A Rap/Phosphatidylinositol 3-Kinase Pathway Controls Pseudopod Formation

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GbpD, a Dictyostelium discoideum guanine exchange factor specific for Rap1, has been implicated in adhesion, cell polarity, and chemotaxis. Cells overexpressing GbpD are flat, exhibit strongly increased cell-substrate attachment, and extend many bifurcated and lateral pseudopodia. Phg2, a serine/threonine-specific kinase, mediates Rap1-regulated cell-substrate adhesion, but not cell polarity or chemotaxis. In this study we demonstrate that overexpression of GbpD in pi3K1/2-null cells does not induce the adhesion and cell morphology phenotype. Furthermore we show that Rap1 directly binds to the Ras binding domain of PI3K, and overexpression of GbpD leads to strongly enhanced PIP3 levels. Consistently, upon overexpression of the PIP3-degrading enzyme PTEN in GbpD-overexpressing cells, the strong adhesion and cell morphology phenotype is largely lost. These results indicate that a GbpD/Rap/PI3K pathway helps control pseudopod formation and cell polarity. As in Rap-regulated pseudopod formation in Dictyostelium, mammalian Rap and PI3K are essential for determining neuronal polarity, suggesting that the Rap/PI3K pathway is a conserved module regulating the establishment of cell polarity.

INTRODUCTION

Chemotaxis or directional movement toward a chemical compound is an essential property of many cells (Van Haastert and Devreotes, 2004). It plays a role in diverse functions such as the sourcing of nutrients by prokaryotes, the formation of multicellular structures in protozoa, the tracking of bacterial infections by neutrophils, and the organization of the embryo in metazoa (Baggiolini, 1998; Campbell and Butcher, 2000; Crone and Lee, 2002; Iijima et al., 2002). The mechanism of chemotaxis is essentially identical in all eukaryotes, and because of its genetically tractability, the social amoeba Dictyostelium discoideum is often used as model organism to study chemotaxis (Devreotes and Zigmond, 1988; Van Haastert and Devreotes, 2004). During the vegetative state, Dictyostelium are single-celled amoeba that feed on bacteria. On starvation, cells undergo a tightly regulated developmental process in which they secrete and chemotax toward cAMP, resulting in multicellular fruiting bodies.

During random movement in buffer, Dictyostelium cells repeatedly extend and retract pseudopodia. In response to cAMP cells rapidly polarize due to actin filaments in the front that induce the formation of local pseudopodia and an acto-myosin–containing uropod at the back (Van Haastert and Devreotes, 2004). An important response for establishing cell polarity and chemotaxis is the formation and accumulation of phosphatidylinositol-3,4,5-triphosphate [PIP3] at the leading edge (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003). This PIP3 gradient is achieved by the reciprocal localization of phosphatidylinositol 3-kinase (PI3K), which translocates to the leading edge and produces PIP3 by the phosphorylation of PI(4,5)P2 (phosphatidylinositol 4,5-bisphosphate), and PTEN, which catalyzes the reverse reaction and localizes at the back of the cell (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003). Recent studies have shown that the PI3K pathway is not essential for chemotaxis, but rather that chemotaxis depends on several interconnected pathways (Loovers et al., 2006; Hoeller and Kay, 2007; Takeda et al., 2007). Two studies identified phospholipaseA2 as a component of a chemotactic pathway that acts in parallel to the PI3K pathway (Chen et al., 2007; Van Haastert et al., 2007), whereas Veltman et al. (2008) have shown the presence of a third soluble guanylyl cyclase-dependent pathway in cells starved for longer times. Kamimura et al. (2008) recently described another PIP3-independent pathway regulating chemotaxis, involving activation of TorC2 and protein kinase B (PKB). However, although PI3K is not essential for chemotaxis, it is clear that it plays an important role in directional sensing, especially in shallow gradients (Takeda et al., 2007; Van Haastert et al., 2007).

