Characterization of the GbpD-activated Rap1 Pathway Regulating Adhesion and Cell Polarity in Dictyostelium discoideum*

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The regulation of cell polarity plays an important role in chemotaxis. GbpD, a putative nucleotide exchange factor for small G-proteins of the Ras family, has been implicated in adhesion, cell polarity, and chemotaxis in Dictyostelium. Cells overexpressing GbpD are flat, exhibit strongly increased cell-substrate attachment, and extend many bifurcated and lateral pseudopodia. These cells overexpressing GbpD are severely impaired in chemotaxis, most likely due to the induction of many protrusions rather than an enhanced adhesion. The GbpD-overexpression phenotype is similar to that of cells overexpressing Rap1. Here we demonstrate that GbpD activates Rap1 both in vivo and in vitro but not any of the five other characterized Ras proteins. In a screen for Rap1 effectors, we overexpressed GbpD in several mutants defective in adhesion or cell polarity and identified Phg2 as Rap1 effector necessary for adhesion, but not cell polarity. Phg2, a serine/threonine-specific kinase, directly interacts with Rap1 via its Ras association domain.

Rap proteins belong to the Ras superfamily of small GTPases. Rap was first identified as an antagonist of Ras-induced transformation and is capable of inhibiting multiple Ras signaling pathways by binding to downstream targets of Ras (1, 2); however, Rap has since been shown to act in numerous Rap-mediated signaling pathways. In mammalian cells Rap is involved in processes like adhesion, exocytosis, differentiation, and cell proliferation (3, 4).

Small GTPases act as molecular switches in numerous signaling pathways. They switch between an active GTP- and inactive GDP-bound state. Ras activity is regulated by guanine nucleotide exchange factors (GEFs)3 that catalyze the exchange of GDP for GTP, thereby activating the Ras protein. The activation is catalyzed by GTPase-activating proteins (GAPs), which accelerate the intrinsic slow GTP hydrolysis by many orders of magnitude (5).

In the social amoeba Dictyostelium discoideum Ras proteins are involved in a wide variety of processes, including cell movement, polarity, cytokinesis, chemotaxis, macropinocytosis, and multicellular development (6–8). During the vegetative state, Dictyostelium are single-celled amoebae that feed on bacteria. Upon starvation, cells undergo a tightly regulated developmental process in which they secrete and chemotax toward cAMP, resulting in multicellular fruiting bodies.

Recently the assembly of the Dictyostelium genome was completed (9), and 25 sequences encoding putative Ras-GEFs were identified (10), 5 of which have been characterized to some extent. RasGEFA, formerly referred to as AleA, and RasGEFM have a role in chemotaxis, and RasGEFB is involved in endocytosis (11–13). Two proteins, GbpC and GbpD (also referred to as RasGEFT and RasGEFU, respectively), that were identified in a screen for cGMP binding domains also contain CDC25 homology domains (14). GbpC belongs to the family of ROC GTPases and has a unique domain architecture: it contains a GTPase domain, a kinase domain, a CDC25-homology domain, a Ras exchange motif (REM) domain, a GRAM domain, and two cyclic nucleotide binding (cNB) domains (15).

Mutant studies showed that GbpC is the high affinity cGMP-binding protein involved in regulation of myosin II (16). GbpD is homologous to the C-terminal half of GbpC and also contains a CDC25 homology domain, GRAM, and two cyclic nucleotide binding domains. Contrary to gbpC-null mutants, which show a strongly reduced chemotaxis, gbpD-null mutants display improved chemotaxis and appear hyperpolar because cells make very few lateral pseudopodia while still retaining the ability to remodel the leading edge. Overexpression of GbpD

