Rac-like protein of unknown function.

In mammalian cells, Rac proteins regulate the organization of polymerized actin associated with exploratory filipodia and lamellipodia (Nobes and Hall, 1995; Ridley et al., 1992) critical to defining morphology and movement. They also have been reported to be associated with microtubules (Best et al., 1996). The Rac-like Rho1Ps of pea appears to be associated with vesicle docking in the growing pollen tube and with the periphery of its mobile generative cell (Lin and Yang, 1997). In this study, cross-reacting protein(s) appear(s) to be associated with intracellular organelles.

In conclusion, further identification of putative plant Rho proteins and their functions are needed and are underway in our laboratory. The effects of toxins, known in mammalian cells for their specific catalysis of ADP-ribosylation or UDP-glucosylation of the Rho family (Just et al., 1994; Popoff et al., 1996), are currently studied on plant cells. Although these projects were initiated with a broader representation of the Rho family, the present results suggest a certain focus. Plant proteins recognized by Cdc42Hs antibodies should be studied in the context of cell morphogenesis with, surprisingly, emphasis upon tubulin dynamics. A plant Rac-like protein might be studied in the context of endo-

Fig. 6. Western blot of total protein extracts of tobacco and lucerne cells. (A) BY-2 protein extracts: YOL1/34 antibody (lane 1), Cdc42Hs antibody (lane 2); (B) A2 protein extracts: Cdc42Hs antibody; (C) BY-2 protein extracts: Rac1 antibody. (A) Lane 1: YOL1/34 reveals one band of 50 kDa. Lane 2: Cdc42Hs antibody reveals one band of approximately 55 kDa Mr. (B) Cdc42Hs antibody reveals three bands of approximately 54, 35 and 19 kDa. (C) Rac1 antibody reveals one band of approximately 19 kDa Mr. These results are typical of many repetitions. 50 μg of proteins were loaded in each lane.

Fig. 7. [γ32P]GTP overlays of tobacco proteins. The autoradiogram shows the distribution of 32P on putative GTP-binding proteins renatured after SDS-PAGE and electrotransfer: there are three major bands of approximately 54, 35 and 19 kDa Mr. The lane contains 30 μg total proteins.

Fig. 8. Localization of the Rac1 epitopes in BY-2 cells. (A, B) The anti-Rac1 antibodies recognize punctuate subcellular structures dispersed through the cytoplasm of interphase cells (A), and stain the new cell plate during cytokinesis (B). Note that two populations of punctuate structures are recognizable, large organelles and smaller points. (C, D) Localization of Golgi stacks in BY-2 cells by JIM84 antibodies. The staining pattern in BY-2 cells is also punctuate throughout the cytoplasm in interphase cells (C) or associated with the new cell plate in dividing cells (D), suggesting an association between the Rac1 epitopes and Golgi epitopes (A, × 670; B, × 1460; C, × 510; D, × 1250), cp: cell plate.
membrane flow and the homotypic fusion seen, for instance, at the phragmoplast. The quality of these antibodies favours cloning of plant homologues via screening of a plant-DNA bacterial expression library. For both candidates there are appropriate yeast mutants that may be tested for complementation with a plant-derived yeast expression library.

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