Rap belongs to the Ras subfamily of small G-proteins and is involved in processes like adhesion, exocytosis, differentiation, and cell proliferation (Bos et al., 2001; Bos, 2005). Small G-proteins, acting as molecular switches, which cycle between an active GTP-bound and inactive GDP-bound state. Activation is regulated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP...
for GTP, and inactivation is regulated by GTPase-activating proteins (GAPs) that stimulate the hydrolysis of bound GTP to GDP (Bourne et al., 1991). Recently, the completed assembly of the *Dictyostelium* genome has led to the identification of 14 Ras subfamily members, an unusually large number (Eichinger et al., 2005; Weeks, 2005). Thus far, six Ras subfamily proteins have been characterized and have been shown to be involved in a wide variety of processes including cell movement, polarity, cytokinesis, chemotaxis, macropinocytosis, and multicellular development (Chubb and Insall, 2001; Wilkins and Insall, 2001; Weeks, 2005; Kortholt and Van Haastert, 2008). RasC and RasG are the best characterized *Dictyostelium* Ras proteins; both are activated in response to cAMP (Kae et al., 2004), are involved in regulation of the cAMP relay, and are important for cAMP-dependent chemotaxis (Bolourani et al., 2006; Bolourani et al., 2008). Disruption of both rasC and rasG results in a total loss of cAMP-mediated signaling, suggesting that all cAMP signal transduction in early development is partitioned between pathways that use either RasC or RasG (Bolourani et al., 2006, 2008). The only thus far characterized Rap subfamily member, Rap1, is essential and is involved in proliferation, growth, adhesion, development, and regulation of the cytoskeleton (Rebstein et al., 1993; Seastone et al., 1999; Kang et al., 2002; Kortholt et al., 2006; Jean et al., 2007b; Parkinson et al., 2009).

Previously we have characterized GbpD as a Rap-specific *D. discoideum* GEF important for adhesion, cell polarity, and chemotaxis. GbpD contains a CDC25-homology domain, a Ras exchange motif (REM) domain, a GRAM domain, and two cyclic nucleotide-binding (CNB) domains (Goldberg et al., 2002). Overexpression of GbpD results in an increased level of active Rap1 and subsequently in increased cell flattening, increased cell-substrate attachment, and severely reduced chemotaxis, as these cells extend many bifurcated and lateral pseudopodia (Bosgraaf et al., 2005; Kortholt et al., 2006). Phg2, a serine/threonine-specific kinase, directly interacts with active Rap1 via its Ras association (RA) domain and is necessary for GbpD/Rap1-regulated adhesion but is not essential for GbpD/Rap1-regulated cell polarity (Kortholt et al., 2006). Jean et al. (2007a,b) showed that Phg2 mediates cell adhesion at the leading edge by regulating myosin II disassembly. In this study we characterized the role of GbpD/Rap1 in cell polarity and chemotaxis in more detail. Together our data indicate that a GbpD/Rap1/P13K pathway helps controlling pseudopod formation in *Dictyostelium*.

**MATERIALS AND METHODS**

**Cell Culture**

All cell lines were grown either on 9-cm dishes or in shaking culture containing HG medium (143 g/1 of peptone, 7.15 g/1 of glucose, 0.49 g/1 of KH₂PO₄, and 1.36 g/1 of Na₂HPO₄·2H₂O). To select for transformants with one of the extrachromosomal plasmids described below, cells were grown in HG-5 supplemented with 10 μg/ml G418 (Gibco BRL, Rockville, MD) and/or hygromycin B (Invitrogen). The *pi3K2*- null strain was obtained from the *Dictyostelium* Stock Center (DSC, Columbia University, New York, NY).

**Aggregation Test**

*Dictyostelium* cells were grown to an amount of 2 × 10⁷ cells per 9-cm dish. Cells were collected, washed two times in 10 mM phosphate buffer (PB), pH 6.5, and suspended in 500 μl of PB. Subsequently cells were plated on nonnutrient agar plates (150 g/1 agar in PB), and pictures were taken after 24 h with an Olympus DP10 camera (Melville, NY).

**Construction of Plasmids**

For expression of GbpD in *Dictyostelium* the previously described MB74HYC GbpD vector and MB74CgbpD-GFP were used (Bosgraaf et al., 2005). The PIP3 detector P11cracGFP and GbpD were expressed from a single vector. For this the pleckstrin homology (PH) domain of CRAC was subcloned in the previously described shuttle vector pDM329 (Veltman et al., 2009a). Subsequently the expression cassette with the P11 promoter was amplified using Ng0MI and ligated into the unique *Ng0MI* site of MB74HYC GbpD. For expression and purification of the RBD domain of PI3K (amino acids 757-918) the encoding DNA fragment was cloned in pGEX-4T3 (Pharmacia, Piscataway, NJ) leading to an N-terminal glutamycin-resistance cassette (Bosgraaf et al., 2005), resulting in the plasmid pDM115-RalGDS-RBD-GFP. For the regulated expression of Rap1G12V and Rap1S17N the previously described tetracycline-controlled inducible expression system was used (Veltman et al., 2008b). The Rap1 mutants were amplified using the forward primers RapG12VF (5′-GGATGCAAAGTTCTTGATCAAAATCTGCCTTTGATTTAGGTTCAGTTGGTGTAGGTAAATCTGCTTTG-3′) and RapG12VR (5′-GGATGCAAAGTTCTTGATCAAAATCTGCCTTTGATTTAGGTTCAGTTGGTGTAGGTAAATCTGCTTTG-3′) and the reverse primer RapR (5′-CCGGGACAAAGTCATAG-3′). The fragment was digested with BamHI and NotI and cloned in pGEX-4T3 (Pharmacia, Piscataway, NJ) leading to an N-terminal glutathione-S-transferase PH domain of CRAC was subcloned in the previously published protocol (Fey et al., 2002) with a few modifications. Briefly, cells were detached by repeated pipetting. The number of cells in the two samples was determined in triplicate using a hemocytometer. The number of nonadhered cells in the first sample was divided by the total number of cells (first plus second sample) to yield the percentage of loose-adhered cells.