3 The abbreviations used are: GEF, nucleotide exchange factor; GAP, GTPase-activating protein; REM, Ras exchange motif; RA, Ras association; RBD, Ras binding domain; GDI, guanine nucleotide dissociation inhibition; GST, glutathione S-transferase; mGDP, 2′-3′-O-[(N′-methylanthraniloyl)-guanosine diphosphate; GppNHp, guanosine-5′-[(β,γ)-imido]triphosphate; mGppNHp, 2′-3′-O-[(N′-methylanthraniloyl)-guanosine-5′-[(β,γ)-imido]triphosphate.
results in severely reduced chemotaxis as these cells extend many bifurcated and lateral pseudopodia, resulting in the absence of a leading edge. Furthermore, GbpD-overexpressing cells (GbpD\(^{145}\)) are flat and adhesive due to an increased number of substrate-attached pseudopodia (17). In mutant cells, which have no detectable cAMP and cGMP, overexpression of GbpD still induces strong adhesion and loss of cell polarity, suggesting that the activity of GbpD is not dependent on intracellular cGMP or cAMP. In support of these data, radioactive cyclic nucleotide binding assays revealed no detectable binding of cAMP or cGMP to GbpD (16). The flattened morphology and increased adhesion of the GbpD-overexpressing cells is similar to that of cells overexpressing Rap1, suggesting that both proteins function in the same pathway (18). In this paper we show by \textit{in vivo} pulldown assays and \textit{in vitro} nucleotide exchange measurements that GbpD is a Rap-specific GEF involved in substrate attachment and cell polarity. So far, the Rap signaling pathway has not been characterized in \textit{Dictyostelium}. To identify downstream targets of GbpD/Rap in \textit{Dictyostelium}, GbpD was overexpressed in null mutants of genes involved in adhesion or cell polarity. Using this assay, Phg2 was identified as a Rap1 effector, essential for regulation of substrate attachment but not for cell polarity. Phg2 is a serine/threonine-specific kinase, previously identified in a screen for mutants defective in phagocytosis, that plays a role in phagocytosis, cellular adhesion, actin cytoskeleton organization, and motility (19). Beside the kinase domain, Phg2 contains proline-rich repeats and a Ras association (RA)/Ras binding domain (RBD) (19). Using guanine nucleotide dissociation inhibition (GDI) measurements, we demonstrated a direct binding of Rap1 to the RA domain of Phg2. These results suggest that GbpD and Rap1 are upstream activators of Phg2 in cell adhesion. Although \textit{phg2}-null mutants overexpressing GbpD lacked the strong cell-substratum attachment, they still displayed defects in chemotaxis, indicating that the involvement of GbpD in cell polarity is important for chemotaxis of migrating \textit{Dictyostelium} cells.

**EXPERIMENTAL PROCEDURES**

\textbf{Cell Culture—AX3 (wild-type), DH1 (a uracil auxotroph wild type, kindly provided by Dr. Cosson), and the indicated mutant cell lines were grown in HG5 medium (14.3 g/liter peptone, 7.15 g/liter glucose, 0.49 g/liter KH\(_2\)PO\(_4\), and 1.36 g/liter Na\(_2\)HPO\(_4\) \(\cdot\)H\(_2\)O) to a density of no more than 2 \(\times\) 10\(^6\) cells/ml. Depending on the antibiotic resistance cassette present in the plasmids, the medium was supplemented with 10 \(\mu\)g/ml G418 (Invitrogen), 10 \(\mu\)g/ml Blasticidin S (ICN), and/or 10 \(\mu\)g/ml hygromycin B (Invitrogen). Rap1S17N cells were grown in the presence or absence of 10 \(\mu\)g/ml tetracycline (Roche Applied Science). The \textit{phg2}-, \textit{talina}-, and \textit{myosin VII}-null cell lines were kindly provided by Dr. Cosson (19).}

\textbf{Construction of Plasmids—For expression of GbpD in \textit{Dictyostelium}, the previously described MB74 GbpD vector was used (17). For the regulated expression of Rap1S17N, the previously described tetracycline-controlled inducible expression system was used (20). The Rap1 mutant was amplified from cDNA using the primers RapS17NF (5’-GGATCCAAAAATG-GCTTTAGAGAATTCAAAAATCGTCGTTTATGGTTCA- GGTGGTGAAGTAAAATGCTTGTG-3’) and RapRmut (5’-GCATGCTTACAAATAGCAGCATTTT-3’). Subsequently, the BamHI-Sphl fragment was cloned into the BglII-Sphl of the response plasmid MB38 (20). Five \(\mu\)g of plasmid DNA was transfected in \textit{tet7} cells, which already contain the transactivator plasmid MB35 (20). For expression and purification of the GEF domain of GbpD (amino acids 1-587), Phg2RA (amino acids 583-680), full-length RasG and the C-terminal-truncated RasC (amino acids 1-170), and Rap1 (amino acids 1-169) proteins, the encoding DNA fragments were cloned in pGEX-4T3 (GE Healthcare) leading to the corresponding N-terminal GST fusion proteins. The GEF domain was amplified using the forward primer (5’-TGGATCCGAAAAATGACAGATTACCCA-TTC-3’) and the reverse primer (5’-GTTATCTAGCTGC- AGTGATGATGTCCTCTTCTCTAATC-3’), using the forward primer (5’-GGATCCATGTTCAACAAAAAGATATTAG-3’) and reverse primer (5’-GAGCGGCCGCTTATTGTTGTGTTATG- TGTGAC-3’). The RasG gene was amplified from cDNA using the forward primer (5’-GAGGATCCATGACAGATACAAATTAG-3’) and reverse primer (5’-CAGCGGGCCTTTATAAAAAAGTCAAAGC-3’). The C-terminal-truncated RasC was amplified from cDNA using the primers 5’-GGATCCATGTCAAATTTATAAAAAATTAG-3’ and reverse primer (5’-GAGCGGCCGCGTTAATTATTTTAC- CATAAGC-3’). The C-terminal-truncated Rap1 was amplified using the forward primer (5’-GGATCCAAAATGCTTATTAGAATG-3’) and reverse primer (5’-GAGCGGCCGTTAACCGCTTGTGTTAC-GGATTAG-3’) with genomic DNA as the template. The fragments were digested with BamHI and NotI and cloned in pGEX4T-3. The expression vectors were checked by sequencing.

