Rac Homologues and Compartmentalized Phosphatidylinositol 4, 5-Bisphosphate Act in a Common Pathway to Regulate Polar Pollen Tube Growth

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Abstract. Pollen tube cells elongate based on actin-dependent targeted secretion at the tip. Rho family small GTPases have been implicated in the regulation of related processes in animal and yeast cells. We have functionally characterized Rac type Rho family proteins that are expressed in growing pollen tubes. Expression of dominant negative Rac inhibited pollen tube elongation, whereas expression of constitutive active Rac induced depolarized growth. Pollen tube Rac was found to accumulate at the tip plasma membrane and to physically associate with a phosphatidylinositol monophosphate kinase (PtdIns P-K) activity. Phosphatidylinositol 4, 5-bisphosphate (PtdIns 4, 5-P_2), the product of PtdIns P-Ks, showed a similar intracellular localization as Rac. Expression of the pleckstrin homology (PH)-domain of phospholipase C (PLC)-δ_1, which binds specifically to PtdIns 4, 5-P_2, inhibited pollen tube elongation. These results indicate that Rac and PtdIns 4, 5-P_2 act in a common pathway to control polar pollen tube growth and provide direct evidence for a function of PtdIns 4, 5-P_2 compartmentalization in the regulation of this process.

Key words: pollen tube • polarity • actin • Rac • PtdIns 4, 5-P_2

Rho, Rac, and Cdc42 homologues, which constitute the Rho family of Ras related small GTPases, are signaling molecules with key roles in the regulation of a variety of cellular processes in animal and yeast cells (Hall, 1998). They act as molecular switches by transducing signals in the GTP-bound conformation and by returning to an inactive GDP-bound state after GTP hydrolysis (Bourne et al., 1991). The best characterized function of Rho family proteins is the regulation of actin organization. Rho, Rac, and Cdc42 homologues each control the formation of distinct actin structures in different cell types (Hall, 1998). Drastic rearrangement of the cortical actin cytoskeleton accompanies the induction of secretion in animal cells (Aunis, 1998). Rho and Rac were found to regulate mast cell secretion, both via controlling actin organization and in an actin-independent manner (Price et al., 1995; Mariot et al., 1996; Norman et al., 1996). *Saccharomyces cerevisiae* Cdc42 plays an essential role in bud formation during vegetative reproduction. Together with a group of other proteins, including several Rho homologues, Cdc42 is thought to control the assembly of polarized, actin-containing complexes at the cell surface that mediate targeted secretion required for bud growth (Mata and Nurse, 1998). Although the function of *Schizosaccharomyces pombe* Rho homologues is less well characterized, these proteins are also known to localize to the cell cortex specifically at growth sites (Arellano et al., 1997; Hirata et al., 1998).

Phosphatidylinositol monophosphate kinases (PtdIns P-Ks),[1] which synthesize phosphatidylinositol 4, 5-bisphosphate (PtdIns 4, 5-P_2), are among the proteins that have

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1. *Abbreviations used in this paper:* ARNO, ADP-ribosylation factor nucleotide-binding site opener; α-At-Rac, affinity-purified polyclonal anti–At-Rac antibody; GFP, green fluorescent protein; GST, glutathione *S*-transferase; GUS, β-glucuronidase; GroPIns, glycerophospho-inositol; Ins, inositol; K, kinase; ORF, open reading frame; PH, pleckstrin homology; PLC, phospholipase C; PNS, post-nuclear supernatant; PtdIns, phosphatidylinositol; TcdB, *Clostridium difficile* toxin B.

been identified as Rac and Rho effectors (Chong et al., 1994; Hartwig et al., 1995). Stimulation of PtdIns 4, 5-P_2 synthesis appears to be a pathway that could allow Rho family GTPases to control targeted secretion by regulating actin organization and exocytotic membrane traffic in a coordinated manner (Martin, 1997; Van Aelst and D'Souza-Schorey, 1997). PtdIns 4, 5-P_2 is a ligand for a large number of regulatory proteins, including actin-binding proteins and proteins with pleckstrin homology (PH-) or C_2-domains. Binding to PtdIns 4, 5-P_2 may affect the activities of these proteins and/or recruit them to target membranes (Lemmon et al., 1997; Kopka et al., 1998; Toker, 1998). It is well established that PtdIns 4, 5-P_2 regulates actin organization via its interaction with key actin-binding proteins such as profilin, gelsolin, and vinculin (Janmey, 1994). Synthesis of PtdIns 4, 5-P_2 has also been demonstrated to be essential for regulated secretion in animal cells (Hay et al., 1995). Although the precise function of PtdIns 4, 5-P_2 in this process is not known, Hay and coworkers suggested that it acts by controlling actin organization, by altering the lipid composition of membrane microdomains, and/or by recruiting proteins that mediate membrane fusion. Compartmentalized synthesis of PtdIns 4, 5-P_2 and other membrane lipids was proposed to be a key step in the organization of localized signaling events (Carpenter and Cantley, 1996; Martin, 1997; Irvine, 1998). However, only limited direct evidence for the accumulation of particular lipids in specific membrane domains has been reported to date.

A surprisingly large number of homologues of Rho family small GTPases has been cloned from different plant species. Interestingly, most of the cloned genes encode proteins that are closely related to each other and to mammalian Rac (Winge et al., 1997; Li et al., 1998). Little information is currently available on the cellular functions of these proteins. A pea Rac homologue (Rop1Ps) was found to be specifically expressed in pollen and in pollen tubes. Microinjection of an antibody against this protein inhibited pea pollen tube elongation (Lin and Yang, 1997). Using immunofluorescence techniques, Rop1Ps was determined to localize to the pollen tube plasma membrane in the tip region (Lin et al., 1996). These results indicated a possible role of Rac homologues in the regulation of pollen tube growth.

Pollen tubes are highly specialized, extremely elongated cells with a diameter of 10–20 μm and length of up to several centimeters. Male generative cells are enclosed in the pollen tube cytoplasm. Growing through the style, pollen tubes transport generative cells from the stigma to the ovule, where fertilization occurs. Pollen tubes extend in a strictly polar manner with rates of several micrometers per minute and are among the fastest growing cells known to exist (Bedinger et al., 1994).

The structure of pollen tube cells has been examined in numerous studies (Pierson and Cresti, 1992; Taylor and Hepler, 1997). The living protoplast is located in a 1–2-mm-long region at tip, whereas the rest of the pollen tube consists of nothing but cell wall material. The pollen tube protoplast shows a characteristic longitudinal zonation. An apical cytoplasmic "clear zone" packed with post-Golgi secretory vesicles is followed by more granular cytoplasm that contains other cell organelles and male generative cells. Large vacuoles are located at the basal end. Longitudinally oriented, thick actin bundles are found throughout the cytoplasm with the exception of the apex, where actin filaments are sparse and fine (Miller et al., 1996; Kost et al., 1998).

The following mechanisms have been implicated in polar pollen tube extension (Derksen et al., 1995; Taylor and Hepler, 1997). Pollen tube elongation is thought to be based on a process known as "tip growth." Post-Golgi secretory vesicles are believed to fuse with a small area of the plasma membrane at the tube apex and to deliver cell membrane as well as cell wall material required for growth exclusively to this location. As observed in other tip growing cells, rapid translocation of pollen tube organelles along the longitudinal axis ("cytoplasmic streaming") is essential for sustained secretion and tube elongation. Pollen tube growth is very sensitive to drugs that interfere with actin polymerization and insensitive to microtubule inhibitors. Actin is believed to mediate cytoplasmic streaming and may also have a function in the secretory processes at the pollen tube tip.

Several reports have demonstrated an important role of Ca^{2+} in the regulation of pollen tube growth. A tip-focused Ca^{2+} gradient with highest concentrations in the cell cortex at the extreme apex has been detected in pollen tube cells. The steepness of the gradient was found to correlate with the speed of pollen tube elongation (Pierson et al., 1996). Interestingly, reorientation of pollen tube growth was induced by photoactivated local release of caged Ca^{2+} in the flanks of the apical dome (Malhó and Trewavas, 1996). Pharmacological experiments in combination with analysis of effects of local release of caged inositol 1, 4, 5-triphosphate (Ins-P_3) have provided evidence suggesting that phospholipase C (PLC)-mediated Ins-P_3 production from PtdIns 4, 5-P_2 is involved in Ca^{2+} regulation in pollen tubes (Franklin-Tong et al., 1996).

Here, we describe the cloning of an *Arabidopsis thaliana* Rac type small GTPase that is preferentially expressed in pollen and in pollen tubes. The function of this protein and its tobacco homologues was analyzed in cultured tobacco pollen tubes. Our results indicate that Rac proteins and PtdIns 4, 5-P_2 both localize to the tip plasma membrane and act in a common pathway to regulate polar pollen tube growth. Possible links between Rac and Ca^{2+} signaling in growing pollen tubes are discussed.

Materials and Methods

Cloning of At-Rac cDNA and Genomic Sequences

An *At-Rac1* cDNA (Xia et al., 1996) probe was used to screen an *A. thaliana* whole-plant Uni-ZAP XR cDNA library (Stratagene). From phages containing hybridizing sequences, pBluescriptSK$^-$ phagemids were excised according to the manufacturer's protocol. By sequencing, a phagemid with an insert containing a full-length cDNA encoding an At-Rac1 homologue was identified. The sequence of this cDNA was deposited in the database (GenBank accession number AF107663) and the encoded protein was designated At-Rac2. The MegAlign software package (DNASTAR Inc.) and the Clustal method were used to align the amino acid sequences of At-Rac2 and of related proteins.

An *A. thaliana* genomic library (CD 4–8; Voytas et al., 1990; obtained from the *Arabidopsis* Biological Resource Center, Columbus, OH) was screened with *At-Rac1* and *At-Rac2* cDNA probes. Genomic fragments that specifically hybridized with each probe were subcloned and partially

sequenced. Clones containing sequences identical to *At-Rac1* and *At-Rac2* coding sequences were identified. These clones were later found to overlap with BAC clones sequenced as part of the ESSAII project. Clones M4E13 (GenBank accession number AL022023) and T19K4 (GenBank accession number AL022373) contain the genomic sequences of *At-Rac1* or *At-Rac2*, respectively.

