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-P₂) showed a similar intracellular localization as Rac. Expression of the pleckstrin homology (PH)-domain of phospholipase C (PLC)-d₁, which binds specifically to PtdIns 4, 5-P₂, inhibited pollen tube elongation. These results indicate that Rac and PtdIns 4, 5-P₂ act in a common pathway to control polar pollen tube growth and provide direct evidence for a function of PtdIns 4, 5-P₂ compartmentalization in the regulation of this process.

Key words: pollen tube • polarity • actin • Rac • PtdIns 4, 5-P₂

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 et al., 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 (Arelano et al., 1997; Hirata et al., 1998).

Phosphatidylinositol monophosphate kinases (PtdIns P-Ks), ¹ which synthesize phosphatidylinositol 4, 5-bisphosphate (PtdIns 4, 5-P₂), are among the proteins that have

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 regulat-
ing actin organization and exocytotic membrane traffic in a coordinated manner (Martin, 1997; Van Aelst and D'Souza-Schorey, 1997). PtdIns 4, 5-P2 is a ligand for a large number of regulatory proteins, including actin-binding proteins with pleckstrin homology (PH-) or C2-domains. Binding to PtdIns 4, 5-P2 may affect the ac-
tivities of these proteins and/or recruit them to target membranes (Lemmon et al., 1997; Kopka et al., 1998; Toke,
1998). It is well established that PtdIns 4, 5-P2 regulates actin organization via its interaction with key actin-
binding proteins such as profilin, gelsolin, and vinculin (J. Janmey, 1994). Synthesis of PtdIns 4, 5-P2 has also be-
ned demonstrated to be essential for regulated secretion in an-
cimal cells (H.ay et al., 1995). A though the precise function of PtdIns 4, 5-P2 in this process is not known, H.ay and co-
workers suggested that it acts by controlling actin organi-
ization, by altering the lipid composition of membrane mi-
crodomains, and/or by recruiting proteins that mediate
membrane fusion. Compartmentalized synthesis of PtdIns 4, 5-P2 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 accumula-
tion 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 mam-
nalian Ras (Winge et al., 1997; Li et al., 1998). Little infor-
mation is currently available on the cellular functions of
these proteins. A pea Ras homologue (Rop1Ps) was found
to be specifically expressed in pollen and in pollen tubes.
Microinjection of an antibody against this protein inhibi-
ted pea pollen tube elongation (Lin and Yang, 1997). Us-
ing immunofluorescence techniques, Rop1Ps was deter-
mined to localize to the pollen tube plasma membrane in
the tip region (Lin et al., 1996). These results indicated a
possible role of Ras homologues in the regulation of pol-
en tube growth.
Pollens are highly specialized, extremely elongated
cells with a diameter of 10–20 μm and length of up to se-
veral 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 con-
sists of nothing but cell wall material. The pollen tube pro-
toplast 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. Longi-
tudinally oriented, thick actin bundles are found through-
out 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 po-
lar pollen tube extension (Derkson 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
eclusively to this location. A observed in other tip grow-
ing 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 inter-
fere with actin polymerization and insensitive to microtu-
bule inhibitors. A cell 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
Ca2+ in the regulation of pollen tube growth. A tip-
focused Ca2+ gradient with highest concentrations in the
cell cortex at the extreme apex has been detected in pollen
tubes. The steepness of the gradient was found to corre-
late 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 Ca2+
in the flanks of the apical dome (Mahló and Trewavas,
1996). Pharmacological experiments in combination with
analysis of effects of local release of caged inositol 1, 4,
5-trisphosphate (Ins-P3) have provided evidence suggesting
that phospholipase C (PLC)-mediated Ins-P3 production
from PtdIns 4, 5-P2 is involved in Ca2+ 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-P2 both localize to the tip plasma membrane and
act in a common pathway to regulate polar pollen tube
growth. Possible links between Rac and Ca2+ signaling in
growing pollen tubes are discussed.