\textbf{Protein Preparation—The GST fusion constructs were expressed in BL21(DE3)codonplus-RIL cells (Stratagene). The cells were grown in Standard I medium (Merck) containing 50 \(\mu\)g/ml ampicillin and 25 \(\mu\)g/ml chloramphenicol, induced at an \(A_{600}\) of 0.8 with 0.1 mM isopropyl-1-thio-\(\beta\)-D-galactopyranoside and incubated overnight at 25 °C. After protein production, the cells were pelleted (15 min, 4000 \(\times\) g, 4 °C), washed in 0.9% NaCl, and resuspended in lysis buffer (5 mM 1,4-dithiothreitol, 50 mM NaCl, 5 mM MgCl\(_2\), 30 mM Tris-HCl pH 7.9). To inhibit protein degradation, 1 mM phenylmethylsulfonyl fluoride was added. Cells were lysed in a microfluidizer (Microfluidizer Inc.), and 0.1 mg/ml DNase I was added. Lysates were cleared by centrifugation (45 min, 100,000 \(\times\) g at 4 °C), and fusion proteins were purified using a glutathione-Sepharose (GSH) affinity column (GE Healthcare). RasG and the C-truncated RasC and Rap1 proteins were eluted from the column as GST fusion proteins in lysis buffer containing 20 mM glutathione. For GbpDGEF, the GST tag was cleaved on the column using 200 units of thrombin (SERVA) followed by elution of the protein in lysis buffer. Isolated proteins were analyzed using...
SDS-PAGE, and the protein concentration was determined using the Bradford method (Bio-Rad).

**Guanine Nucleotide Exchange Assays**—For the in vitro measurement of GEF activity, the GTP-binding proteins were loaded with the fluorescent GDP analogue mGDP by incubation in the presence of 10 mM EDTA and a 20-fold excess of mGDP for 2 h at room temperature (21). The mGDP-loaded GTPases were incubated at 25 °C in assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 50 mM NaCl, and 5 mM 1,4-dithioerythritol) containing a 200-fold excess of unlabeled GDP, with the indicated amounts of GEF protein. The nucleotide exchange activity was measured in real time as decay in fluorescence using a Spex1 spectrofluorometer (Spex Industries), with excitation and emission wavelengths of 366 and 450 nm, respectively. The obtained data were fitted to a single exponential decay using the program Grafit (Erithacus software).

**GDI Measurements (Assay)**—For the GDI measurements, Rap1 was loaded with mGppNHp by incubating in ammonium sulfate buffer (200 mM (NH₄)₂SO₄, 50 mM 1,4-dithioerythritol, 10 μM ZnCl₂, pH 7.6) containing alkaline phosphatase (2 units/mg Rap1 protein) and a 1.5 μM excess of the fluorescent GTP analogue mGppNHp (22). The protein-nucleotide complex was separated from unbound nucleotides by size exclusion chromatography (Superdex 75 16/20, GE Healthcare).

The affinity between Phg2-RA and Rap1 was determined using the inhibition of the mGppNHp release from Rap1 as described (23). Shortly, 200 nm mGppNHp-loaded Rap1 in the presence of varying concentrations of Phg2-RA and a 200-fold excess of unlabeled GppNHp was incubated in assay buffer at 25 °C. The decay in fluorescence was measured in a Spex1 spectrofluorometer (Spex Industries), with excitation and emission wavelengths of 366 and 450 nm, respectively. The observed rate constants (kₐ) were single exponential-fitted using the program Grafit (Erithacus software). The dependence of the observed rate constants on the effector concentration was fitted to yield the dissociation constant (Kᵯ) as described (23).