Expression Constructs

pUCAP-GUS was constructed by cloning an expression cassette consisting of the *35S* promoter, a *GUS* (β-glucuronidase) coding sequence interrupted by an intron (*GUS-intron*), and the *nos* poly A addition signal as an SalI fragment from pTJK136 (Kapila et al., 1997) into pUCAP (van Engelen et al., 1995). Sequences upstream of the *At-Rac1* and *At-Rac2* coding regions were amplified by PCR from genomic clones using a polymerase with proofreading capability (*Pfu* polymerase; Stratagene). Primers were designed to amplify the entire *At-Rac1* (~1.1 kb) or *At-Rac2* (~1.9 kb) promoter regions from the ends of adjacent open reading frames (ORFs) to the beginning of the coding sequences and to introduce NcoI sites at the start codons. Amplified *At-Rac* promoters were fused via the NcoI site to the *GUS-intron* sequence by cloning restricted PCR fragments into pUCAP-GUS. Insertion of the *At-Rac* promoters replaced the *35S* promoter originally present in this construct. The resulting plasmids were used to analyze promoter activity in growing pollen tubes in transient expression experiments. *At-Rac1::GUS-intron::nos* and *At-Rac2::GUS-intron::nos* expression cassettes were subcloned from these constructs as HindIII or XbaI fragments, respectively, into the binary vector pPZP211 (Hajdukiewicz et al., 1994). Resulting plasmids were used to generate stably transformed *A. thaliana* lines.

Sequences encoding At-Rac2 and At-Rac2ΔCSIL were amplified by PCR from the *At-Rac2* cDNA in pBluescriptSK$^-$. A DNA fragment encoding the NH_2-terminal catalytic domain of *Clostridium difficile* toxin B (TcdB; amino acids 1–546) was also amplified by PCR using the construct "CDB1-546 in pGEX-2T" (Hofmann et al., 1997) as a template. Primers were designed to create an ATG context optimal for gene expression in dicot plants (AACAATG; Lütcke et al., 1987) and to introduce stop codons at the 3′ ends of the *At-Rac2ΔCSIL* and *TcdB* sequences. PCR fragments, after confirmation of error-free amplification by sequencing, as well as a *GUS* cDNA (derived from pBI121; CLONTECH Laboratories Inc.) were inserted into the pUCAP-based expression vector pLAT52MCS (Kost et al., 1998) between the *lat52* promoter and the *nos* poly A addition signal. QuikChange™ (Stratagene) PCR-based mutagenesis was used to create mutant sequences encoding G^{15}V-At-Rac2, T^{20}N-At-Rac2, and Q^{64}E-At-Rac2. After sequence confirmation, mutated *At-Rac2* cDNAs were subcloned into nonamplified pLAT52MCS.

The cloning of sequences encoding green fluorescent protein (GFP) and GFP fused to the NH_2 terminus of the mouse talin f-actin binding domain into pLAT52MCS was described earlier (Kost et al., 1998). Into the same expression vector, sequences were inserted that encoded GFP fused to the NH_2 terminus of the following polypeptides: At-Rac2, At-Rac2ΔCSIL, and G^{15}V-At-Rac2 (sequences subcloned from the vectors described above); the PH-domain of human PLC-δ_1 (amino acids 2–175 of PLC-δ_1; sequence subcloned from "PLC-δ1-PH in Hiro3"; Stauffer et al., 1997); and the PH-domain of ADP-ribosylation factor nucleotide-binding site opener (ARNO) (amino acids 262–399 of ARNO; sequence amplified from "ARNO in pEGFP-C1", Venkateswarlu et al., 1998. The DraIII-SalI fragment at the 3′ end of the PCR product was found to contain a point mutation and was replaced by corresponding wild-type sequences from "ARNO-PH in pEGFP-C1"; the final construct was free of sequence errors). The *GFP-PLC-δ_1-PH* sequence in pLAT52MCS was altered by QuikChange™ mutagenesis to sequences encoding GFP-PLC-δ_1-PH-K^{32}L and GFP-PLC-δ_1-PH-K^{32}E. Correct amplification of the regions encoding mutated versions of PLC-δ_1-PH was confirmed by sequencing.

A. thaliana Transformation

Transgenic *A. thaliana* ecotype Landsberg *erecta* lines were generated as described in Kost et al. (1998) using the *Agrobacterium* vacuum-infiltration method developed by Bechtold et al. (1993). T1 seeds were plated on MS medium (Murashige and Skoog, 1962) containing 50 mg/liter kanamycin and 100 mg/liter cefotaxime. In vitro grown, kanamycin-resistant T1 plants at different developmental stages as well as pollen collected from such plants were analyzed for GUS expression as described below.

Transient Gene Expression in Cultured Tobacco Pollen Tubes

For transient expression, genes were transferred into cultured tobacco pollen tubes by particle bombardment as described in Kost et al. (1998). Coexpression of two genes was achieved by coating particles with equal amounts of each expression vector.

Histochemical Analysis of GUS Expression

To assay for GUS activity, *A. thaliana* plants and pollen grains were immersed for 12–18 h in a X-Gluc substrate solution. Assayed plants were extracted with ethanol to remove chlorophyll and improve visibility of the blue GUS reaction product. Destained plants were examined under a dissection microscope. Plant organs and pollen grains were mounted in water between slides and coverslips for observation at higher magnifications.

To visualize transient GUS expression, X-Gluc solution (1 ml per plate) was evenly distributed on the surface of solid culture medium covered with pollen tubes grown from bombarded grains. After 2 h of incubation, squares of solid medium were cut out and flipped upside-down (pollen tubes in direct contact with the glass) onto coverslips.

Stained plant organs, pollen grains, and pollen tubes were analyzed by bright-field transmitted light microscopy using an Axioscope (Carl Zeiss Inc.) microscope. Images were taken by 35-mm photography (64T film; Eastman Kodak Co.).

Incubation in X-Gluc solution was performed at 37°C. All X-Gluc solutions were buffered with 0.1 M sodium phosphate at pH 7.0. Plants were incubated in a solution containing 0.2% X-Gluc (Jersey Lab and Gloves Supply), 0.1% Triton X-100, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 6% *N*,*N*-dimethyl-formamide (DMF). The same solution supplemented with 5% mannitol was used for pollen tubes. Pollen grains were assayed in 0.1% X-Gluc, 0.1% Triton X-100, 3% DMF, and 5% mannitol.

Fluorescence Microscopy

Epifluorescent images of GFP-expressing pollen tubes were taken through a 4× lens using a standard FITC filter set and an Axioscope microscope equipped with a 100-W mercury lamp. All images were obtained by exposing 400 ASA 35 mm film (EliteII; Eastman Kodak Co.) for 10–15 s. Confocal analysis of GFP expression was performed using an LSM410 inverted confocal microscope (Carl Zeiss Inc.) as outlined in Kost et al. (1998). Methods used to determine pollen tube growth rates and techniques used for digital image processing are described in the same report. Intensity plots were created using the Scion Image software package (Scion Corp.).

Preparation of Recombinant Protein

Recombinant glutathione S-transferase (GST) and GST fusion proteins were prepared as described by Lemichez et al. (1997) with some modifications. Synthesis of recombinant protein in *Escherichia coli* BL21 was induced by treatment with 0.5 mM IPTG for 2 h at 30°C. Cells were lysed and recombinant proteins were purified in PB buffer (50 mM Tris-HCl at pH 7.4; 250 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT). After purification, recombinant proteins on agarose beads were resuspended in PB buffer containing 50% glycerol, frozen in liquid nitrogen, and stored at −80°C. For GTP binding and kinase binding assays, recombinant proteins on agarose beads were loaded with nucleotides for 20 min at room temperature in loading buffer (50 mM Tris-HCl at pH 7.4; 25 mM NaCl, 1 mM EDTA, 0.5 mM DTT) containing 0.1 mM [γ-^{32}P]GTP (6,000 Ci/mmol; New England Nuclear), GTPγS, or GDPβS (both Boehringer Mannheim), followed by addition of $MgCl_2$ to a final concentration of 20 mM. GTP binding and GTPase activity assays were performed using protocols established by Self and Hall (1995a and 1995b, respectively).

Preparation of Pollen Tube Extracts

Tobacco pollen tubes (1 g fresh weight) grown for 3 h in liquid PT medium (Kost et al., 1998) were washed twice with a solution containing 150 mM potassium phosphate (pH 7.2), 0.4 M mannitol, and 150 mM NaCl. After resuspension in 5 ml lysis buffer, pollen tubes were passed twice through a Dounce homogenizer. If nothing else is indicated, a solution containing 10 mM Hepes (pH 7.4) and 0.8 M sorbitol was used as lysis buffer. All lysis buffers used were supplemented with a protease inhibitor cocktail (Com-

plete™, EDTA-free; Boehringer Mannheim) according to the manufacturer's instructions.

Preparation of Affinity-purified Anti–At-Rac Antibody (α-At-Rac), SDS-PAGE, and Immunoblotting

Polyclonal anti–At-Rac antiserum was obtained from rabbits immunogenized with GST-At-Rac1. Standard methods described in Harlow and Lane (1988) were used to affinity purify specific anti–At-Rac antibodies (α-At-Rac) on nitrocellulose membranes loaded by blotting with thrombin-treated, GST-free At-Rac1. Pollen tube and recombinant proteins were resolved by SDS-PAGE according to Laemmli (1970) under reducing conditions using 12% gels. For immunoblotting, proteins were transferred onto PVDF membranes (Schleicher & Schuell). Membranes were incubated with primary antibodies, affinity-purified α-At-Rac (diluted 1:200) or a monoclonal mouse anti-actin antibody (Boehringer Mannheim; diluted 1:2,500), and secondary antibodies, peroxidase-conjugated goat anti–rabbit or goat anti–mouse antibodies (Boehringer Mannheim; diluted 1:5,000), for 1 h at room temperature in Tris-buffered saline (TBS; 25 mM Tris-HCl at pH 7.4; 137 mM NaCl, 5 mM KCl) supplemented with 4% BSA (A-3059; Sigma Chemical Co.). After incubation with primary and secondary antibodies, membranes were washed twice for 10 min with TBS containing 4% BSA and 0.02% Tween 20. After the final wash, membrane-associated peroxidase activity was visualized using the ECL kit (Boehringer Mannheim) according to the manufacturer's instructions.