Materials and Methods
Cloning of At-Rac1 cDNA and Genomic Sequences
A N A r a - R a c 1 c D N A ( X i a e t a l . , 1 9 9 7 ) p r o b e w a s u s e d t o s c r e e n a A r a-
thaliana whole-plant U n i Z A P X R c D N A l i b r a r y ( S t r a t a g e n e ) . F r o m
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 A t-
Rac1 homologue was identified. The sequence of this cDNA was depos-
it in the database (GenBank accession number A F107663) and the en-
coded protein was designated A t-Rac2. The Megalign software package
(DNA ST A R R . I n c . ) a n d t h e C l u s t a l m e t h o d w e r e u s e d t o a l i g n t h e a m i n o
acid sequences of A t-Rac2 and of related proteins.
A N A t-haliana genomic library (C D 4-8; V o y t a s a l . , 1 9 9 0 ) o b t a i n e d f r o m t h e A r a b i d o p s i s B i o l o g i c a l R e s o u r c e C e n t e r , C o l u m b u s , O H ) w a s
screened with A t-Rac1 and A t-Rac2 cDNA probes. Genomic fragments that
specifically hybridized with each probe were subcloned and partially

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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 A L022023) and T19K4 (GenBank accession number A L022373) contain the genomic sequences of At-Rac1 or At-Rac2, respectively.

Expression Constructs
puCA-P-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 Sall fragment from ptjK136 (K apila et al., 1997) into puCA-P (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 include nci site at the start codons. Amplified At-Rac promoters were fused via the Ncol site to the GUS-intron sequence by cloning restricted PCR fragments into puCA-P-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 Xbal fragments, respectively, into the binary vector pFP2P211 (Hajdukiewicz et al., 1994). Resulting plasmids were used to generate stably transformed A. thaliana lines.

Sequences encoding At-Rac2 and At-Rac2CSIL were amplified by PCR from the At-Rac2 cDNA in pBluescriptSK−. A DNA fragment encoding the NH2-terminal catalytic domain of Closstridium difficile toxin B (TcdB; amino acids 1-546) was also amplified by PCR using the construct “CD3-546 in GEX-2T” (Hoffmann et al., 1997) as a template. Primers were designed to create an ATG context optimal for gene expression in dicot plants (A AC TGT; Lütcke et al., 1987) and to introduce stop codons at the 3’ ends of the At-Rac2CSIL and TcdB sequences. PCR fragments, after confirmation of error-free amplification by sequencing, as well as a GUS cDNA (derived from pB121; CLONTECH Laboratories Inc.) were inserted into the puCA-P-based expression vector pLAT52MCS (Kost et al., 1998) between the nci site and the nos poly A addition signal. QuikChange™ (Stratagene) PCR-based mutagenesis was used to substitute mutated sequences encoding G4V-At-Rac2, T27N-At-Rac2, and G46E-At-Rac2. A fter sequence confirmation, mutated At-Rac2 cDNAs were subcloned into nonamplified pLAT52MCS.

The cloning of sequences encoding green fluorescent protein (GFP) and GTP fused to the NH2-terminus of the class B t-actin-binding domain into pLAT52MCS was described earlier (Kost et al., 1998). Into the same expression vector, sequences were inserted that encoded fused GTP and FUSI, fused to the NH2-terminus of the following polypeptides: At-Rac1, At-Rac2CSIL, and G46V-At-Rac2 (sequences subcloned from the vectors described above); the PH-domain of human PLC-β1 (amino acids 2-175 of PLC-β1); G-protein subcloned from “PLC-61-PH in Hi ro3”; Stauffer et al., 1997); and the PH-domain of Adenosine-binding factor nucleotide-binding site opener (A R N O in pEGFP-C1, Y enkateswarlu et al., 1998). The DraIII-Sall 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 “A R N O in pEGFP-C1”.