**RBD Pulldown Assay**—The assay was performed as described previously (24). In brief, cells were grown by shaking in HL5 media supplemented with 50 μg/ml streptomycin (Sigma), resuspended at 5 × 10⁷ cells/ml in KK₅ (20 mM potassium phosphate, pH 6.1), and lysed after mixing with an equal volume of 2× HK-LB (20 mM sodium phosphate, pH 7.2, 2% Triton X-100, 20% glycerol, 300 mM NaCl, 20 mM MgCl₂, 2 mM EDTA, 2 mM Na₃VO₄, 10 mM NaF, with two tablets of protease inhibitors (Roche Applied Science Complete) added per 50 ml of buffer). Lysates were incubated on ice for 5 min and cleared by centrifugation for 10 min. Protein concentrations were determined using the DC protein assay (Bio-Rad). Dictyostelium lysates (800 μg of protein) were incubated with 100 μg of GST-RBD protein coupled to GSH beads at 4 °C for 1 h. Beads were harvested by centrifugation and washed 3 times in 1× HK-LB. 40 μl of 1× SDS gel loading buffer (0.5% β-mercapto-ethanol, 0.5% SDS, 50 mM Tris-HCl pH 6.8, 12.5% glycerol, and 0.04% bromphenol blue) was added to the pelleted beads, and the mixture was boiled for 5 min. Samples were fractionated by SDS-PAGE, blotted onto nitrocellulose, blocked with nonfat milk, and probed with either RasC (25) or RasG (26) antibody. The amounts of bound RasC and RasG were determined by an ECL (Amerham Biosciences) reaction.

**Adhesion Assay**—To quantify cell adhesion to the surface, we used a previously published protocol (27) with a few modifications. Briefly, cells were grown in HG-5 medium on 6-well plates (Nunc) to a maximum of 70% confluence. The medium was replaced and incubated for 1 h. Then, plates were rotated on a rotary shaker at 150 rpm, and after 1 h a sample of 150 μl of the medium was taken. The remaining medium was removed and replenished with fresh HG-5, and the remaining adhering cells were detached by repeated pipetting. The number of cells in the two samples was determined in triplicate using a hemocytometer. The amount of loose cells in the first sample was divided by the total number of cells (first plus second sample) to yield the percentage of loose cells.

**Chemotaxis Assay**—Chemotaxis toward cAMP was tested using micropipettes filled with 10⁻⁴ M cAMP applied to a field of aggregation competent cells with an Eppendorf femtotip at a pressure of 25 hectaropascals. Cells were starved in 10 mM phosphate buffer, pH 6.5 (PB) for 6–8 h, resuspended 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 (28). Briefly, images were recorded every 10 s for a period of 15 min. The contour of the cells and position of the cell centroids were determined at 1-min intervals for DH1 and phg2⁻ and at 3-min intervals for DH1/GbpDOE and phg2⁻/GbpDOE.

**RESULTS**

**GbpD Is a Guanine Nucleotide Exchange Factor for Rap1**—GbpD contains a CDC25 homology domain and a REM and is, therefore, predicted to possess guanine nucleotide exchange activity on members of the Ras subfamily of GTPases (14). Fourteen Ras subfamily proteins have been identified in the Dictyostelium genome, an unusually large number (7). Thus far, six Ras subfamily members have been characterized, and they all appear to have important roles in cell physiology (6–8, 10). RasC and RasG are the best characterized Dictyostelium Ras proteins, and both are activated in response to cAMP (24). RasG regulates cell motility and the cytoskeleton during vegetative state (29), and both RasC and RasG are involved in regulation of the cAMP relay and cAMP-dependent chemotaxis (25). RasB is linked to the progression through the cell cycle (30), and RasD is involved in both phototaxis and thermotaxis (31), whereas RasS is involved in endocytosis and the regulation of cell motility (32). The only thus far characterized Rap subfamily member, Rap1, is essential in Dictyostelium and involved in proliferation, growth, adhesion, and regulation of the cytoskeleton (18, 33, 34).

