A dual role for Rac1 GTPases in the regulation of cell motility

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Summary

Rac proteins are the only canonical Rho family GTPases in *Dictyostelium*, where they act as key regulators of the actin cytoskeleton. To monitor the dynamics of activated Rac1 in *Dictyostelium* cells, a fluorescent probe was developed that specifically binds to the GTP-bound form of Rac1. The probe is based on the GTPase-binding domain (GBD) from PAK1 kinase, and was selected on the basis of yeast two-hybrid, GST pull-down and fluorescence resonance energy transfer assays. The PAK1 GBD localizes to leading edges of migrating cells and to endocytotic cups. Similarly to its role in vertebrates, activated Rac1 therefore appears to control de novo actin polymerization at protruding regions of the *Dictyostelium* cell. Additionally, we found that the IQGAP-related protein DGAP1, which sequesters active Rac1 into a quaternary complex with actin-binding proteins cortexillin I and cortexillin II, localizes to the trailing regions of migrating cells. Notably, PAK1 GBD and DGAP1, which both bind to Rac1-GTP, display mutually exclusive localizations in cell migration, phagocytosis and cytokinesis, and opposite dynamics of recruitment to the cell cortex upon stimulation with chemoattractants. Moreover, cortical localization of the PAK1 GBD depends on the integrity of the actin cytoskeleton, whereas cortical localization of DGAP1 does not. Taken together, these results imply that Rac1 GTPases play a dual role in regulation of cell motility and polarity in *Dictyostelium*.

Key words: Rac1, Cell motility, Cell polarization, FRET, Actin cytoskeleton, Rho GTPases, *Dictyostelium*

Introduction

A major role of canonical small GTPases from the Rho subfamily in regulation of the actin cytoskeleton was first demonstrated in 1995 (Nobes and Hall, 1995). In agreement with results of these seminal studies, a general notion prevails that Rac and Cdc42 are primarily engaged in the initiation of protrusive structures such as lamellipodia and filopodia at the front of motile cells, whereas Rho predominantly regulates contractile structures in the back. However, evidence obtained in various cell types indicates that the spatial distribution of these proteins can be more complex (Heasman et al., 2010; Machacek et al., 2009; Pestonjamasp et al., 2006).

In mammalian cells, actin polymerization appears to be induced by two major routes: GTP-bound Rac1 and Cdc42 trigger Scar/WAVE- and WASP-mediated activation of the Arp2/3 complex (Iden and Collard, 2008), whereas activated Rho proteins release autoinhibition of Diaphanous-related formins (Faix and Grosse, 2006). In addition to their regulatory role in cell protrusion, Rac1 and Cdc42 have been implicated in numerous other processes involving actin polymerization, such as endocytosis, vesicular transport, secretion, cell polarization and cytokinesis (Heasman and Ridley, 2008). Small GTPases are activated by guanine nucleotide exchange factors (GEFs) and deactivated by GTPase-activating proteins (GAPs), and each of these two groups of regulating proteins is represented by more than 70 members encoded in the human genome (Rossman et al., 2005; Tcherkezian and Lamarche-vane, 2007). Deactivation of small GTPases is also attributed to a less numerous group of

guanine nucleotide dissociation inhibitors (GDIs) that sequester the GTPases in a GDP-bound state in the cytoplasm, rendering them inaccessible to activators at the plasma membrane (DerMardirossian and Bokoch, 2005).

Highly motile cells of *Dictyostelium discoideum* represent a rewarding model in which regulation of the actin cytoskeleton dynamics is investigated (Noegel and Schleicher, 2000). *Dictyostelium* genome analysis revealed 18 Rho GTPases, among them several members of the Rac subfamily, whereas no typical Rho and Cdc42 subfamily members were identified (Vlahou and Rivero, 2006). Several Rac proteins have been investigated, indicating that their ubiquitous role in regulation of the actin cytoskeleton is conserved in *Dictyostelium*, but more detailed analyses of their dynamics and activity in living cells are still lacking (Rivero and Somesh, 2002). Such information is of major interest to complement data on related signalling pathways from other cell types and organisms (Heasman and Ridley, 2008; Wang and Zheng, 2007). Moreover, regulation of a rich repertoire of actin-driven activities in *Dictyostelium*, strongly resembling the dynamics of highly motile mammalian cells, has to be mapped to a specific set of canonical Rho GTPase proteins. Consequently, important insights into general principles of regulation of the motile machinery in eukaryotic cells and its diversity, robustness and evolution could be gained.