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 E. 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 MgCl2, 0.1 mM DTT). A per purification, recombinant proteins on agarose beads were suspended 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-32P]GTP (6,000 Ci/mmol; New England Nuclear). GTP, GTPyS, or GDPpS (both from Boehringer Mannheim) followed by addition of MgCl2 to a final concentration of 20 mM. GTP binding and GTPase activity assays were performed using protocols established by Bell and Hall (1994 and 1995b, respectively).

Preparation of Pollen Tube Extracts
Tobacco pollen tubes (1 g fresh weight) grown for 3 h in liquid culture 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. A fter resuspension in 5 ml isosmotic buffer, pollen tubes were passed twice through a 200 micrometer homogenizer. If nothing else is indicated, a solution containing 10 mM Heps (pH 7.4) and 0.8 M sorbitol was used as isosmotic buffer. A suspension of pollen tubes was analyzed by bright-field transmitted light microscopy using an A xioscope (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 (Japan 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 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 A xioscope microscope equipped with a 100-W mercury lamp. A II 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 Affinity-purified Anti–At-Rac Antibody (α-At-Rac), SDS-PAGE, and Immunoblotting

Polyclonal anti–At-Rac antisera 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 threonin-batin-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 15,000), 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.). A filter incubation with primary and secondary antibodies, membranes were washed twice for 10 min with TBS containing 4% BSA and 0.02% Tween 20. A filter 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 EDTA, 5 mM MgCl2, protease inhibitors). TcdB glucosylation reactions were carried out as described in Jüst et al. (1995). In brief, 0.5 μg TcdB (obtained from Ingo Just, Albert-Ludwigs-Universität, Freiburg, Germany) and 0.3 μCi UDP-3H-glucose (300 mCi/mmol; New England Nuclear) in FB buffer were added to extract containing 10 μg total pollen protein or to PB containing 3 μg recombinant GST-At-Rac 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 μg 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 MgCl2, 1 mM EDTA, 1 mM DTT, protease inhibitors). Extracts were clarified by centrifugation at 13,000 g for 15 min at 4°C. A garose beads bound to GST (25 μg protein) or to GST-At-Rac2 (25 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. A filter incubation, beads were washed three times in buffer B (50 mM Tris-HCl at pH 7.4; 0.1% Triton X-100, 100 mM NaCl, 5 mM MgCl2, 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 MgCl2, 0.1 mM EGTA, 1 mM DTT). A filter resuspension in Sigma plus buffer C during this washing step, one-tenth of the total volume of each sample was analyzed by SDSPAGE 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 phospha-
tidic 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-9629; Sigma Chemical Co.) or a mix of phosphoinositides (P6023; 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 MgCl2, 0.5 mM ATP, 10–25 μCi [γ-32P]ATP, 6,000 Ci/mmol; New England Nuclear) to each sample. A filter 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 N2 to a volume of 20 μl and spotted on OXalate-EDTA–impregnated silica gel plates (LKB; Whatman Inc.). Chromatography was performed as described by Pignataro and Acioli (1990).

Radioactivity Detection

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

HPLC Analysis

32P-labeled products of kinase binding assays performed as described above using a mix of phosphoinositides as lipid substrate were deacylated, mixed with 3H-labeled standards, and analyzed by anion-exchange HPLC using a Partisphere SAX column (Whatman) as described by Serunian et al. (1991). Elation of assay products and standards was detected simultaneously using an on-line continuous flow scintillation detector (Radiochromatic® 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 an A. thaliana cDNA 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 (E 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. 1a), 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 turn-over 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,
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; 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 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, Rop1A t 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 A-t-Rac1 (α-A-t-Rac) was found to have similar affinities to recombinant A-t-Rac1 and At-Rac2 (data not shown). Immunoblot analysis showed that in tobacco pollen tube extracts α-A-t-Rac binds to at least one protein with an apparent molecular mass of 21 kD (Fig. 3 a), which corresponds to the size of...
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-[^14C]-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. Rac2 was generally finer and less organized 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 A-t-Rac2 on the tobacco pollen tube actin cytoskeleton were examined. G15V-A-t-Rac2 and T20N-A-t-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 G15V-A-t-Rac2-expressing pollen tubes (Fig. 5 b). Expression of constitutive active A-t-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 T20N-A-t-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).
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**