To characterize the specificity of GbpD, we determined GDP/GTP exchange activity on the six characterized Ras subfamily members using in vitro fluorescence exchange measurements. For these experiments, the catalytic GEF domain of GbpD was expressed in Escherichia coli and isolated as a 66-kDa protein (data not shown). The Ras proteins (RasB, -C, -D, -G, and -S) and Rap1 were isolated from Escherichia coli as C-terminal-truncated proteins. C-terminal-truncated Ras proteins possess the same biochemical and structural properties as the

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4 P. Bolourani, unpublished observation.
full-length protein but are much more stable than the full-length proteins (22). The Ras and Rap proteins were loaded with the fluorescent GDP analogue mGDP and incubated in the presence of excess GDP. Nucleotide exchange was measured in real time as a decay in fluorescence, caused by the release of mGDP, which shows a higher fluorescence intensity when bound in the hydrophobic environment of Rap than in the buffer solution. To control for the stability and folding of the purified Dictyostelium Ras and Rap proteins, the human Ras-specific GEF CDC25 and the highly active Rap-specific GEF C3G were used as controls. As expected, the GDP exchange activity of Dictyostelium Rap1 was enhanced by C3G, but not by CDC25, and exchange activity of RasC and RasG was enhanced by CDC25 but not by C3G (Fig. 1), indicating that the purified proteins are stable and functional. The slower decay of fluorescence of RasC compared with RasG may be due to the lower degree of homology to human Ras that RasC exhibits and, thus, is less sensitive to activation by human CDC25 (6). The addition of GbpDGEF to mGDP-loaded Rap1 resulted in a rapid decrease in fluorescence and, hence, acceleration of mGDP exchange (Fig. 1A). Fig. 1, B and C, show that the addition of GbpDGEF to mGDP-loaded RasG and RasC did not result in a decrease in fluorescence. GbpD was also unable to accelerate nucleotide exchange on RasB, RasD, or RasS, which were activated by human CDC25 (supplemental data). These results suggest that GbpD specifically activates Rap1 in vitro and not RasB, -C, -D, -G, and -S.

It was previously shown that the RBD of human RalGDS can be effectively used to detect the amount of activated Dictyostelium Rap1 in vivo (33). Kae et al. (24) described a similar method in which they used the RBD of Schizosaccharomyces pombe Byr2 to detect the amount of GTP loaded RasC and RasG (24). To further determine the activity and specificity of GbpD, the amount of activated Ras proteins in wild type AX3 cells, in gbpD-null cells, and in GbpD<sup>OE</sup> cells was determined. The amount of Rap1-GTP is significantly higher in GbpD<sup>OE</sup> mutant cells than in wild type and gbpD-null cells, whereas the total amount of Rap1 is similar in all three cell lines (Fig. 2). Seastone et al. (34) have previously shown that modest overexpression of Rap1 results already in the described strong phenotype. These data support the in vitro data and suggest that GbpD acts as a RapGEF in vivo.

![FIGURE 1. Activation of Ras proteins by GbpD in vitro](image1)

![FIGURE 2. Activation of Ras proteins by GbpD in vivo](image2)
The GST-Byr2 pulldowns, analyzed by Western blotting using antibodies against RasC and RasG, showed similar amounts of activated RasG in all 3 cell lines, but reproducibly, an increased amount of GTP-RasC was detected in GbpDOE cells (Fig. 2). Because GbpD is not able to activate RasC in vitro, the increased activation of RasC seen in vivo may be due to an indirect effect.

**Rap1 Mediates GbpD-induced Adhesion and Pseudopodia Formation**—The pulldown assays revealed that Rap1 and possibly RasC are potential targets of GbpD in vivo. To investigate the possible role of RasC in the GbpD^{OE} phenotype, we overexpressed GbpD in rasC-null cells. The rasC^-/GbpD^{OE} mutant possessed the characteristic GbpD^{OE} phenotype, a strong increased attachment to the substratum (Fig. 3C) and a very flat morphology (Fig. 3A), indicating that RasC is not essential for mediating the effect of GbpD.

To investigate if Rap1 indeed mediates the GbpD-induced adhesion and pseudopodia formation, GbpD was expressed in cells expressing dominant negative Rap1 (Rap1S17N). The Rap1S17N substitution restricts the protein to the GDP-bound state and interferes with the endogenous Rap protein (18). RapS17N cells have altered phagocytosis, adhesion, polarity, and viability (18, 20, 33, 34, 35). Because of this strong phenotype, we decided to tightly control the expression of Rap1S17N using the previously described tetracycline-controlled inducible expression system (20). Briefly, this system employs a promoter by which the expression of the downstream gene is negatively regulated by tetracycline. Rap1S17N/GbpDOE mutants grown in the presence of tetracycline (Rap1S17N off) possessed the characteristic GbpD^{OE} phenotype, a strong increased attachment to the substratum (Fig. 3C), and a very flat cell morphology (Fig. 3B). Rap1S17N/GbpDOE cells grown in the absence of tetracycline (Rap1S17N on) lack both the alteration in morphology as well as in adhesion. These data support our conclusion that GbpD is a Rap-GEF, mediating adhesion and pseudopodia formation.