**Confluent Analysis and Chemotaxis Assays**

Chemotaxis toward cAMP was tested using micropipettes filled with 10⁻⁴ M cAMP applied to a field of aggregation competent cells with an Eppendorf micropipette holder (Hamburg, Germany) at a pressure of 25 hPa. Cells were starved in HG medium for 6–8 h, suspended in PB, and monitored by phase-contrast microscopy. The motile behavior of cells in spatial gradients of cAMP was analyzed using computer-assisted methods previously described (Soll, 1999). Briefly, images were recorded every 10 s during 15 min. The contour of the cell and the position of the cell centroid were determined at each time point for ~25 cells at a distance of 50–100 μm from the pipette.

**Protein Preparation**

The GST fusion constructs were expressed in *B. subtilis* and purified using glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ) leading to an N-terminal glutamylic-acid-resistance cassette (Bosgraaf et al., 2005), resulting in the plasmid pDM115-RalGDS-RBD-GFP. For the regulated expression of Rap1G12V and Rap1S17N the previously described tetracycline-controlled inducible expression system was used (Veltman et al., 2008b). The Rap1 mutants were amplified using the forward primers RapG12VF (5′-GGATGCAAAGTTCTTGATCAAAATCTGCCTTTGATTTAGGTTCAGTTGGTGTAGGTAAATCTGCTTTG-3′) and RapG12VR (5′-GGATGCAAAGTTCTTGATCAAAATCTGCCTTTGATTTAGGTTCAGTTGGTGTAGGTAAATCTGCTTTG-3′) and the reverse primer RapR (5′-CCGGGACAAAGTCATAG-3′). The fragment was digested with BamHI and NotI and cloned in pGEX-4T3 (Pharmacia, Piscataway, NJ) leading to an N-terminal glutathione-S-transferase PH domain of CRAC was subcloned in the previously published protocol (Fey et al., 2002) with a few modifications. Briefly, cells were detached by repeated pipetting. The number of cells in the two samples was determined in triplicate using a hemocytometer. The number of nonadhered cells in the first sample was divided by the total number of cells (first plus second sample) to yield the percentage of loose-adhered cells.
Guanine Nucleotide Dissociation Inhibition

For the guanine nucleotide dissociation inhibition (GDI) measurements, Rap1 was loaded by incubating in ammonium sulfate-buffer (200 mM (NH₄)₂SO₄, 50 mM DTE, 10 μM ZnCl₂, pH 7.6) containing alkaline phosphatase (2 U/mg protein) and a 1.5 M excess of the fluorescence GTP analogue mGppNHp (John et al., 1990). The protein-nucleotide complex was separated using unbound nucleotides by size exclusion chromatography (Superdex 75 16/20, Pharmacia). The affinity between PI3K-RBD and the indicated G-proteins was determined using the inhibition of the mGppNHp release as described (Herrmann et al., 1996). Briefly, mGppNHp-loaded G-proteins were incubated in the presence of varying concentrations of PI3K-RBD and a 200-fold excess of unlabeled GppNHp in assay buffer at 25°C. The decay in fluorescence was measured in a Spectrofluorometer (SpectraIndustries, Edison, NJ), with excitation and emission wavelengths of 366 and 450 nm, respectively. The observed rate constants (kobs) were single exponential fitted using the program Grafit (Eiritheus Software, West Sussex, United Kingdom). The dependence of the observed rate constants on the effector concentration was fitted to yield the dissociation constant (Kd) as described (Herrmann et al., 1996).