TcdB Glucosylation Assays

Pollen tube extracts were prepared as described above with homogenization performed in FB (50 mM triethanolamine at pH 7.8; 150 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, protease inhibitors). TcdB glucosylation reactions were carried out as described in Just et al. (1995). In brief, 0.5 μg TcdB (obtained from Ingo Just, Albert-Ludwigs-Universität, Freiburg, Germany) and 0.3 μCi UDP-[^{14}C]-glucose (300 mCi/mmol; New England Nuclear) in FB buffer were added to extract containing 10 μg total pollen protein or to PB containing 1 μg recombinant GST-At-Rac2 to obtain a final volume of 20 μl. The reaction mix was scaled up to a total volume of 200 μl for immunoprecipitation experiments. Reactions were carried out at 37°C for 1 h. Products were resolved by SDS-PAGE either directly or after immunoprecipitation with affinity-purified α-At-Rac (diluted 1:20). Gels were incubated for 10 min in 1 M salicylic acid before drying.

Pollen Tube Fractionation

Pollen tube extracts were centrifuged for 10 min at 10,000 *g* to obtain a post-nuclear supernatant (PNS) with a protein concentration of ~0.25 mg/ml. The resulting PNS was centrifuged at 100,000 *g* for 1 h (SW-55 Ti rotor; Beckman Instruments Inc.) to separate cytoplasmic (supernatant) and membrane (pellet) fractions. Pelleted membranes were resuspended in lysis buffer. Proteins contained in 20 μl of each PNS, cytoplasmic, and resuspended membrane fractions were analyzed by immunoblotting. TcdB glucosylation assays were carried out as described above using 5 μl of each fraction.

Kinase Binding Assays

Kinase binding assays were performed as described by Ren et al. (1996) with some modifications. Pollen tube extracts were prepared as described above with homogenization performed in buffer A (50 mM Tris-HCl at pH 7.4; 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, protease inhibitors). Extracts were clarified by centrifugation at 13,000 *g* for 15 min at 4°C. Agarose beads bound to GST (25 μg protein) or to GST-At-Rac2 (2.5 μg protein) loaded with nucleotides as described above were added to 500 μl clarified extract (0.5 mg/ml total protein) and incubated for 2 h at 4°C. After incubation, beads were washed three times in buffer B (50 mM Tris-HCl at pH 7.4; 0.1% Triton X-100, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) and once in buffer C (50 mM Tris-HCl at pH 7.4; 0.02% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT). After resuspension in buffer C during the last washing step, one-tenth of the total volume of each sample was analyzed by SDS-PAGE and Coomassie brilliant blue staining to verify concentration and quality of the recombinant proteins used. Washed beads were resuspended in 35 μl buffer C. Samples used to investigate effects of phosphatidic acid on At-Rac2–associated kinase activity were treated similarly, but Triton X-100 was omitted from all buffers except for buffer A. Beads in 35 μl buffer C were supplemented with 20 μg lipid substrates in 10 μl 10 mM Tris-HCl, pH 7.4, and incubated at room temperature for 10 min. Either PtdIns 4-P (P-9628; Sigma Chemical Co.) or a mix of phosphoinositides (P-6023; Sigma Chemical Co.) was used as a substrate. Some samples were supplemented with phosphatidic acid (P-9511; Sigma Chemical Co.) to a final concentration of 40 μM. Kinase reactions were started by adding 5 μl ATP buffer (50 mM Tris-HCl at pH 7.4; 5 mM $MgCl_2$, 0.5 mM ATP, 10–25 μCi [γ-^{32}P]ATP, 6,000 Ci/mmol; New England Nuclear) to each sample. After 7 min, reactions were stopped with 80 μl HCl (1 N). Lipids were extracted in 160 μl methanol/chloroform (1:1) by vigorous mixing for 1 min. Organic and aqueous phases were separated by centrifugation. The lower organic phase was washed with 260 μl HCl (1 N)/methanol/chloroform (48:47:3), concentrated under N_2 to a volume of 20 μl and spotted on Oxalate-EDTA–impregnated silica gel plates (LK6D; Whatman Inc.). Chromatography was performed as described by Pignataro and Ascoli (1990).

Radioactivity Detection

Radioactivity emitted from membranes, gels, or TLC plates was visualized using a PhosphorImager detection system (Molecular Dynamics).

HPLC Analysis

^{32}P-labeled products of kinase binding assays performed as described above using a mix of phosphoinositides as lipid substrate were deacylated, mixed with ^{3}H-labeled standards, and analyzed by anion-exchange HPLC using a Partisphere SAX column (Whatman) as described by Serunian et al. (1991). Elution of assay products and standards was detected simultaneously using an on-line continuous flow scintillation detector (Radiomatic® FSA; Packard Instrument Co.).

Results

Cloning of Sequences Encoding an A. thaliana Rac Homologue That Is Preferentially Expressed in Growing Pollen Tubes

Previously, we reported the cloning of *At-Rac1*, which was identified in a screen for *A. thaliana* cDNAs that cause morphological changes when expressed in yeast (Xia et al., 1996). Using *At-Rac1* as a probe, *At-Rac2* was isolated from an *A. thaliana* cDNA library. The corresponding genomic sequences were cloned by screening an *A. thaliana* library with *At-Rac1* or *At-Rac2* cDNA probes. Genomic sequences upstream of the *At-Rac1* and *At-Rac2* ORFs, including the ends of adjacent ORFs and the entire *At-Rac* 5′ untranslated regions, were fused to a cDNA encoding GUS (Jefferson et al., 1987). Transgenic *A. thaliana* lines containing the resulting *At-Rac1* or *At-Rac2* promoter-*GUS* fusion constructs (*At-Rac1::GUS*, *At-Rac2::GUS*) were generated and histochemically analyzed for GUS expression. Four independent lines transformed with *At-Rac2::GUS* showed GUS expression confined to pollen (Fig. 1 a), to growing pollen tubes, to stipules, and to a small region of the vascular tissue below the cotyledons (data not shown). In contrast, six lines transformed with *At-Rac1::GUS* were all found to express GUS in the vascular tissues of all organs. Only one of these lines showed a weak GUS expression in pollen (data not shown).

GUS is known to be a stable protein with a slow turnover rate in plant cells (Martin et al., 1992). To confirm that GUS activity detected in transgenic pollen tubes resulted at least partially from gene expression during pollen germination and tube growth, promoter-*GUS* fusion constructs were also transiently expressed in growing tobacco (*Nicotiana tabacum*) pollen tubes. Under these conditions,

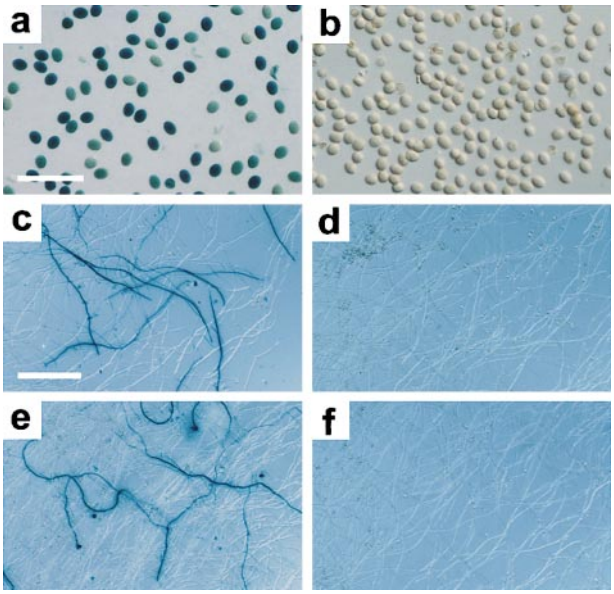

Figure 1. The *At-Rac2* promoter confers *GUS* expression in transgenic pollen grains and in transiently transformed pollen tubes. (a and b) *A. thaliana* ecotype Landsberg *erecta* pollen grains assayed for GUS activity. (a) Transgenic *At-Rac2::GUS* pollen. (b) Wild-type pollen. (c–f) Tobacco pollen tubes assayed for GUS activity 16 h after gene transfer into germinating pollen grains. Pollen grains were bombarded with expression constructs containing the following sequences: (c) *At-Rac2::GUS*; (d) *At-Rac1::GUS*; (e) *lat52::GUS*; and (f) *lat52::GFP*. Bars, a and b, 250 μm; c–f, 500 μm.

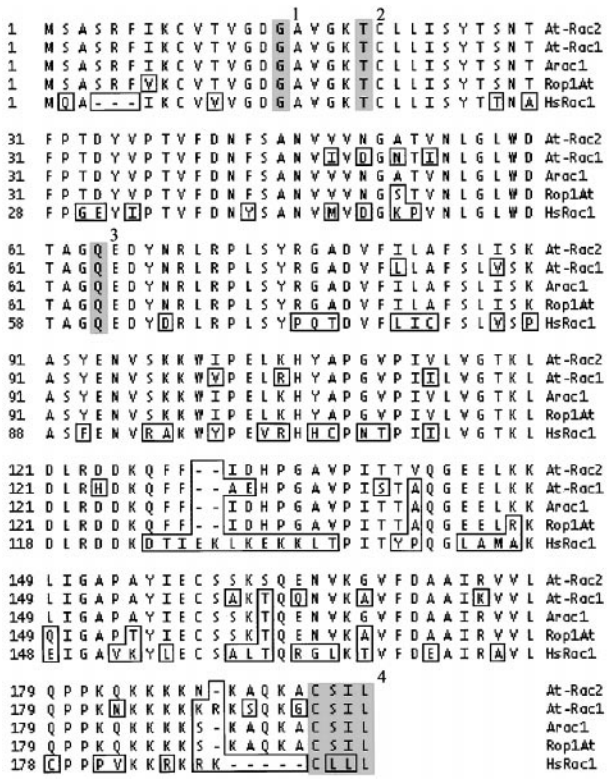

Figure 2. Alignment of the amino acid sequences of At-Rac2 and of homologous proteins. Amino acids different from the At-Rac2 sequence are boxed. Conserved amino acids which have been mutated are shaded: [1]constitutive active G^{15}V-At-Rac2: Gly^{15} replaced by Val; [2]dominant negative T^{20}N-At-Rac2: Thr^{20} replaced by Asn; [3]constitutive active Q^{64}E-At-Rac2: Gln^{64} replaced by Glu; [4]At-Rac2ΔCSIL: prenylation domain Cys-Ser-Ile-$Leu^{194–197}$ deleted. GenBank accession numbers: U62746 (At-Rac1), AF107663 (At-Rac2 cDNA), U41295 (Arac1 cDNA), U49971 (Rop1At), and M29870 (HsRac1).

the *At-Rac2* promoter was found to confer strong GUS expression (Fig. 1 c) at a level similar to that obtained with the pollen-specific *lat52* promoter (Twell et al., 1989; Fig. 1 e). No GUS expression was observed in pollen tubes transiently transfected with *At-Rac1::GUS* (Fig. 1 d) or with a control construct containing a cDNA encoding GFP (Prasher et al., 1992) fused to the *lat52* promoter (Fig. 1 f). These results indicate that *At-Rac2*, but not *At-Rac1*, is preferentially expressed in pollen and in growing pollen tubes.