**Phg2 Mediates GbpD-induced Adhesion but Not Pseudopodia Formation**—The strong GbpD^{OE} phenotype provides a powerful tool to screen for downstream targets of GbpD and Rap1 in Dictyostelium. GbpD was overexpressed in mutants with deletions in genes known to be involved in cell adhesion, talinA, myosin VII, and phg2. TalinA is an actin-binding protein necessary for phagocytosis and adhesion (36), myosin VII is involved in both cell-cell and cell-substrate adhesion (36), and Phg2 plays a role in the modeling of actin-rich focal sites and in cell motility (19, 37). First, the morphology of the mutants was analyzed microscopically. Wild type (DH1, top) and DH1/GbpD^{OE} (bottom) cells are shown in the left panel of Fig. 4A. The GbpD^{OE} mutants extended more pseudopodia and were flat and large compared with the parent cell lines, and a similar phenotype was obtained when GbpD was overexpressed in phg2^-/-, talinA^-/-, or myosin VII-null. However, although starved phg2^-/-, talinA^-/-, and myosin VII-null cells aggregate and
develop morphologically normal, GbpDOE cells were unable to aggregate (data not shown). These data indicate that Phg2, talinA, and myosin VII are not essential downstream components of GbpD for its role in cell morphology (Fig. 4A).

A second characteristic of the strain overexpressing GbpD is its strong attachment to the substratum; mutants lacking GbpD are less adherent to the substratum than wild type cells. Therefore, the adhesive capacities of the mutants were tested via an adhesion assay. As shown in Fig. 4B, ~65% of the DH1 cells were detached after shaking compared with only 18% for the DH1-overexpressing GbpD. These values are slightly higher than those published previously, which may reflect the different parental cell line used in these experiments and the use of HG-5 instead of phosphate buffer (17). In the mutants lacking talinA, myosin VII, or phg2, the percentage of loose cells after 1 h of shaking was about 70%, slightly higher than that of wild type. These defects in adhesion are smaller than those published before, which may reflect the different method used and the use of Nunc plates instead of glass (19). Overexpressing GbpD in cells deficient of myosin VII or talinA resulted in a strong increase in cell attachment (Fig. 4B), similar to that observed with the overexpression of GbpD in wild type cells. In contrast, overexpression of GbpD in phg2-null cells had no effect on cell attachment (Fig. 4B). These data suggest Phg2 is a downstream component of the GbpD pathway, essential for its role in adhesion.

Phg2 Interacts Directly with Rap1 via Its RA Domain—Phg2 is a serine/threonine-specific kinase involved in phagocytosis, adhesion, and organization of the actin cytoskeleton (19). In addition to a kinase domain, the protein contains two proline-rich domains, through which it may interact with other cellular proteins, and a RA or RBD (19). RA/Ras binding domains have an ubiquitin fold and interact tightly only with GTP bound and not GDP-bound Ras-like proteins (38). Gebbie et al. (19) used yeast two-hybrid analysis to characterize the interaction between Phg2-RA and three members of the Dictyostelium Ras family, RasG, RasS, and Rap1. They showed that Phg2-RA is able to bind all three GTPases but preferentially binds Rap1 (19). To quantify the ability of Rap1 to bind directly to Phg2-RA, we performed a GDI assay (23). The interaction of an effector with the GTP-bound G-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. Incubating Rap1 loaded with mGppNHp, a hydrolysis-resistant fluorescent GTP analogue, with an excess of unlabeled GppNHp results in the exchange of mGppNHp for GppNHp. This exchange can be monitored as a decay in fluorescence, and the resulting $k_{obs}$ is a measure of effector binding. Adding increasing concentrations of the effector results in a concentration-dependent decrease of $k_{obs}$ (Fig. 5A). The affinity between Rap1 and the effector ($K_d$) can be determined from this dependence (23). For the interaction of Rap1 with the RA domain of Phg2, we determined a $K_d$ of 30 μM (Fig. 5B). From this data we conclude that Phg2 is able to interact with Rap-GTP via its RA domain. We also investigated the GDI effect of Phg2-RA of RasC and observed no inhibition on nucleotide exchange, suggesting the absence of an interaction between RasC and Phg2-RA in vitro (data not shown).
GbpD/Rap-regulated Cell Morphology Is Important for Proper Chemotaxis in Dictyostelium—GbpDOE cells are characterized by an increased attachment to the substratum and increased numbers of pseudopodia, resulting in a severe defect in chemotaxis. Expression of GbpD in phg2-null cells also resulted in an increased amount of pseudopodia but did not result in an increased attachment to the substratum, nor in HG-5 medium (see Fig. 4) or 10 mM phosphate buffer, pH 6.5 (data not shown). Therefore phg2−/GbpDOE cells provide a tool to discriminate between the effects of GbpD on the adhesion versus the cell polarity components for chemotaxis. DH1, DH1/GbpDOE, phg2−, and phg2−/GbpDOE cells were starved for several hours, and chemotaxis toward cAMP was measured. In GbpDOE cells developed for similar periods of time it previously has been shown that the cAMP-stimulated accumulation of filamentous actin was essentially normal, indicating that the cells develop a competent signal transduction pathway (17). When a pipette filled with cAMP is placed in the surrounding of wild-type cells, cells rapidly respond and chemotax persistently toward the source of cAMP, as depicted in Fig. 6. Cells lacking Phg2, although moving slower, exhibit a chemotaxis response that is essentially identical to the response of wild type cells (Fig. 6, lower left panel). Overexpression of GbpD in wild type cells or in phg2−null cells negatively affects chemotaxis as these cells showed little movement toward the pipette. These results suggest that the chemotaxis defect of GbpDOE cells is not due to the increased cell adhesion to the substratum but more likely due to defects in maintaining proper cell polarity.