GST-Ras-binding Domain Pulldown Assay

The GST-Ras-binding domains (RBDs) of PI3K1–3 and Byr2 were expressed in Escherichia coli and purified as described previously (Kae et al., 2004). After cAMP pulsing, AX-2 cells were harvested by centrifugation and resuspended at a density of 2 × 10⁶ cells/ml in PB containing 1 mM caffeine. After 30 min, aliquots (2 ml) of cell suspension were stimulated by addition of cAMP to 15 μM. Cell suspensions (350 μl) were lysed at the indicated times by mixing with an equal volume of 2× lysis buffer (20 mM sodium phosphate, pH 7.2, 2% Triton X-100, 20% glycerol, 300 mM NaCl, 20 mM MgCl₂, 2 mM EDTA, 2 mM Na₂VO₄, 10 mM NaF, containing two tablets of Roche Complete protease inhibitor per 50 ml buffer; Mannheim, Germany). The lysates were centrifuged for 10 min, and the protein concentrations of the supernatants were determined using the Bio-Rad protein assay from 400 μg of lysis protein. Then, 100 μg of GST-RBD on glutathione-Sepharose beads (Amersham Biosciences, Freiburg, Germany) at 4°C for 1 h. The glutathione-Sepharose beads were harvested by centrifugation and washed three times in 1× lysis buffer. Gel loading buffer (50 μl of 1× SDS) was then added to the pelleted beads, and the suspension boiled for 5 min. Samples were subjected to SDS-PAGE, and Western blots probed with anti-Rap1- or anti-RasG-specific antibody. (Lim et al., 2001). Briefly, 100-μl aliquots of cAMP-stimulated cell suspension were removed at the indicated time points and immediately lysed as described above for the RBD pulldown assays. Protein samples (10 μg) were fractionated by SDS-PAGE, blotted onto nitrocellulose, and probed with the phospho-threonine antibody (Cell Signaling Technologies, Beverly, MA).

RESULTS

PI3K Is Essential for GbpD/Rap1-mediated Cell Protrusions and Adhesion

Previously the strong GbpDŒ phenotype was used to screen for downstream targets of GbpD and Rap1 in Dictyostelium (Kortholt et al., 2006). GbpD was overexpressed in mutants defective in adhesion or cell polarity, and Phg2, a serine/threonine-specific kinase, was identified as a Rap1 effector necessary for adhesion but not for cell polarity and chemotaxis (Kortholt et al., 2006).

PI3K is known to be a strong inducer of pseudopod formation (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003). To investigate the possible role of PI3K in the increased extension of pseudopodia by GbpDŒ cells, we overexpressed GbpD in cells lacking PI3K1/PI3K2 (p3ki1/2-null). Disruption of PI3K1/PI3K2, results in cells that have a strongly reduced amount of PI3P, a 90% reduced AKT/PI3K activation in response to cAMP, defects in polarity, and a reduced chemotaxis speed (Zhou et al., 1998; Funamoto et al., 2001, 2002). First, the morphology of the mutants was analyzed microscopically. Wild-type cells overexpressing GbpD (AX3/GbpDŒ) extended more pseudopodia and were very large and flat compared with wild-type cells (AX3; Figure 1A). Confoical fluorescence microscopy revealed that GFP-tagged GbpD was expressed in p3ki1/2-null cells at levels approximately equal to that of wild-type cells (data not shown). Although expressing similar amounts of GbpD, p3ki1/2-null cells overexpressing GbpD (p3ki1/2/GbpDŒ) did not show an increased amount of pseudopodia or a flattened cell morphology (Figure 1, A and B). Furthermore, starved p3ki1/2-null and p3ki1/2-null/GbpDŒ cells, although defective in cell polarity, are able to aggregate and showed normal development, whereas AX3/GbpDŒ cells were unable to aggregate (Figure 1C).

Second, the adhesive capacities of the cell lines were tested via an adhesion assay. As shown in Figure 1D, 77% of the p3ki1/2-null cells were detached from the substratum after 1 h of shaking, a slightly higher percentage than that for wild-type cells (68%). AX3/GbpDŒ cells showed a large increase in cell-substrate adhesion, because only 18% of the cells were detached after 1 h of shaking. In contrast, cell attachment in p3ki1/2/GbpDŒ cells is similar to that for p3ki1/2-null cells (76% detached). These results suggest that PI3K is essential for GbpD-mediated adhesion. To investigate the role of Rap1 in a more direct way, we expressed constitutive active (Rap1G12V) and dominant negative (Rap1S17N) mutants in wild-type cells and p3ki1/2-null cells. Cell attachment is substantially enhanced in AX3/Rap1G12V cells and is reduced in AX3/Rap1S17N cells as previously described (Figure 1E; Jeon et al., 2007b). Consistent with an essential role for PI3K in GbpD/Rap1-mediated adhesion, expression of Rap1G12V or Rap1S17N does not alter cell-substratum attachment of p3ki1/2-null cells.