At-Rac1 and *At-Rac2* encode proteins of 21 kD that show high homology to human HsRac1 (62.0 and 61.3% identical amino acids, respectively; Fig. 2). Recombinant At-Rac1 and At-Rac2 were both found to bind and to hydrolyze GTP (data not shown), confirming that the two proteins are indeed functional small GTPases. They belong to a large family of Rac-like proteins with unknown or poorly characterized cellular functions whose genes have been cloned recently from different plant species. The protein sequences of Arac1 (Winge et al., 1997) and Rop1At (Li et al., 1998), two *A. thaliana* Rac-like proteins that share very high sequence homology with At-Rac2 (98.5 and 95.4% identical amino acids, respectively), are shown in Fig. 2. Interestingly, Rop1At has been shown to be expressed in pollen and was proposed to have a function in the regulation of pollen tube growth (Li et al., 1998).

We found that in situ analysis of At-Rac2 function in cultured *A. thaliana* pollen tubes is not feasible. These cells elongate slowly, show severe morphological abnormalities, and cease growing only a few hours after germination (Taylor and Hepler, 1997; Kost, B., and N.-H. Chua, unpublished observations). By contrast, tobacco pollen tubes can grow rapidly and morphologically normal in vitro for up to 48 h (Read et al., 1993). Methods have been established that allow analysis of transient gene expression in cultured tobacco pollen tubes after gene transfer into germinating pollen grains by particle bombardment (Kost et al., 1998). We have used these methods, along with biochemical techniques, to study the function of At-Rac2 and its tobacco homologues in the regulation of pollen tube growth.

Growing Tobacco Pollen Tubes Express at Least One At-Rac2 Homologue with an Essential Function in Tube Elongation

An antibody raised against At-Rac1 (α-At-Rac) was found to have similar affinities to recombinant At-Rac1 and At-Rac2 (data not shown). Immunoblot analysis showed that in tobacco pollen tube extracts α-At-Rac binds to at least one protein with an apparent molecular mass of 21 kD (Fig. 3 a), which corresponds to the size of

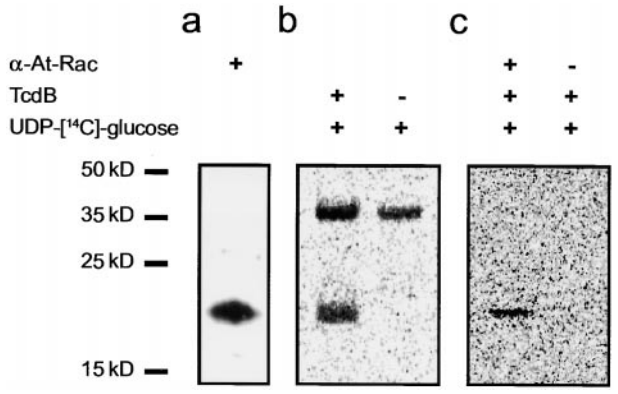

Figure 3. Tobacco pollen tubes express at least one 21-kD protein that is recognized by an anti–At-Rac antibody (α-At-Rac) and glucosylated by TcdB. (a) Immunoblot analysis of pollen tube extract using α-At-Rac. (b) The left lane shows tobacco pollen tube total protein assayed for glucosylation by TcdB and resolved by SDS-PAGE, and the right lane is a control reaction without TcdB. Note that pollen tube extracts apparently contain an enzyme that transfers glucose to a protein of ~40 kD. (c) The left lane is SDS-PAGE analysis of proteins immunoprecipitated by α-At-Rac from pollen tube extracts assayed for glucosylation by TcdB, and the right lane is a control sample treated with preimmune serum. Migration positions of molecular mass markers are indicated on the left.

Rac. *Clostridium difficile* toxin B (TcdB) is known to glucosylate and thereby inactivate specifically mammalian Rho family proteins including Rac (Aktories, 1997). Treatment with TcdB and UDP-[^{14}C]-glucose resulted in glucosylation and labeling of recombinant At-Rac2 (data not shown), showing that plant Rac homologues can serve as substrates of this toxin. Radiolabeled proteins of 21 kD were detected in tobacco pollen tube extracts assayed for glucosylation by TcdB, but not in control samples treated with UDP-[^{14}C]-glucose alone (Fig. 3 b). To investigate whether the detected pollen tube TcdB targets are recognized by α-At-Rac, proteins interacting with this antibody were immunoprecipitated from tobacco pollen tube extracts assayed for glucosylation by TcdB. In these experiments, at least one 21-kD pollen tube protein glucosylated by TcdB was found to interact with α-At-Rac (Fig. 3 c) and was therefore identified as an At-Rac2 homologue. No radiolabeled proteins were precipitated from assayed extracts treated with preimmune serum (Fig. 3 c).

A cDNA encoding the catalytic domain of TcdB (Hofmann et al., 1997) was transiently expressed under the control of the *lat52* promoter in tobacco pollen tubes. Fluorescence emitted by coexpressed GFP was used to identify successfully targeted pollen tubes among an excess of untransformed, nonfluorescent tubes. Transient expression of marker genes did not affect pollen tube growth. Control pollen tubes transfected with expression vectors containing *GFP* and *GUS* coding sequences fused to the *lat52* promoter showed normal morphology (Fig. 4, a–d) and elongated at the same rate as untransformed pollen tubes (data not shown). By contrast, transient expression of the catalytic domain of TcdB clearly inhibited tobacco pollen tube growth (Fig. 4, e–h). Together, these results indicate that tobacco pollen tubes contain at least one At-Rac2 homologue with an essential function in tube elongation.

Transient Expression of Wild-Type and Mutant Forms of At-Rac2 Interferes Drastically with the Polar Extension of Tobacco Pollen Tubes

The function and the intracellular localization of Ras related small GTPases can be predictably altered by introducing specific mutations in conserved domains (Diekmann et al., 1991; Farnsworth and Feig, 1991; Chardin, 1993). Sequences encoding wild-type and mutant forms of At-Rac2 (Fig. 2) were transiently expressed in growing tobacco pollen tubes under the control of the *lat52* promoter. Coexpression of GFP was used to identify targeted tubes. Expression of dominant negative T^{20}N-At-Rac2 strongly inhibited pollen tube extension (Fig. 4, i–l). After reaching a total length (tip to grain) of several 100 μm, T^{20}N-At-Rac2–expressing pollen tubes stopped elongating. By contrast, expression of constitutive active G^{15}V-At-Rac2 or Q^{64}E-At-Rac2 caused germinating pollen tubes to form large, spherical balloons instead of elongating tubes (Fig. 4, m–p, and data not shown, respectively). Apparently, a complete loss of polarity of cell extension was induced. Wild-type At-Rac2 had similar although somewhat weaker effects (Fig. 4, q–t). After germination, pollen tubes elongated normally for a short while before tips started to form balloons. The balloons often had irregular shapes instead of being spherical, presumably because the potential for polarized growth was not completely abolished. At-Rac2ΔCSIL, lacking the COOH-terminal consensus prenylation domain known to be required for the membrane association of Ras related proteins, still induced depolarized tip growth but was clearly less effective than wild-type At-Rac2 (Fig. 4, u–x). These results demonstrate a key role of At-Rac2 homologous proteins in the regulation of polar pollen tube growth and indicate that membrane localization is essential for their activity.

The best characterized function of Rho family proteins in animal and yeast cells is the regulation of actin organization (Hall, 1998). Therefore, effects of transient expression of mutant At-Rac2 on the tobacco pollen tube actin cytoskeleton were examined. G^{15}V-At-Rac2 or T^{20}N-At-Rac2 were coexpressed in pollen tubes with a GFP-mouse talin fusion protein, which we have shown to label plant actin filaments in vivo in a specific and noninvasive manner (Kost et al., 1998). The actin cytoskeleton in normally growing tobacco pollen tubes consists of thick, longitudinally oriented actin bundles in the shank and fine filamentous structures close to the tip (Fig. 5 a; Kost et al., 1998). An excessive number of thick actin cables arranged in a helical pattern was observed in balloons formed by G^{15}V-At-Rac2–expressing pollen tubes (Fig. 5 b). Expression of constitutive active At-Rac2 apparently induced actin polymerization or bundling and, possibly, reorientation of actin cables in the pollen tube tip. By contrast, actin bundles in pollen tubes expressing dominant negative T^{20}N-At-Rac2 were generally finer and less organized than in control tubes, indicating that inhibition of pollen tube Rac activity may reduce actin bundling (Fig. 5 c). As in other cell