To study the dynamics of Rac proteins in *Dictyostelium*, we designed a fluorescent probe specific for the activated form of Rac1 GTPases and monitored its localization in migrating cells. Our probe displayed a clear temporal and spatial coincidence

with structures that are propelled by actin polymerization, such as protruding leading edges and expanding endocytotic cups. Comparison with another Rac1-GTP-binding protein, IQGAP-related DGAP1, which localizes to retracting trailing regions of migrating cells, suggests that activated Rac1 plays at least two different roles in the regulation of the actin cytoskeleton.

Results
Rac1A is distributed homogenously in the cell cortex
When expressed as GFP fusion proteins in *Dictyostelium*, wild-type forms of highly similar GTPases Rac1A, Rac1B and Rac1C show a slight enrichment in the cell cortex compared with a prominent cytoplasmic background (Dumontier et al., 2000). We decided to take a closer look at Rac1A localization in processes that involve remodelling of the actin cytoskeleton. GFP-tagged Rac1A is homogenously distributed throughout the cell cortex during migration of vegetative *Dictyostelium* cells, showing no preference for either protruding or retracting regions (Fig. 1A). GFP–Rac1A is also constantly present in the perinuclear region and in endocytotic cups during macropinocytosis and phagocytosis (Fig. 1A,C). Its cortical localization is, however, less prominent during cytokinesis, and barely discernible during chemotaxis of aggregation competent cells (Fig. 1B,D).

Distribution and dynamics of the GFP–Rac1A fusion protein in living cells presumably mirrors the distribution and dynamics of endogenous Rac1A. However, information conveyed in this way bears two important limitations. First, the total population of Rac1A molecules in the cell represents a mixture of an active, GTP-bound form and an inactive, GDP-bound form. Therefore, distribution and dynamics of GFP–Rac1A per se does not provide spatial and temporal information about the intracellular activity of the GTPase. Additionally, a more than tenfold overexpression level of the fusion protein compared with endogenous Rac1A probably leads to a high degree of its retention in the cytoplasm

and, consequentially, to an exceedingly strong cytoplasmatic fluorescent background (Dumontier et al., 2000).

PAK1 GBD interacts with the active form of Rac1A in vitro
To obtain information about the intracellular distribution of Rac1 activity, we decided to generate a fluorescent probe based on a domain that interacts specifically with the active, GTP-bound Rac1A. As a first step in the selection of an appropriate GTPase-binding domain, we performed a yeast two-hybrid (Y2H) screen using the following four candidate domains as baits: a full-length and a truncated form of the Rac1A-interacting protein DGAP1 (Faix et al., 1998), the GTPase-binding domain of *Dictyostelium* DPAKa kinase (Müller-Taubenberger et al., 2002) and the regulatory, GTPase-binding domain of rat PAK1 kinase (Buchwald et al., 2001). In addition to constitutively active forms of 11 *Dictyostelium* Rac proteins, we included the constitutively inactive forms of Rac1A, Rac1C and RacC as preys in the Y2H screen. The PAK1 GBD (amino acid residues 57–200) interacted with active forms of Rac1A, Rac1C and RacC, but not with their inactive forms, under both low-stringency and high-stringency conditions (Fig. 2A). We therefore chose this probe to further study its interaction with active Rac1A.