Thirdly we analyzed the chemotaxis properties of the mutants. When a pipette filled with cAMP is placed in the surrounding of starved wild-type cells, cells rapidly respond and chemotax persistently toward the source of cAMP, as depicted in Figure 2. p3ki1/2-null cells exhibit good chemotaxis toward the pipette (Funamoto et al., 2002). Overexpression of GbpD in wild-type cells strongly inhibits chemotaxis as these cells showed little movement toward the pipette. In contrast p3ki1/2/GbpDŒ cells exhibit a chemotaxis response that is nearly identical to the response of the parent p3ki1/2 cell line (Figure 2).

Overexpression of GbpD in wild type affects cell morphology, adhesion, and chemotaxis. The results presented here show that none of these effects are observed upon overexpression of GbpD in p3ki1/2-null cells or addition of the PI3K-inhibitor LY294002 (data not shown), indicating that PI3K is essential for all these GbpD-mediated signaling pathways.

Enhanced PI3P Levels in GbpDOE Cells

To investigate if GbpD is able to regulate PI3K in vivo, the effect of GbpD overexpression on PI3P levels was measured by expressing the PI3P detector PHcracGFP in GbpDŒ cells (Parent et al., 1998). Nonstimulated, differentiated AX3/PHcracGFP-expressing cells moved in random directions and showed an evenly distributed PHcrac localization in the cytosol (Figure 3A, Movie 1). On the contrary, nonstimulated GbpDŒ/PHcracGFP cells made multiple and broad PI3P patches along the entire plasma membrane, from which multiple pseudopodia are extended (Figure 3A, Movie 2). Both AX3/PHcracGFP and GbpDŒ/PHcracGFP cells were stimulated with cAMP. Similar to previous investigations (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003), introduction of a pipette filled with cAMP in the surroundings of wild-type cells induced a strong translocation of PHcracGFP from the cytosol to the leading edge (Figure 3A, Movie 1). Pseudopodia are extended from PHcracGFP-containing areas of the plasma membrane, and cells move persistently toward the pipette. On the contrary, GbpDŒ/PHcracGFP cells make multiple and broad PI3P...
patches along the entire plasma membrane. Cells extend multiple, functional pseudopodia at the front and at lateral sides and showed little movement toward the pipette (Figure 3A, Movie 2). In *Dictyostelium* the activity of Akt/PKB is transiently stimulated in response to cAMP, in a PIP3-dependent manner (Meili et al., 1999; Lim et al., 2005). Akt/PKB is activated by phosphorylation at a conserved threonine residue, which can be detected by using a phospho-threonine specific antibody (Lim et al., 2005; Kortholt et al., 2007).

Wild-type and GbpD OE cells were stimulated with cAM, and cell lysates were analyzed by Western blotting. In lysates from wild-type cells, a 51-kDa protein was transiently phosphorylated, peaking at 10 s after stimulation (Figure 3, B and C). GbpDOE cells show about twofold higher basal level of active PKB; the response to cAMP is similar as in wild type, but it recovers to the elevated basal activity.

These experiments show that GbpDOE cells have enhanced PIP3 formation. Consistently, cells expressing dominant active RapG12V also make broad PIP3 patches and show poor chemotaxis (Figure 3A). These data suggest a stimulatory role for GbpD and Rap1 in the regulation of PI3K activity.

Subcellular Distribution of Activated Rap1 Coincides with PI3K Activation and PIP3 Formation