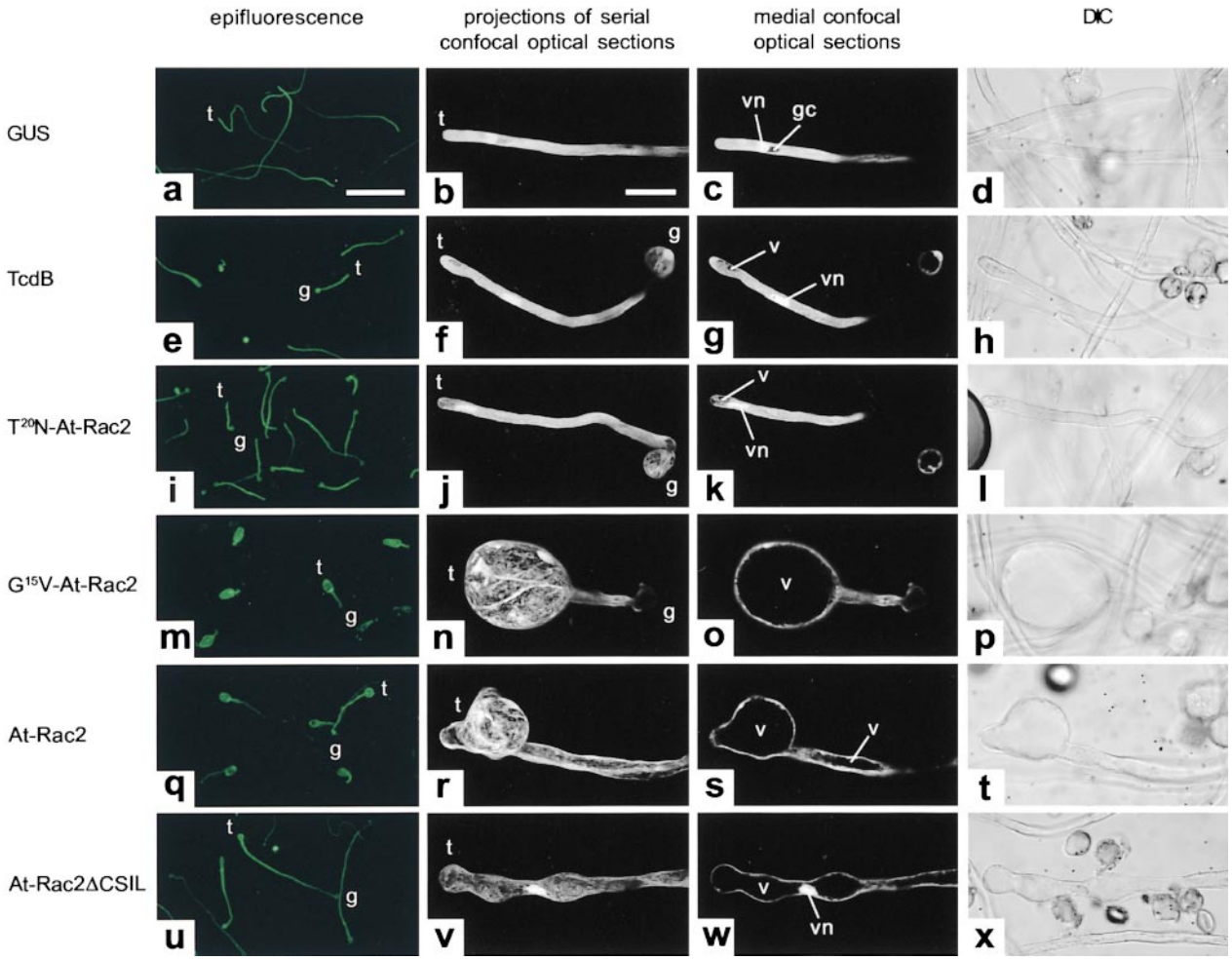

Figure 4. Transient expression of TcdB activity and of wild-type or mutant At-Rac2 affects tobacco pollen tube growth. Sequences encoding the indicated polypeptides were transiently expressed in tobacco pollen tubes by particle bombardment. Coexpression of GFP was used to identify targeted tubes. Epifluorescence pictures of GFP fluorescence were taken at low magnification (first column). Representative pollen tubes were also imaged at higher magnification using confocal (second column: projections of serial optical sections; third column: medial optical sections) and transmitted light differential interference contrast (DIC; fourth column) microscopy. All images were taken 12–18 h after bombardment. (a–d) GUS. (e–h) NH_2-terminal catalytic domain of TcdB. (i–l) $T^{20}N$-At-Rac2. (m–p) $G^{15}V$-At-Rac2. (q–t) Wild-type At-Rac2. (u–x) At-Rac2ΔCSIL. Imaging of GFP fluorescence visualized the living protoplast at the tip of targeted pollen tubes, whereas the empty shank of tubes with a length of more than ~1.5 mm remained invisible. GUS-expressing control pollen tubes (a–d) had reached a total length of ~4 mm at the time of the imaging. Most pollen tubes shown in e, i, m, q, and u are depicted at full length from the tip to the germinated grain. t, pollen tube tip; g, germinated pollen grain; vn, vegetative nucleus; gc, generative cell; v, vacuole. Bars, column 1, 500 μm; columns 2–4, 50 μm.

types, one of the functions of pollen tube Rac appears to be the regulation of the actin cytoskeleton.

Pollen Tube Rac Localizes to the Plasma Membrane at the Tip and to the Cytoplasm

Analysis of pollen tube extracts revealed that a significant portion of the 21-kD tobacco pollen tube protein that interacts with the α-At-Rac antibody cofractionates with membranes, whereas the majority of the protein remains in the cytoplasmic fraction (Fig. 6 a). A similar distribution of radiolabeled protein with the same size was observed when pollen tube extracts assayed for glucosylation by TcdB were fractionated (Fig. 6 c). In control experiments, pollen tube actin showed a typical distribution between cytoplasmic and membrane fractions (Fig. 6 b).

To investigate the intracellular localization of pollen tube Rac in more detail, sequences encoding wild-type and mutant forms of At-Rac2 fused to the COOH terminus of GFP were transiently expressed in tobacco pollen tubes under the control of the *lat52* promoter. A GFP cDNA was also expressed from the same promoter. Although somewhat weaker, effects of expressing the fusion proteins on pollen tube growth (Fig. 7, a–c) were very similar to those observed with untagged wild-type or mutant At-Rac2 (Fig. 4, m, q, and u). This indicates that the fusion proteins were functional and that analysis of their localization provided relevant information on the intracellular distribution of pollen tube Rac proteins.

Tobacco pollen tubes expressing GFP or GFP fused to different forms of At-Rac2 were examined by confocal microscopy. GFP was found to be evenly distributed in the

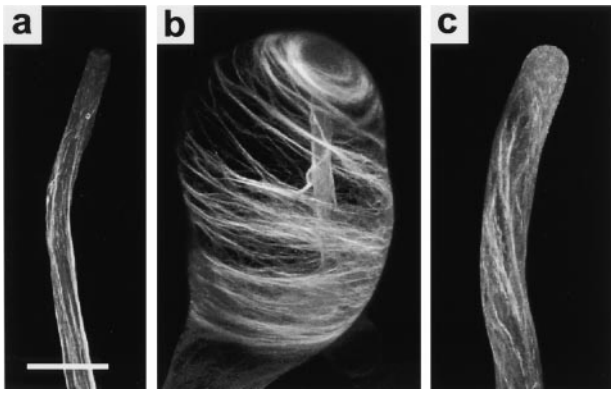

Figure 5. Transient expression of mutant At-Rac2 alters actin organization in tobacco pollen tubes. Sequences encoding the indicated proteins were transiently expressed in tobacco pollen tubes by particle bombardment. Coexpression of GFP fused to the f-actin binding domain of mouse talin was used to visualize the actin cytoskeleton in targeted tubes. Projections of serial confocal optical sections through representative pollen tubes are shown. Images were taken 6–10 h after bombardment. (a) GUS. (b) $G^{15}V$-At-Rac2. (c) $T^{20}N$-At-Rac2. Bar, 25 μm.

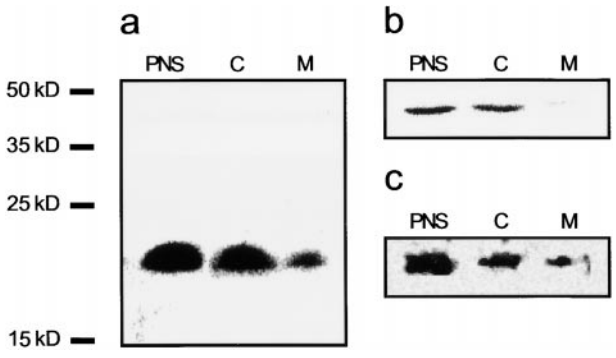

Figure 6. Tobacco pollen tube 21-kD proteins recognized by the α-At-Rac antibody and glucosylated by TcdB partially cofractionate with membranes. Pollen tube extracts were fractionated by centrifugation. Proteins in different fractions were analyzed by application of the indicated techniques. (a) Immunoblotting using α-At-Rac. (b) Immunoblotting using a monoclonal mouse antiactin antibody. (c) TcdB glucosylation assay. Migration positions of molecular mass markers are indicated on the left. C, cytoplasmic fraction; M, membrane fraction.

pollen tube cytoplasm (Fig. 4, b and c, and Fig. 7, h and l). Confocal optical sections through pollen tubes expressing GFP fused to constitutive active (Fig. 7, e and i) or to wild-type (Fig. 7, f and j) At-Rac2 revealed that these fusion proteins accumulated at the cell cortex exclusively in the tube tip, indicating that they associated with the plasma membrane specifically in this pollen tube region. The rest of the fusion proteins was evenly distributed in the cytoplasm (Fig. 7, e, f, i, and j). GFP fused to At-Rac2ΔCSIL did not show membrane association (Fig. 7, g and k) and was localized to the cytoplasm similar to GFP (Fig. 7, h and l).

In summary, the results described here indicate that At-Rac2 homologues localize specifically to the plasma membrane at the pollen tube tip and confirm that the COOH-terminal prenylation domain is essential for their proper localization and function.

Pollen Tube Rac Physically Associates with a PtdIns P-K Activity That Produces PtdIns 4, 5-P_2

PtdIns P-Ks and PtdIns 4, 5-P_2 were identified as effectors of mammalian Rho family small GTPases (Van Aelst and D'Souza-Schorey, 1997). Rac and Rho, both in the GTP- and in the GDP-bound conformation, have been demonstrated to physically interact with PtdIns P-K activity in mammalian cell extracts (Ren et al., 1996; Tolias et al., 1998).