A nalysis of pollen tube extracts revealed that a significant portion of the 21-kD tobacco pollen tube protein that interacts with the α-A-t-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 A-t-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 A-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 A-t-Rac2 were examined by confocal microscopy. GFP was found to be evenly distributed in the...
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 A t-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 A t-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-K s and PtdIns 4, 5-P 2 act as Rac effectors also in pollen tubes. Experiments were performed to investigate whether A t-Rac2 and its tobacco homologues bind to PtdIns P-K activity present in tobacco pollen tube extracts. Recombinant A t-Rac2 fused to the COOH terminus of GST was loaded with the nonhydroyzable nucleotide analogues GTPγS or GDPγS. Proteins interacting with nucleotide-bound GST-A t-Rac2 were purified from tobacco pollen tube extracts and assayed for lipid kinase activity using [γ-32P]ATP and a mix of phosphoinositides as substrates. A n 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 GDPγS-loaded GST-A t-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 A t-Rac2 homologues from tobacco pollen tube extracts by the α-A t-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, 32P-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), 32P-labeled, deacylated assay products coeluted from HPLC columns with 3H-labeled GroPIns 4, 5-P 2 used as a standard (Fig. 8 b).

The described results demonstrate that recombinant A t-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 and to inhibit pollen tube growth. This domain uses a mechanism involving the binding of PtdIns 4, 5-P 2 to the PH-domain, the formation of PtdIns P-K activity that produces PtdIns 4, 5-P 2, and the activation of PLC-δ 1, which results in the liberation of inositol trisphosphate, calcium, and the inhibition of pollen tube growth. The current study suggests that the PH-domain of PLC-δ 1 may be used as a tool to study the role of PtdIns 4, 5-P 2 in pollen tube growth.
Under the control of the lat52 promoter, a sequence encoding a PLC-δ1 PH-domain GFP fusion protein (GFP-PLC-δ1-PH) was transiently expressed in tobacco pollen tubes. Consistent with a key role of PtdIns 4,5-P2 in the regulation of pollen tube growth, moderate levels of GFP-PLC-δ1-PH expression were sufficient to strongly inhibit tobacco pollen germination and tube growth (Fig. 9a). Only weakly fluorescent GFP-PLC-δ1-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. 9a. Confocal optical sections through such pollen tubes revealed that GFP-PLC-δ1-PH accumulated at the plasma membrane in the tube tip (Fig. 9e and i). Although GFP-PLC-δ1-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-δ1-PH labeling as shown in Fig. 9e were indistinguishable from untransformed control tubes in terms of morphology (Fig. 9e; data not shown) and growth rate (the average growth rate of 20 weakly fluorescent, GFP-PLC-δ1-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-δ1-PH with PtdIns4,5-P2 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-δ1 PH-domain has been shown to form a direct hydrogen bond to the 4-phosphate of PtdIns4,5-P2 (Ferguson et al., 1995). Replacement of this basic lysine residue by neutral leucine (K32L) or even by glutamic acid (K32E) was demonstrated to abolish the ability of PLC-δ1 to bind PtdIns4,5-P2 in membranes (Yagisawa et al., 1998). Neither GFP-PLC-δ1-PH-K32L (Fig. 9f and j) nor GFP-PLC-δ1-PH-K32E (Fig. 9g and k) detectably accumulated at the tip membrane when transiently expressed in tobacco pollen tubes. Whereas high level expression of GFP-PLC-δ1-PH-K32L still severely inhibited pollen tube growth (Fig. 9b), even brightly fluorescent tubes expressing GFP-PLC-δ1-PH-K32E could elongate rapidly and showed an essentially normal morphology (Fig. 9c; data not shown). The PH-domain of ARNO has been demonstrated to bind with a high degree of specificity to PtdIns3,4,5-P3 (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. 9d). No accumulation of the GFP-ARNO-PH fusion protein at the plasma membrane was observed (Fig. 9h and l).