To examine the localization of Rap1 activation, a GFP-fused RalGDS-RBD construct (GFP-RBD$_{RalGDS}$) was expressed in...
Previously it has been shown that GFP-RBD RalGDS only interacts with the GTP-bound form of Rap and can be used as a reporter to study dynamic changes in Rap activation in human cells (Bivona et al., 2004). Like PHcracGFP, GFP-RBD RalGDS is mainly localized in the cytosol of nonstimulated wild-type cells (Figure 4A, Movie 3), whereas in GbpD OE cells multiple and broad patches of GFP-RBD RalGDS along the entire plasma membrane were observed (Figure 4B). Because GbpD does not activate RasC or RasG, these results indicate that the GFP-RBD RalGDS probe can be used to visualize Rap activation, although it may not be completely specific. As shown in the right panel of Figure 4A, introduction of a pipette filled with cAMP in the surroundings of wild-type cells resulted in the rapid translocation of cytosolic GFP-RBD RalGDS to the membrane, similar to the translocation of the PIP3 detector PH-cracGFP (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003). Jeon et al. (2007) previously presented a similar localization of activated Rap in wild-type cells to that presented here. On the contrary, GbpD OE cells make multiple and broad GFP-RBD RalGDS patches along the entire plasma membrane (Figure 4B), similar to the distribution of PH-cracGFP. These data show that the localization of Rap1 activation coincides with the localization of PI3K activation and PIP3 formation.

**Rap1 and RasG Bind to the RBD Domain of PI3K**

PI3K consist of an N-terminal membrane-targeting region, an RBD, a C2 lipid-binding domain, a PI3K catalytic association domain (PI3Ka), and a PI3K catalytic domain (PI3Kc; Figure 5A). RBD domains have an ubiquitin fold and interact tightly only with the GTP-bound but not with the GDP-bound conformation of Ras-like proteins (Herrmann, 2003). Previous studies have shown that the RBD of PI3K is essential for its activation and has been shown to interact weakly with RasD and RasC and strongly with RasG (Funamoto et al., 1998).
RasG with similar properties. Jeon et al., 2006) showed that Rap1 is rapidly activated upon cAMP stimulation. Consistent with these results, more active Rap1 was detected in cell lysates stimulated with cAMP than in lysates from unstimulated cells (Figure 5B). To further characterize the PI3K/Rap1 and PI3K/RasG interaction, in vitro GDP assays were performed. The interaction of the GTP-bound G-protein with an effector protein stabilizes the interaction between the G-protein and the nucleotide. This stabilization results in decreased dissociation of the nucleotide from the G-protein/nucleotide/effector complex (Herrmann et al., 1996). Incubating G-protein loaded with mGppNHP, a hydrolysistraincent GTP analogue, with an excess of unlabeled GppNHP results in the exchange of mGppNHP for GppNHP. This exchange is monitored as decay in fluorescence, from which the rate constant $k_{\text{obs}}$ is calculated. The addition of increasing concentrations of the effector results in a concentration dependent decrease of $k_{\text{obs}}$. The affinity ($K_d$) between the G-protein and the effector is determined from this dependency (Herrmann et al., 1996). For the PI3K/Rap1 and PI3K/RasG interaction we determined a $K_d$ of 40 and 24 µM, respectively (Figure 5, C and D). This affinity is similar to that described for the interaction between Rap1 and Phg2 (Kortholt et al., 2006) and the interaction of human Rap with several effectors (Woehlgeuth et al., 2005). Together our data show that both in vivo and vitro Rap1 and RasG bind with approximately the same affinity and specificity to PI3K.

The GbdD$^{OE}$ Phenotype Is Lost upon Overexpression of the PIP3-degrading Enzyme PTEN

The data presented in the previous sections suggest that GbdD/Rap regulated PIP3 formation is important for cell morphology, adhesion, and chemotaxis. If PIP3 formation is essential for GbdD mediated signaling, expression of the PIP3 degrading enzyme PTEN should interfere with GbdD-mediated processes. To investigate this hypothesis, PTEN was expressed in wild-type (AX3) and GbdD$^{OE}$ cells. Expression of PTEN in wild-type cells did not influence cell morphology (Figure 1A) or adhesive capacity (Figure 1D). In contrast, the strong phenotype of GbdD$^{OE}$ cells was lost upon expression of PTEN. Overexpression of GbdD in AX3 cells induced a ∼125% increase of the surface contact area and the number of protrusions (Figure 1B). Overexpression of PTEN-GFP in these cells (AX3/GbdD$^{OE}$/PTEN-GFP) resulted in a pronounced reduction of the GbdD$^{OE}$-induced increase of the surface contact area and the number of protrusions (Figure 1B). Furthermore, the strong substrate attachment of GbdD$^{OE}$ cells (only 18% of cells detached from the substratum after 1-h shaking) is almost completely lost upon expression of PTEN (64% detached cells; Figure 1D). Finally, although AX3/GbdD$^{OE}$ cells fail to aggregate upon starvation, overexpression PTEN in these cells allows cell aggregation (Figure 1C). These data reveal that the PIP3-degrading enzyme PTEN largely reverses all the phenotypic defects induced by overexpression of GbdD.