We have tested the possibility that PtdIns P-Ks and PtdIns 4, 5-P_2 act as Rac effectors also in pollen tubes. Experiments were performed to investigate whether At-Rac2 and its tobacco homologues bind to PtdIns P-K activity present in tobacco pollen tube extracts. Recombinant At-Rac2 fused to the COOH terminus of GST was loaded with the nonhydrolyzable nucleotide analogues GTPγS or GDPβS. Proteins interacting with nucleotide-bound GST-At-Rac2 were purified from tobacco pollen tube extracts and assayed for lipid kinase activity using [γ-^{32}P]ATP and a mix of phosphoinositides as substrates. An excess of GST (Fig. 8 a, bottom) was used in control experiments. Labeled lipids produced in kinase assays were analyzed by TLC and autoradiography. Fig. 8 a (top) shows that significant amounts of radiolabeled PtdIns P_2 were synthesized when proteins associated with GTPγS- or GDPβS-loaded GST-At-Rac2 were assayed, whereas minimal levels of PtdIns P_2 production were detected in GST control samples. Similar results were obtained when purified PtdIns 4-P was used as a substrate instead of a mixture of phosphoinositides or when proteins coimmunoprecipitated with At-Rac2 homologues from tobacco pollen tube extracts by the α-At-Rac antibody were assayed (data not shown).

The different PtdIns P_2 isoforms identified to date in plant and/or animal cells, including PtdIns 4, 5-P_2, PtdIns 3, 4-P_2 and PtdIns 3, 5-P_2 (Fruman et al., 1998; Munnik et al., 1998), are difficult to separate on TLC plates. Therefore, ^{32}P-labeled lipid products of kinase binding assay performed as described above using a mix of phosphoinositides as substrates were deacylated and subjected to HPLC. Under conditions that allow clear separation of deacylated PtdIns P_2 isoforms (glycerophospho-inositol-bisphosphates, GroPIns P_2s; Rameh et al., 1997), ^{32}P-labeled, deacylated assay products coeluted from HPLC columns with ^{3}H-labeled GroPIns 4, 5-P_2 used as a standard (Fig. 8 b).

The described results demonstrate that recombinant At-Rac2, both in the GTP- and in the GDP-bound conformation, as well as at least one of its tobacco homologues, physically associates with tobacco pollen tube PtdIns P-K activity, which synthesizes specifically PtdIns 4, 5-P_2.

Expression of GFP Fused to the PH-Domain of PLC-δ_1 Inhibits Tobacco Pollen Tube Growth and Labels the Plasma Membrane at the Tube Tip

The isolated PH-domain of PLC-δ_1 has been shown to bind PtdIns 4, 5-P_2 integrated into lipid membranes specifically and with high affinity, both in vitro (Lemmon et al.,

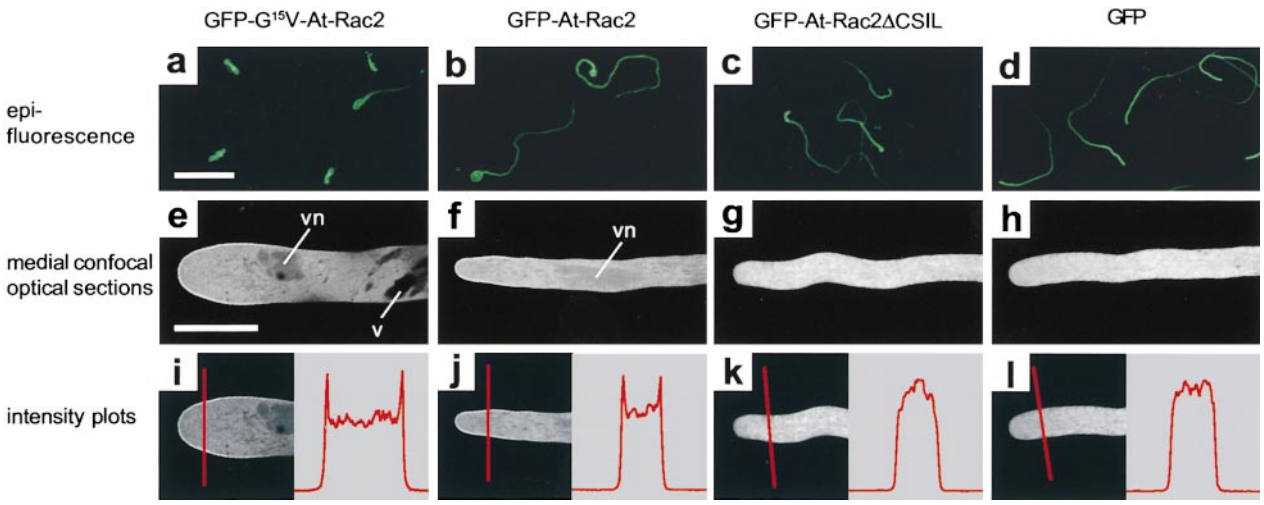

Figure 7. Wild-type and mutant At-Rac2 fused to GFP are functional and localize to the plasma membrane at the tip of tobacco pollen tubes. Sequences encoding the indicated GFP-fusion proteins or untagged GFP were transiently expressed in tobacco pollen tubes by particle bombardment. The first row shows low magnification epifluorescence pictures taken 12–18 h after bombardment. The second row shows medial confocal optical sections through representative pollen tubes imaged 3–4 h after particle bombardment, when expression of the GFP-fusion proteins started to affect tube morphology. The third row is the quantification of fluorescence intensities along the indicated lines. (a, e, and i) GFP-G^{15}V-At-Rac2. (b, f, and j) GFP-At-Rac2. (c, g, and k) GFP-At-Rac2ΔCSIL. (d, h, and l) GFP. vn, vegetative nucleus; v, vacuole. Bars, a–d, 500 μm; e–l, 25 μm.

1995, Tall et al., 1997) and in vivo (Stauffer et al., 1997; Várnai and Balla, 1998). Under the control of the *lat52* promoter, a sequence encoding a PLC-δ_1 PH-domain GFP fusion protein (GFP-PLC-δ_1-PH) was transiently expressed in tobacco pollen tubes. Consistent with a key role of PtdIns 4, 5-P_2 in the regulation of pollen tube growth, moderate levels of GFP-PLC-δ_1-PH expression were sufficient to strongly inhibit tobacco pollen germination and tube growth (Fig. 9 a). Only weakly fluorescent GFP-PLC-δ_1-PH–expressing pollen tubes were able to grow normally. Fluorescence emitted by these pollen tubes was too weak to be visible on micrographs like the one shown in Fig. 9 a. Confocal optical sections through such pollen tubes revealed that GFP-PLC-δ_1-PH accumulated at the plasma membrane in the tube tip (Fig. 9, e and i). Although GFP-PLC-δ_1-PH appeared to label a somewhat smaller area, its localization in tobacco pollen tubes was strikingly similar to that of transiently expressed GFP-At-Rac2 fusion proteins (Fig. 7). Pollen tubes with GFP-PLC-δ_1-PH labeling as shown in Fig. 9 e were indistinguishable from untransformed control tubes in terms of morphology (Fig. 9 e; data not shown) and growth rate (the average growth rate of 20 weakly fluorescent, GFP-PLC-δ_1-PH–expressing pollen tubes was 5 μm/s, which is identical to the average growth rate of untransformed pollen tubes), demonstrating that the observed localization of the fusion protein provides information on a physiologically normal situation.

A number of control experiments supported the view that the specific interaction of GFP-PLC-δ_1-PH with PtdIns 4, 5-P_2 caused inhibition of pollen tube growth and was responsible for the localization of the fusion protein to the plasma membrane at the tip. The free amino group of the lysine residue at position 32 in the PLC-δ_1 PH-domain has been shown to form a direct hydrogen bond to the 4-phosphate of PtdIns 4, 5-P_2 (Ferguson et al., 1995). Replacement of this basic lysine residue by neutral leucine (K^{32}L) or even by glutamic acid (K^{32}E) was demonstrated to abolish the ability of PLC-δ_1 to bind PtdIns 4, 5-P_2 in membranes (Yagisawa et al., 1998). Neither GFP-PLC-δ_1-PH-K^{32}L (Fig. 9, f and j) nor GFP-PLC-δ_1-PH-K^{32}E (Fig. 9, g and k) detectably accumulated at the tip membrane when transiently expressed in tobacco pollen tubes. Whereas high level expression of GFP-PLC-δ_1-PH-K^{32}L still severely inhibited pollen tube growth (Fig. 9 b), even brightly fluorescent tubes expressing GFP-PLC-δ_1-PH-K^{32}E could elongate rapidly and showed an essentially normal morphology (Fig. 9 c; data not shown). The PH-domain of ARNO has been demonstrated to bind with a high degree of specificity to PtdIns 3, 4, 5-P_3 (Irvine, 1998; Venkateswarlu et al., 1998), a lipid that has not been identified in plant cells to date (Munnik et al., 1998). Transient expression from the *lat52* promoter of a cDNA sequence encoding the ARNO PH-domain fused to GFP did not significantly affect tobacco pollen tube growth (Fig. 9 d). No accumulation of the GFP-ARNO-PH fusion protein at the plasma membrane was observed (Fig. 9, h and l).

These results provide strong evidence for an essential function of PtdIns 4, 5-P_2 in pollen tube elongation. Rac proteins, which physically interact with PtdIns 4, 5-P_2 synthesizing PtdIns P-K activity, and PtdIns 4, 5-P_2 both localize to the plasma membrane at the pollen tube tip. This suggests that Rac proteins, PtdIns P-K activity, and PtdIns 4, 5-P_2 act together in a common pathway to regulate polar pollen tube growth.