These results provide strong evidence for an essential function of PtdIns4,5-P2 in pollen tube elongation. Rac proteins, which physically interact with PtdIns4,5-P2 synthesizing PtdInsP-K activity, and PtdIns4,5-P2 both localize to the plasma membrane at the pollen tube tip. This suggests that Rac proteins, PtdInsP-K activity, and PtdIns4,5-P2 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
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 is impeded by the formation of rigid cell wall structures which depends on constant depositions 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 A t-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 A t-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-A t-Rac2 fusion proteins and of corresponding untagged A t-Rac proteins had similar effects on pollen tube growth. This demonstrates that the fusion proteins were functional and valid indicators of A t-Rac2 localization. A few hours after particle bombardment, when effects on pollen tube morphology started to become apparent, GFP-A t-Rac2 fusion proteins were associated with an extended area of the plasma membrane at the tip. A t this stage, their localization was very similar to the intracellular distribution of pea pollen tube Rac as observed by immunofluorescence. A t 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-A t-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 355 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-A t-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.
as compared with GFP-A t-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 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 A t-Rac2CSIL 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-

**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 A t-Rac2CSIL 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.

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**Figure 9.** GFP fused to the PH-domain of PLC-δ1 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-δ1-PH; (b, f, and j) GFP-PLC-δ1-PH-K32L; (c, g, and k) GFP-PLC-δ1-PH-K32E; (d, h, and l) GFP-ARNO-PH. Bars, a–d, 500 μm; e–l, 10 μm.
tially constant after pollen germination, was estimated to be ~20 times smaller than the volume of balloons formed by constitutive active A t-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-P2 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-P2 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-P2 cooperate in a common pathway to regulate polar pollen tube growth. Our results indicate that PtdIns P-K and PtdIns 4, 5-P2 may act as Rac effectors in pollen tubes, as they do in mammalian cells. Rac activation 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-P2 with its downstream targets was disrupted by transient expression of GFP-PLC-δ1-PH, which binds strongly and specifically to this lipid. Recombinant A t-Rac2 and its endogenous tobacco homologues were demonstrated to physically associate in pollen tube extracts with PtdIns 4-P activity that synthesizes specifically PtdIns 4, 5-P2. Rac and PtdIns 4, 5-P2 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 A t-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 NH2-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-P2 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 A t-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-P2 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-P2 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 4-P 5-K s 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-K s 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-K s activity in pollen tubes.

**Function of Compartmentalized PtdIns 4, 5-P2 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-P2 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. Homology between mammalian PtdIns 4-P 5-Ks and PtdIns 4, 5-P2 kinases and the availability of 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 4-P 5-K s 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-K s 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-K s activity in pollen tubes.

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at the pollen tube tip may also function as a precursor for the generation of other signaling molecules. Hydrolysis of PtdIns 4, 5-P2 by PLC, which results in the formation of inositol 1, 4, 5-trisphosphate (InsP3) and diacylglycerol, followed by InsP3-mediated Ca2+ 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-P2 and InsP3-mediated Ca2+ influx into the cytoplasm may be involved in the establishment of the tip-focused Ca2+ 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 InsP3- sensitive Ca2+ stores. A putative, InsP3-regulated Ca2+ channels, which allow Ca2+ 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-P2 to regulate polar pollen tube growth. We present direct evidence for PtdIns 4, 5-P2 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-P2 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 InsP3 production by PLC activity, which could have a function in establishing the tip-focused Ca2+ gradient known to be involved in the regulation of pollen tube elongation (Mahló and Trewavas, 1996; Pierson et al., 1996).

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