DISCUSSION

Dictyostelium GbpD was previously characterized as a CDC25-homology domain-containing protein involved in cell attachment and cell polarity (17). In this study the function of GbpD was explored in more detail. First the specificity of the guanine nucleotide exchange activity was tested by in vivo pulldown and in vitro fluorescence exchange assays. Both experiments showed activity of GbpD on Dictyostelium Rap1, whereas no exchange activity was detected for RasB, -C, -D, -G, and -S. In vivo (as measured by a Byr2-RBD pulldown assay) an increase of both Rap1-GTP and RasC-GTP levels was observed when GbpD was overexpressed. However, because GbpD does not activate RasC in vitro, we suspect the effect on RasC to be indirect. Further-
more, a rasC^-/GbpDOE cell line still possesses the characteristic GbpDOE phenotype, indicating that RasC is not an essential component downstream of the GbpD signaling pathway. The RapS17N/GbpDOE cell line does not have the GbpD^OЕ phenotype, confirming our conclusion that Dictyostelium GbpD possesses an active GEF that is specific for Rap1.

The mechanism of regulation of GbpD remains unclear. Although GbpD contains two cyclic nucleotide binding domains, so far no binding of cAMP or cGMP to GbpD could be detected (16). Additionally, the strong GbpDOE phenotype is independent of the presence of cAMP/cGMP, indicating that the activity of GbpD is at least not strictly regulated by cyclic nucleotides (17). Mammalian PDZ-GEF1 and -2, which also contain a CDC25-homology domain and a cyclic nucleotide binding (cNB) domain, also seem to function without cAMP or cGMP (39). Interestingly, like GbpD, PDZ-GEF is a Rap-specific GEF (39, 40).

Cells overexpressing Rap1 are flat, extend many substrate-attached pseudopodia, and are highly adhesive (18, 35), phenotypes strikingly similar to that of the GbpDOE mutant. Multiple attempts to generate a rapA-null cell line failed and antisense rapA mRNA resulted in cells with defects in growth and viability, suggesting an essential role for Rap1 in Dictyostelium (33). Because, a disruption in gbpD is not lethal and gbpD-null cells still contain active Rap (Fig. 2), there must be at least one other GEF for Rap1.