DISCUSSION

Overexpression of GbdD in wild-type cells results in a very strong phenotype; cells have a flat morphology and exhibit enhanced adhesion, many protrusions, and impaired chemotaxis (Kortholt et al., 2006). In our previous study we showed that GbdD is a GEF specific for Rap1. In a screen for effectors, Phg2 was identified as a Rap1 effector necessary for adhesion (Kortholt et al., 2006). Overexpression of GbdD in phg2-null cells still resulted in a flattened cells with many protrusions and defective chemotaxis (Figure S1), indicating that Dictyostelium cells have at least two Rap1 effectors: Phg2 mediating cell substrate attachment and another effector mediating cell polarity (Kortholt et al., 2006). In this study we demonstrate that PI3K is the second Rap1 effector in Dictyostelium and is essential for the adhesion, cell morphology, and chemotaxis phenotypes of GbdD$^{OE}$ cells.

GbdD/Rap1-mediated PIP3 Production

Dictyostelium contains eight putative PI3K family members (Janetopoulos et al., 2005). It has been shown that PI3K1 and PI3K2 contribute most to the regulated PI3K activity (Huang et al., 2003). We show here that GbdD/Rap1 activates PI3K, which is essential for GbdD/Rap1-mediated signaling, because 1) GbdD$^{OE}$ cells contain strongly enhanced basal and cAMP-stimulated level of PIP3; 2) the strong GbdD$^{OE}$ phenotype is lost in pi3k1/2-null cells and upon overexpression of the PIP3 degrading enzyme PTEN; 3) expression of Rap1G12V or Rap1S17N does not alter cell-substratum attachment of pi3k1/2-null cells; 4) Rap1 both in vivo and vitro directly interacts with the RBD domain of PI3K, and 5) GDP-RBD$_{\text{Rap1,RS}}$; the detector of Rap1-GTP, colocalizes with PI3K and PIP3 at the sides of enhanced pseudopod formation.

Rap1 Regulates PI3K Activation in Conjunction with Ras Proteins

Recent studies on the regulation of Dictyostelium PI3K by small G-proteins have suggested that RasG is the main regulator of PI3K signaling (Bolourani et al., 2006). GbdD$^{OE}$ cells, like cells overexpressing constitutive active RasG12V, have enhanced basal and cAMP-stimulated PIP3 formation. GbdD$^{OE}$ cells do not exhibit elevated levels of RasG-GTP or RasC-GTP (Kortholt et al., 2006). In addition, the characteristic GbdD$^{OE}$ phenotype, including the elevated levels of PIP3, is still present in rasC$^{-}$/GbdD$^{OE}$, rasG$^{-}$/GbdD$^{OE}$, rasC$^{-}$/rasG$^{-}$/GbdD$^{OE}$, and RasG12V/GbdD$^{OE}$ cells (unpublished data; Kortholt et al., 2006), but is completely lost...
upon expression of dominant negative Rap1S17N (Figure S1). These results indicate that Rap1 does not activate PI3K indirectly, by activating RasC or RasG. Binding studies with different PI3K RBD domains show that Rap1 and RasG bind with approximately the same affinity and specificity to PI3K. Together these data suggest that RasG-GTP and Rap1-GTP may independently activate PI3K. These two proteins may have a different contribution in PI3K activation for basal movement, cAMP-stimulation chemotaxis, and feedback mechanisms, which we will discuss in more detail below.

**Rap1 Regulates Basal Pseudopod Formation in Conjugation with Ras Proteins**

A recent study by Sasaki et al. (2007) showed that a Gβ-independent PI3K/Ras pathway is important for the regulation of random cell movement and the extension of F-actin projections in the absence of extracellular stimuli. Gβ- cells exhibit normal random movement and show spontaneous Ras activation and PIP3 accumulation at sites of actin protrusion. In contrast, wild-type cells treated with LY294002 or pi3k1/2/3-null cells lack spontaneous Ras activation and show defects in random movement. Active RasG is localized at sites of actin protrusion, and rasG-null cells have decreased random cell movement, suggesting that RasG is one of the Ras isoforms that plays a role in the Ras/PI3K regulatory circuit (Tuxworth et al., 1997; Sasaki and Firtel, 2006; Sasaki et al., 2007). However, because rasG-null cells only show modest defects in random movement, most likely other Ras/Rap isoforms are also involved. GbpD-OE cells moving randomly in buffer make multiple and broad PIP3 patches along the entire plasma membrane, from which multiple pseudopodia are extended, a phenotype similar to that described for cells expressing membrane-bound PI3K2 (Funamoto et al., 2002; Sasaki et al., 2007). Furthermore, randomly moving gbpD-null cells are, like pi3k1/2/3-null cells, much rounder and extend fewer pseudopodia than wild-type cells. Therefore, our data suggest that Rap1 is the second Ras isoform involved in regulation of random cell movement. The GbpD/Rap1/PI3K pathway, which regulates local activation of PIP3 and the basal formation of pseudopodia, apparently escalates in GbpD-OE cells leading to many intense PIP3 patches and multiple pseudopodia. Because, nonstimulated wild-type cells do not extend multiple pseudopodia and show an even distribution of PHcrac,