Discussion

Rac Homologues Control Polar Pollen Tube Growth

Transient expression of wild-type or constitutive active At-Rac2 in tobacco pollen tubes resulted in the formation

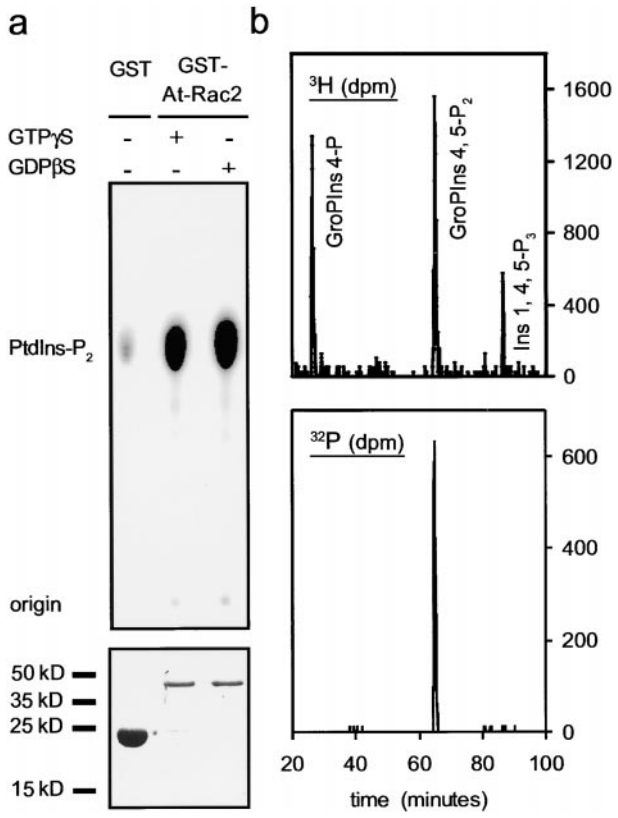

Figure 8. GTPγS- and GDPβS-bound At-Rac2 physically associate in tobacco pollen tube extracts with a PtdIns P-K activity that synthesizes specifically PtdIns 4, 5-P_2. Proteins interacting with GST and with GST-At-Rac2 bound to GTPγS or to GDPβS were purified from tobacco pollen tube extracts and assayed for lipid kinase activity using [γ-^{32}P]ATP and a mix of phosphoinositides as substrates. ^{32}P-labeled phospholipid products of the kinase binding assays were analyzed as indicated. (a) The top panel is analysis by TLC and autoradiography. The TLC origin as well as the migration position of nonradioactive PtdIns 4, 5-P_2 used as a standard is indicated on the left. The bottom panel shows proportional amounts of the recombinant proteins used for the assay shown resolved by SDS-PAGE and stained with Coomassie brilliant blue. Migration positions of molecular mass markers are indicated on the left. (b) Analysis by HPLC after deacylation and mixing with ^{3}H-labeled standards. Elution of ^{3}H-labeled standards (top) and of ^{32}P-labeled assay products (bottom) was detected simultaneously and plotted against time. Under the conditions used, GroPIns 3, 4-P_2 and GroPIns 3, 5-P_2 elute several minutes before GroPIns 4, 5-P_2 (not shown). dpm, disintegrations per minute; Ins 1, 4, 5-P_3, inositol 1, 4, 5-triphosphate.

of large balloons instead of elongated tips. The cell wall of pollen tubes, similar to that of most plant cells, must withstand turgor pressure built up in the protoplast by osmotic water uptake. Certain treatments, including incubation in hypotonic media, can induce some swelling of pollen tubes at the tip, where the newly formed cell wall is relatively elastic and has not yet attained its ultimate rigidity. However, pollen tube tips generally burst before the swelling induced by such treatments results in a substantial increase of their diameter (Steer and Steer, 1989; Benkert et al., 1997; Kost, B., and N.-H. Chua, unpublished observations). Extensive balloon formation as induced by Rac overexpression requires the formation of rigid cell wall structures which depends on constant deposition of new cell wall material. Balloons induced by Rac overexpression clearly resulted from organized but depolarized growth. Whereas Rac overexpression depolarized pollen tube extension, inhibition of endogenous Rac activity by transient expression of a dominant negative At-Rac2 or of TcdB completely inhibited the growth of these cells. These observations demonstrate that pollen tube Rac is essential for growth and plays a key role in the determination of growth polarity.

Intracellular Localization of Pollen Tube Rac

A pea pollen tube Rac homologue was determined to be localized to the plasma membrane at the tip using immunofluorescence techniques (Lin et al., 1996). Chemical fixation and permeabilization required for such experiments are known to severely change the structure of pollen tube cells (He and Wetzstein, 1995; Doris and Steer, 1996) and may affect Rac localization. We have chosen to use GFP as a tag to investigate the intracellular localization of At-Rac2 in living pollen tubes. This technique has been successfully employed to analyze the intracellular distribution of related small GTPases in different cell types (Larochelle et al., 1997; Hirata et al., 1998; Vasudevan et al., 1998).

Transient expression of GFP-At-Rac2 fusion proteins and of corresponding untagged At-Rac proteins had similar effects on pollen tube growth. This demonstrates that the fusion proteins were functional and valid indicators of At-Rac2 localization. A few hours after particle bombardment, when effects on pollen tube morphology started to become apparent, GFP-At-Rac2 fusion proteins were associated with an extended area of the plasma membrane at the tip. At this stage, their localization was very similar to the intracellular distribution of pea pollen tube Rac as observed by immunofluorescence. At later stages, the fusion proteins localized to the plasma membrane throughout the balloons formed (Kost, B., and N.-H. Chua, unpublished observations).

Pollen tubes transiently transformed with *GFP-At-Rac2* sequences emitted fluorescence of varying intensities, indicating that they expressed the fusion proteins at different levels. Using a truncated *lat52* promoter (Bate et al., 1996) or the *35S* promoter, which both confer lower expression in pollen tubes as compared with the full-length *lat52* promoter (Lonsdale et al., 1995; Bate et al., 1996; Wilkinson et al., 1997; Kost, B., and N.-H. Chua, unpublished observations), resulted in a reduction of the total number of pollen tubes emitting detectable fluorescence. However, independent of the promoter used, all fluorescent pollen tubes analyzed displayed depolarized growth and localization of GFP-At-Rac2 fusion proteins as described above (Kost, B., and N.-H. Chua, unpublished observations). This indicates that expression of the fusion proteins at minimal levels required for visualization by fluorescence microscopy is sufficient to affect pollen tube growth. Because fluorescence detection may require relatively high concentrations of GFP fusion proteins, it is possible that endogenous Rac in normally growing pollen tubes localizes to a more restricted area of the tip plasma membrane

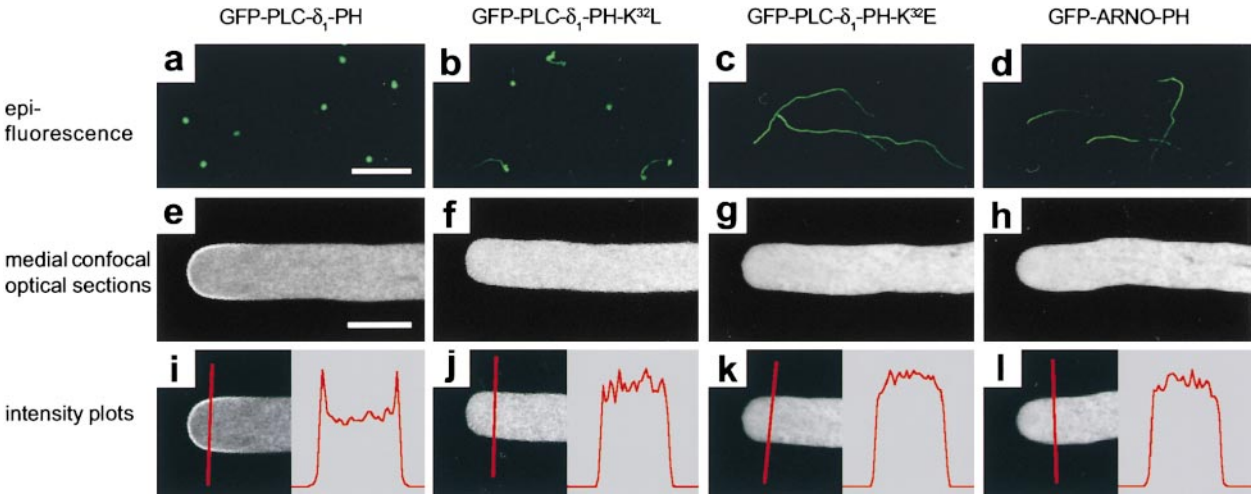

Figure 9. GFP fused to the PH-domain of PLC-δ_1 accumulates at the plasma membrane in the pollen tube tip and strongly inhibits pollen tube growth. Sequences encoding the indicated GFP fusion proteins were transiently expressed in tobacco pollen tubes by particle bombardment. The first row shows low magnification epifluorescence pictures taken 12–18 h after bombardment. The second row shows medial confocal optical sections through representative pollen tubes imaged 7–12 h after particle bombardment. The third row shows quantification of fluorescence intensities along the indicated lines. (a, e, and i) GFP-PLC-δ_1-PH. (b, f, and j) GFP-PLC-δ_1-PH-K^{32}L. (c, g, and k) GFP-PLC-δ_1-PH-K^{32}E. (d, h, and l) GFP-ARNO-PH. Bars, a–d, 500 μm; e–l, 10 μm.

as compared with GFP-At-Rac2 fusion proteins in transiently transformed tubes. Additional experiments, e.g., immunogold labeling of sections through physically fixed pollen tubes, may be required to determine the exact extension of the plasma membrane area that is Rac associated in normally growing pollen tubes. Nevertheless, our results and the earlier immunolocalization study clearly demonstrate that Rac localizes to pollen tube plasma membrane specifically in the tip region.

Pollen Tube Rac Regulates Actin Organization

In normally elongating pollen tubes, the actin cytoskeleton consists essentially of longitudinally oriented thick actin cables that mediate cytoplasmic streaming and of fine actin structures in the tube apex that may have a direct function in polarized secretion (Miller et al., 1996; Kost et al., 1998). Transient expression of mutant Rac was found to alter pollen tube actin organization. The clearest effect observed was the formation of extensive actin cables in growing balloons induced by expression of constitutive active Rac. Expression of dominant negative Rac resulted in a reduction of actin bundling. Interfering with the activity of Rho type small GTPases in fibroblasts is known to have comparable effects. In these cells, Rho activation leads to the formation of actin cables, whereas its inactivation results in the disappearance of thick actin bundles (Hall, 1998). As its animal and yeast homologues, pollen tube Rac functions in the regulation of actin organization. However, the observed effects on the actin cytoskeleton alone are unlikely to account for the dramatic changes in pollen tube growth induced by the expression of mutant Rac.