So far no GEFs, GAPs or downstream targets of Rap1 have been identified in Dictyostelium. Mammalian Rap, like Dictyostelium Rap1, participates in processes such as phagocytosis, adhesion, and chemotaxis (3). Mammalian Rap is involved in adhesion by regulating integrins, cadherins, and actin dynamics, but whereas the direct downstream effectors mediating these effects have not been unequivocally identified (4), several proteins that interact with activated mammalian Rap have been described. The effector protein RapL, which consists of a RA domain and a coiled-coil domain, is involved in Rap1-induced integrin-mediated cell adhesion (41). Riam is a protein involved in Rap1-mediated actin dynamics, probably by binding to profilin and Ena/Vasp and needs both its RA and PH domain for interaction with Rap (42). Binding of Rap to Arap3, which contains both an ARF-GAP and a RhoGAP domain, might mediate down-regulation of RhoA and subsequently affect regulation of actin dynamics (43). In addition, Rap1 directly interacts with the DH-PH domain of the RacGEFs Vav2 and Tiam1, regulating their translocation to cell protrusions (44). As an indication of the complexity of the Rap signaling pathway, the well characterized Ras effectors Raf1 and B-Raf have also been described as putative Rap effectors, suggesting that the there is a level of cross-talk between the Rap and Ras pathways (45). The Dictyostelium Rap pathway is involved in regulation of similar processes, and because of its genetic tractability, Dictyostelium presents a good model for characterizing the basic interactions of Rap-regulated pathways.

To identify downstream targets of Rap1 in Dictyostelium, GbpD was overexpressed in cells ablated for proteins previously shown to be involved in adhesion. In this way we were able to identify Phg2 as an essential downstream component of GbpD-mediated adhesion. Like many of the mammalian Rap effectors, Phg2 contains a putative RA domain. Using the yeast two hybrid system Gebbie et al. (19) showed that the Phg2-RA domain is capable of interacting with members of the Ras family and preferentially binds to Rap1 (19). We measured the Phg2-RA/Rap1 interaction in further detail by performing a GDI assay, and we determined that Phg2-RA binds to Rap1 with a dissociation constant of \( \sim 30 \, \mu M \). Although this affinity is relatively low, other parts of the Phg2 protein may stabilize the interaction and increase the affinity as observed in other Rap-RA interactions (38). Phg2 also contains a serine/threonine-specific kinase and proline-rich repeats and plays an important role in substrate adhesion, cellular adhesion, phagocytosis, actin cytoskeleton reorganization, and motility (19). Recently BlanC et al. (46) identified a new inositol 4,5-diphosphate binding domain that is essential and sufficient for the membrane localization of Phg2 and that this membrane localization is necessary for proper functioning of the protein. This inositol 4,5-diphosphate binding domain comprises amino acids 81–193, distal from the RA and kinase domains. Because Phg2 is a membrane-bound, binding of Rap1 to Phg2 is likely to allosterically activate Phg2. Such a mechanism of activation has been described for Rho family protein-dependent kinases such as Pak or Rho-associated kinase (ROCK) (47) but, presumably, differs from Ras-regulated kinases such as Raf-1 and B-RAF. Binding of Ras to the Ras binding domain of Raf

![Diagram of GbpD-activated Rap1 Pathway](Image)
results in recruitment of the protein to the membrane rather than in its direct activation (48).

GbpD<sup>OE</sup> mutants are severely impaired in chemotaxis. Previously we proposed that the chemotaxis defect is probably due to the formation of many substrate-attached pseudopodia that leads to an increased adhesion and a concomitant decrease in polarity (17). The data from this report allow for a refinement of this model (Fig. 7). We identified GbpD as one of the GEFs that catalyze the exchange of GDP for GTP, thereby activating Rap1. For the inactivation, not yet identified GAPs are necessary to accelerate the intrinsic slow GTP hydrolysis by many orders of magnitude. Active Rap is involved in regulating adhesion and cell polarity. Phg2, a serine/threonine-specific kinase, directly interacts with Rap1 via its RA domain. Phg2 is necessary for the Rap1-regulated adhesion but not for Rap1-regulated kinase, directly interacts with Rap1 via its RA domain. Phg2 is serine/threonine-specific kinase, directly interacts with Rap1 via its RA domain. Phg2 is necessary for the Rap1-regulated adhesion but not for Rap1-regulated kinase, directly interacts with Rap1 via its RA domain. Phg2 is necessary for the Rap1-regulated adhesion but not for Rap1-regulated kinase, directly interacts with Rap1 via its RA domain. Phg2 is necessary for the Rap1-regulated adhesion but not for Rap1-regulated kinase, directly interacts with Rap1 via its RA domain. Phg2 is necessary for the Rap1-regulated adhesion but not for Rap1-regulated kinase, directly interacts with Rap1 via its RA domain.

In summary, GbpD is a Dictyostelium Rap-GEF involved in adhesion and cell polarity. Phg2, a serine/threonine-specific kinase, directly interacts with Rap1 via its RA domain. Phg2 is necessary for the Rap1-regulated adhesion but not for Rap1-regulated cell polarity.

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