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**Figure 5.** Interaction between the RBD domain of PI3K and Rap1. (A) Schematic showing the domain composition of PI3K. The bracket indicates the isolated fragment. RBD, Ras-binding domain; PI3Ka, PI3K catalytic association domain; PI3Kc, PI3K catalytic domain. (B) To determine the ability of Rap1 and RasG to bind to the RBD domains of PI3K1, PI3K2, and PI3K3, pulldown experiments were performed. At the indicated time points after cAMP stimulation (s), the amounts of activated Rap1 (top) and RasG (bottom) that bound to the indicated GST-RBD constructs were determined, as described in Materials and Methods. In lane 1 the total amount of G-protein in the lysate is shown (L). The previously described RBD domain of Schizosaccharomyces pombe Byr2 was used as a control for activity of the G-proteins (lanes 2–4). The dissociation rate of mGpp(NH)p from Rap1 (C) and RasG (D) was measured in the presence of varying concentration of PI3K2-RBD. These data were used to calculate the observed rate constants, $k_{obs}$. The $k_{obs}$ values were plotted against the indicated effector concentration of PI3K-RBD. The addition of increasing concentrations of the effector results in a concentration dependent decrease of $k_{obs}$. These data were used to calculate the dissociation constant of the Rap1-GTP/PI3K-RBD and RasG-GTP/PI3K-RBD complex, yielding a $K_d$ of 40 and 24 μM, respectively.
there has to be down-regulation or inactivation of the GbpD/Rap pathway in wild-type cells after its initial activation. GbpD/Rap signaling could be blocked at several steps, including inhibition of GbpD activity, inactivation of Rap-GTP levels by a yet unidentified Rap-specific GAP protein, or reduced PI3K activity by, for instance, depletion of PI(4,5)P₂ levels. One of the proteins involved in the down-regulation of the Rap/Pi3K loop could be RapGAP1 (Jeon et al., 2007a). Deletion of RapGAP1 results in a phenotype similar to that described for cells overexpressing GbpD or RapG12V, and the localization of RapGAP1 is regulated by F-actin.

**Role of cAMP-stimulated Rap1 Activation in Chemotaxis**

In response to chemoattractant stimulation, PI3K accumulates at the leading edge of chemotaxing cells (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003). The localized production of PI3P is mediated by PI3K at the leading edge and the PI3P-degrading enzyme PTEN in the back of the cell (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003). The RBD domain of PI3K is essential for its activation by extracellular cAMP (Funamoto et al., 2002). We have shown that cAMP mediates Rap1 activation at the leading edge, that Rap1-GTP binds to the RBD domain of PI3K, and that the Rap1-GTP detector GFP-BRD₇₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉₋₉-_
specific. In some cells, such as human embryonic kidney cells (HEK 293) and thyroid cells (Tsygankova et al., 2001; Mei et al., 2002), Rap activation increases PI3K activity, whereas in PCC3 and B-cells (Lou et al., 2002; Christian et al., 2003) activation of Rap leads to inhibition of PI3K. In terms of cell polarity and cell migration, mammalian Rap plays an essential role in the determination of neuronal polarity (Schwamborn and Puschel, 2004). In addition, mammalian PI3K is essential for axon specification and probably acts both upstream and downstream of Rap (Shi et al., 2003; Schwamborn and Puschel, 2004), a situation similar to that for Rap1 regulated pseudopod formation in Dictyostelium.

In summary, PI3K is the Rap1 effector in Dictyostelium necessary for Rap1-regulated adhesion and cell polarity. We propose a role for PI3P and GbpD in the basal formation of pseudopodia, whereas RasC and RasG may be more important during cAMP stimulation. The Rap pathway that regulates pseudopod formation in Dictyostelium is reminiscent of the pathway that determines neuronal polarity, suggesting that the Rap/PI3K pathway is a conserved module regulating the establishment of cell polarity.

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