Two variants of GST-binding pull-down assay were used to verify that PAK1 GBD interacts with the active form of Rac1A, but not with its inactive form. First, a GST–[PAK1(GBD)]$_2$ construct was expressed in *E. coli* and used to affinity-purify GFP-fusion proteins from lysates of cells that express the following constructs: GFP–Rac1A(WT), GFP–Rac1A(V12), GFP–Rac1A(N17) and GFP–RacC(WT). Immunoprecipitation of bound proteins with antibodies against GFP showed that the PAK1 GBD binds to constitutively active mutant Rac1A(V12), but not to constitutively inactive mutant Rac1A(N17) (Fig. 2B). The PAK1 GBD also interacts with the wild-type forms of Rac1A
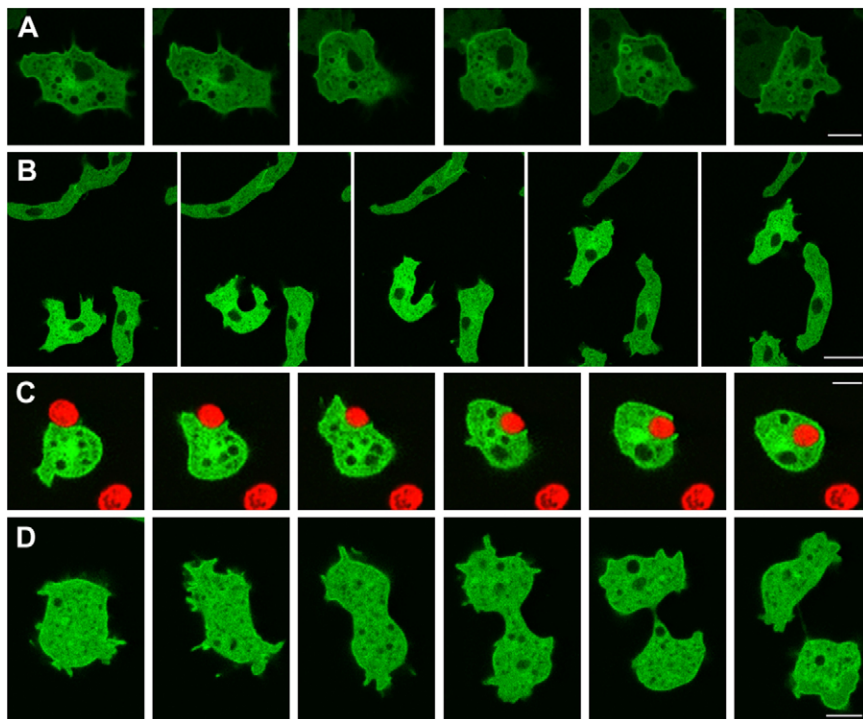

Fig. 1. **Localization of GFP–Rac1A(WT) protein in wild-type *D. discoideum* cells.** (**A**) Random movement. (**B**) Directed migration of aggregation competent cells. (**C**) phagocytosis. (**D**) Cytokinesis. During chemotaxis assay (B), the source of chemoattractant was located 30 μm above the upper right corner of the displayed frame. Phagocytosis was induced using fluorescent, TRITC-labelled yeast particles. Scale bars: 5 μm (A,C,D); 10 μm (B). Time spans: 190 seconds (A); 130 seconds (B); 92 seconds (C); 255 seconds (D).
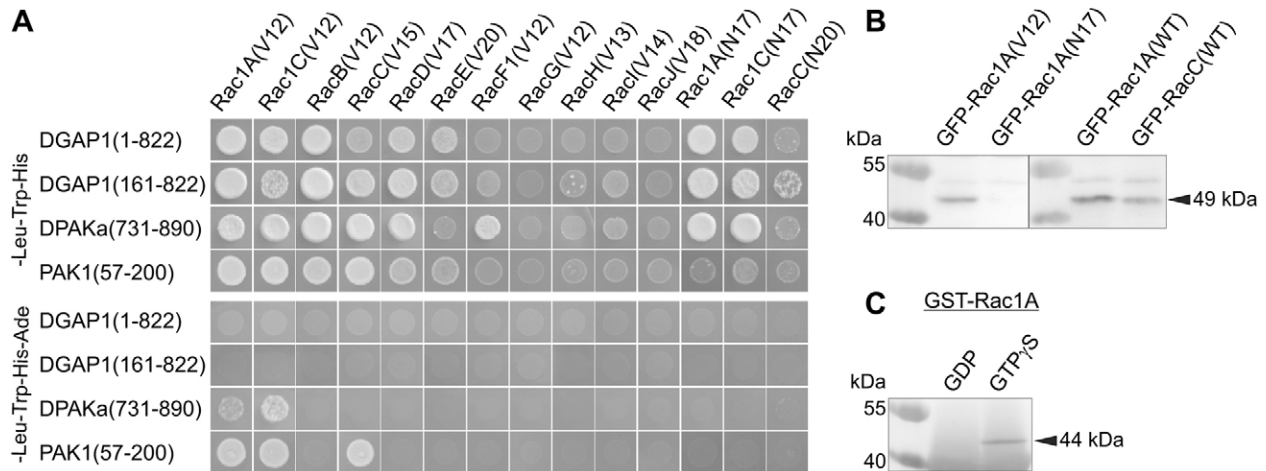
Fig. 2. The regulatory domain of rat PAK1 kinase specifically interacts with active forms of *Dictyostelium* Rac1 and RacC GTPases. (**A**) Yeast two-hybrid assay was used to test interactions between full-length and truncated variants of *Dictyostelium* DGAP1, the GBD of *Dictyostelium* DPAKa kinase, and the regulatory domain of rat PAK1 kinase as baits, with constitutively active forms of 11 *Dictyostelium* Rac proteins and constitutively inactive forms of three of these proteins as preys. Only PAK1 GBD (aa 57–200) interacts with active forms of Rac1A, Rac1C and RacC, but not with their inactive forms, under both low stringency (top panel) and high stringency (bottom panel) conditions. (**B**) GST-binding assay shows interactions of PAK1 GBD with wild-type forms of Rac1A and RacC, and with constitutively active form of Rac1A, but not with constitutively inactive Rac1A. GST–[PAK1(GBD)]$_2$ recombinant protein was bound to glutathione-Sepharose and used to affinity purify GFP fusion proteins from whole-cell lysates, as indicated. After elution proteins were immunoprecipitated with anti-GFP antibodies. (**C**) GST–Rac1A was bound to glutathione-Sepharose and charged with either GDP or GTPγS as indicated. Using whole-cell lysate, GBD probe was immunoprecipitated with anti-PAK1 GBD antibodies exclusively from the column containing GTPγS-loaded GST–Rac1A.