Pollen Tube Rac May Control Targeted Secretion in the Pollen Tube Tip

Pollen tube elongation is thought to be based on polarized secretion restricted to the apex (Steer and Steer, 1989; Taylor and Hepler, 1997). As suggested for some of its animal and yeast homologues, pollen tube Rac may control directed secretion possibly via the coordinated regulation of actin organization and of exocytotic membrane traffic. In normally elongating pollen tubes, activated endogenous Rac associated with the plasma membrane in a restricted area at the tip may organize directed secretion to this site. Inactivation of endogenous Rac by transient expression of TcdB or of dominant negative mutant Rac was found to inhibit pollen tube growth, presumably by blocking secretion. By contrast, expression of constitutive active Rac led to the formation of balloons instead of elongated tips, conceivably because it resulted in an extension of the membrane area associated with activated Rac, which caused depolarized secretion and growth. Overexpressed wild-type Rac was apparently partially activated by endogenous factors and had similar, although somewhat weaker, effects. Deletion of the COOH-terminal CAAX-domain clearly reduced, but did not complete abolish, the potential of wild-type Rac to induce depolarized growth. Even in the absence of a membrane targeting domain, local concentrations of At-Rac2ΔCSIL at the tip plasma membrane achieved by transient expression of a sequence encoding this protein under the control of the strong *lat52* promoter were apparently high enough for some stimulation of ectopic secretion.

Estimations based on simple geometric calculations revealed that the total surface of pollen tubes transiently expressing constitutive active Rac was about four times smaller than that of control pollen tubes at the time of analysis (12–18 h after particle bombardment). Whereas this appears to be in contradiction with a role of activated Rac in the stimulation of secretion, it likely results from the disruption of cytoplasmic organization caused by transient expression of constitutive active Rac. The total volume of the pollen tube cytoplasm, which remains essen-

tially constant after pollen germination, was estimated to be ~20 times smaller than the volume of balloons formed by constitutive active At-Rac2–expressing pollen tubes. As a consequence, the cytoplasm could only form a thin layer at the inner surface of these balloons, with the remainder of the volume filled by large vacuoles. It is conceivable that the proceeding drastic disruption of cytoplasmic organization during balloon formation increasingly interfered with the efficient delivery of secretory vesicles to the plasma membrane.

Rac, PtdIns P-K, and PtdIns 4, 5-P_2 Act in a Common Pathway to Regulate Pollen Tube Growth

Mammalian Rho family small GTPases in the GTP-bound conformation have been found to stimulate PtdIns P-K activity and synthesis of PtdIns 4, 5-P_2 in permeabilized cells and in cell lysates (Chong et al., 1994; Hartwig et al., 1995). Recombinant as well as endogenously produced mammalian Rac and Rho were shown to physically interact with a PtdIns P-K activity in cell extracts (Tolias et al., 1995; Ren et al., 1996). Here, we present compelling evidence that pollen tube Rac, PtdIns P-K, and PtdIns 4, 5-P_2 cooperate in a common pathway to regulate polar pollen tube growth. Our results indicate that PtdIns P-K and PtdIns 4, 5-P_2 may act as Rac effectors in pollen tubes, as they do in mammalian cells. Rac inactivation by transient expression of dominant negative mutant forms of this protein inhibited pollen tube growth. The same effect was observed when the interaction of PtdIns 4, 5-P_2 with its downstream targets was disrupted by transient expression of GFP-PLC-δ_1-PH, which binds strongly and specifically to this lipid. Recombinant At-Rac2 and its endogenous tobacco homologues were demonstrated to physically associate in pollen tube extracts with PtdIns P-K activity that synthesizes specifically PtdIns 4, 5-P_2. Rac and PtdIns 4, 5-P_2 were both observed to localize to the plasma membrane specifically at the pollen tube tip.

Interestingly, recombinant mammalian Rac and Rho (Tolias et al., 1995; Ren et al., 1996) as well as pollen tube At-Rac2 were found to interact with a PtdIns P-K activity both in the activated GTP-bound and in the inactive GDP-bound form. In a recent report, evidence was presented indicating that the COOH-terminal end of mammalian Rac is mainly responsible for the interaction of this protein with PtdIns P-K and not the NH_2-terminally localized effector domain, which is known to undergo drastic conformational changes upon GTP binding (Tolias et al., 1998). As was suggested for its mammalian homologues, activated pollen tube Rac may stimulate PtdIns P-K activity and PtdIns 4, 5-P_2 synthesis via GTP-dependent binding of an additional, unidentified cofactor (Ren et al., 1996) or by translocating a constitutively associated PtdIns P-K from the cytoplasm to the plasma membrane (Tolias et al., 1998). In vivo and in vitro experiments with mutant At-Rac2 are currently being performed to further characterize the interaction between Rac and PtdIns P-K activity in pollen tubes.

Recent results have established that two different PtdIns P-K–dependent pathways contribute to the synthesis of PtdIns 4, 5-P_2 in mammalian cells. In addition to phosphorylation of PtdIns 4-P by PtdIns 4-P 5-K, which represents a well established pathway, phosphorylation of PtdIns 5-P at position 4 of the inositol ring was found to be catalyzed by PtdIns 5-P 4-Ks, formerly known as type II PtdIns 4-P 5-K (Rameh et al., 1997). Rac-associated pollen tube lipid kinase activity generated PtdIns 4, 5-P_2 when PtdIns 4-P was used as a substrate. Because commercially available PtdIns 4-P preparations may contain traces of PtdIns 5-P (Rameh et al., 1997), this does not entirely rule out the possibility that pollen tube Rac interacts with PtdIns 5-P 4-Ks activity. However, the activity of the Rac-associated pollen tube lipid kinase could be stimulated by phosphatidic acid (Lemichez, E., and N.-H. Chua, unpublished observation), which is considered to be characteristic for PtdIns 4-P 5-Ks in mammalian systems (Fruman et al., 1998; Toker, 1998). Therefore, it appears likely that we have detected an interaction between Rac and PtdIns 4-P 5-Ks activity in pollen tubes.

Function of Compartmentalized PtdIns 4, 5-P_2 in the Regulation of Pollen Tube Growth

Signaling appears to often involve recruitment of regulatory proteins to specific membrane domains where these proteins form complexes that organize local cellular responses. A large number of regulatory proteins is known to bind PtdIns 4, 5-P_2 or other phosphoinositides specifically and with high affinity. This has led to the idea that localized phosphoinositide synthesis may have a key function in spatially restricted signaling events. However, only limited evidence for lipid compartmentalization in cells has been generated to date and the mechanisms involved in the regulation of localized synthesis of particular membrane lipids are unknown (Martin, 1997; Irvine, 1998; Toker, 1998).

Our results provide direct evidence showing that PtdIns 4, 5-P_2 accumulates in the plasma membrane of living pollen tubes specifically at the tip and indicate that the observed PtdIns 4, 5-P_2 compartmentalization is controlled by Rac homologues. By causing translocation of actin-binding proteins, tip-localized PtdIns 4, 5-P_2 may induce uncapping and bundling of actin filaments, which could stimulate elongation of longitudinally oriented actin cables in growing pollen tubes, and control the formation of actin structures present in the apical dome. In addition to its effect on actin organization, PtdIns 4, 5-P_2 may directly control exocytosis at the pollen tube tip, either by recruiting proteins that regulate membrane fusion or by locally altering membrane lipid composition. PtdIns 4, 5-P_2 potentially represents the main effector of activated Rac in pollen tubes. It may initiate the formation of complexes of regulatory proteins at the plasma membrane in the tube apex which control actin organization, targeted secretion, and polar growth. Activated Rac possibly stabilizes these complexes as well as their interaction with PtdIns 4, 5-P_2. GFP-PLC-δ_1-PH did not detectably accumulate at the tip plasma membrane of pollen tubes showing depolarized growth induced by coexpressed constitutive active At-Rac2 (Kost, B., and N.-H. Chua, unpublished observations). In such pollen tubes, access of the GFP-PLC-δ_1-PH fusion protein to PtdIns 4, 5-P_2 may have been blocked by tightly bound regulatory proteins.

In addition to acting as an effector itself, PtdIns 4, 5-P_2

at the pollen tube tip may also function as a precursor for the generation of other signaling molecules. Hydrolysis of PtdIns 4, 5-P_2 by PLC, which results in the formation of inositol 1, 4, 5-triphosphate (Ins-P_3) and diacylglycerol, followed by Ins-P_3–induced Ca^{2+} release from intracellular stores, is a key element of many known signaling events (Clapham, 1995). Recently published results have indicated an essential role of this pathway in poppy pollen tube elongation (Franklin-Tong et al., 1996). PLC-mediated hydrolysis of tip localized PtdIns 4, 5-P_2 and Ins-P_3–induced Ca^{2+} influx into the cytoplasm may be involved in the establishment of the tip-focused Ca^{2+} gradient, which is known to have an important function in the regulation of pollen tube growth. ER elements are present in the clear zone at the pollen tube tip (Taylor and Hepler, 1997) and could function as Ins-P_3–sensitive Ca^{2+} stores. Alternatively, putative Ins-P_3–regulated Ca^{2+} channels, which allow Ca^{2+} influx from the extracellular matrix, may be present in the pollen tube plasma membrane.

Conclusions

Our results strongly suggest that Rac homologues act in a common pathway with a PtdIns P-K (probably PtdIns 4-P 5-K) activity and PtdIns 4, 5-P_2 to regulate polar pollen tube growth. We present direct evidence for PtdIns 4, 5-P_2 compartmentalization in the plasma membrane at the pollen tube tip, which appears to be derived from Rac-controlled local activation of a PtdIns P-K. PtdIns 4, 5-P_2 localized to the plasma membrane at the tip could potentially act as the main Rac effector in pollen tubes by directly regulating actin-mediated targeted secretion and polarized growth. It may also serve as a substrate for Ins-P_3 production by PLC activity, which could have a function in establishing the tip-focused Ca^{2+} gradient known to be involved in the regulation of pollen tube elongation (Malhó and Trewavas, 1996; Pierson et al., 1996).

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