and RacC. In another GST pull-down assay, recombinant GST–Rac1A was coupled to gluthathione–Sepharose beads and loaded with either GDP or the non-hydrolysible GTP analogue GTPγS. Proteins from lysate of cells expressing PAK1(GBD)–DYFP fusion protein that bound to functionalized beads were immunoprecipitated with antibodies against PAK1 GBD only from the column containing GTPγS-loaded GST–Rac1A (Fig. 2C).

FRET probes demonstrate an interaction between the PAK1 GBD and the active form of Rac1A in living cells

Fluorescence (or Förster) resonance energy transfer (FRET) is an established method for probing binary interactions between fluorescently labelled molecules in living cells (Zhang et al., 2002). In particular, it has been successfully applied to map the sites of intracellular activity of small Rho GTPases in leukocytes, fibroblasts and other cell types (Pertz and Hahn, 2004). We adapted this approach to examine the interaction between PAK1 GBD and Rac1A in living *Dictyostelium* cells. The following unimolecular FRET probes were constructed and expressed in wild-type cells: Rac1A(WT)$_{FRET}$, Rac1A(V12)$_{FRET}$ and Rac1A(N17)$_{FRET}$ (Table 1). These probes encompass the same

PAK1 GBD domain as used in yeast two-hybrid and pull-down assays. A schematic presentation of the Rac1A(WT)$_{FRET}$ probe is shown in Fig. 3A. All probes contain a C-terminal CAAX motif to allow appropriate anchorage of the probe into the plasma membrane analogous to endogenous Rac1 proteins.

To determine the extent of the FRET effect, we measured sensitised emission of the acceptor (mRFPmars) upon excitation of the donor (DYFP). The cells expressing a FRET construct were mixed with cells expressing either only the donor or the acceptor. The recorded images always included at least one cell of each species (Fig. 3C), and corrections for bleed-through and cross-excitation were performed separately for each analyzed image. Calculations of FRET efficiency were performed on a pixel-by-pixel basis (Fig. 3D). Two probes, Rac1A(WT)$_{FRET}$ and Rac1A(V12)$_{FRET}$, were localized in the cell cortex and in the perinuclear region, resembling to a large extent the localization of GFP–Rac1A, but with a lower background level in the cytoplasm (Fig. 3C). The third probe, Rac1A(N17)$_{FRET}$, was localized in the cytoplasm with no significant cortical enrichment (data not shown). For each measured cell, average FRET efficiency E in the cell cortex was calculated (see the Materials and Methods).

Table 1. Nomenclature of fluorescent probes

| Composition of construct	Description of probe	Abbreviation
DYFP–PAK1(GBD)–Rac1A(WT)–mRFPmars	FRET probe for wild-type Rac1A	Rac1A(WT)$_{FRET}$
DYFP–PAK1(GBD)–Rac1A(V12)–mRFPmars	FRET probe for constitutively activated Rac1A	Rac1A(V12)$_{FRET}$
DYFP–PAK1(GBD)–Rac1A(N17)–mRFPmars	FRET probe for constitutively inactivated Rac1A	Rac1A(N17)$_{FRET}$
DYFP–PAK1(GBD)–mRFPmars	FRET probe for wild-type RacC	RacC(WT)$_{FRET}$
DYFP–PAK1_GBD–mRFPmars	FRET probe for endogenous activated Rac1 (negative control)	Neg$_{FRET}$
DYFP-mRFPmars	FRET probe for constitutive FRET (positive control)	Pos$_{FRET}$
PAK1(GBD)–DYFP	Probe for activated Rac1 in the cell front	GBD probe
mRFPmars-DGAP1	Probe for the quaternary complex in the cell back that includes Rac1 (its assembly depends on binding of activated Rac1)	DGAP1 probe
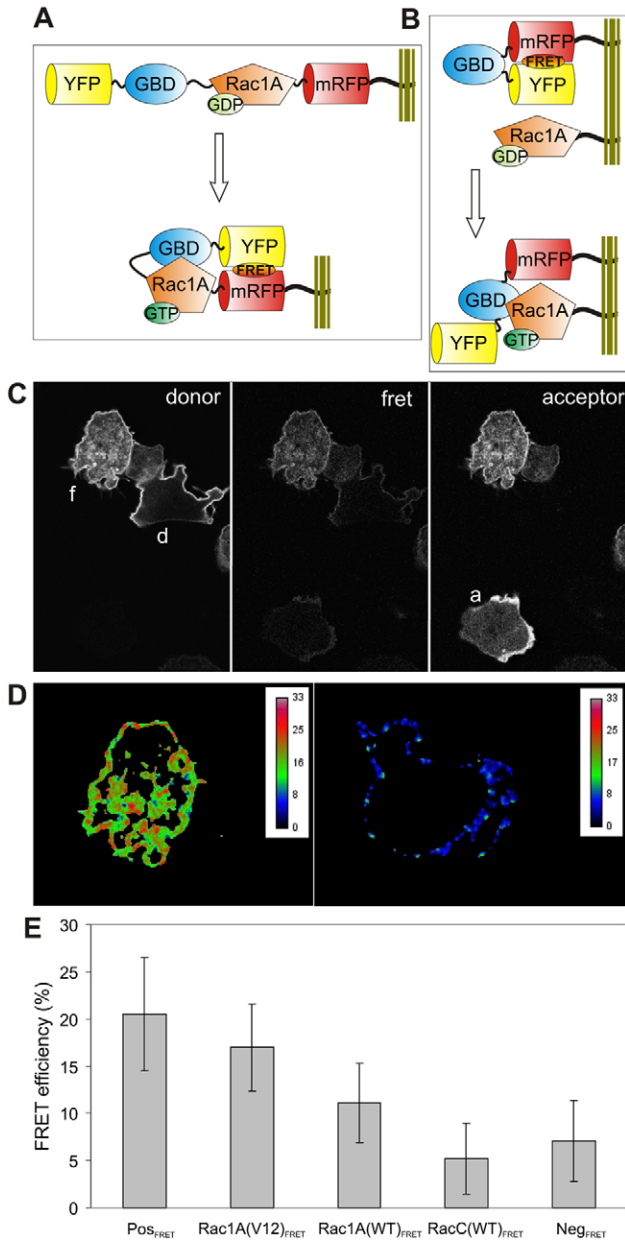
Fig. 3. Intramolecular constructs used for measurements of FRET efficiency. (**A**) Quadruple construct Rac1A(WT)$_{FRET}$ reports FRET upon interaction between Rac1A–GTP and PAK1 GBD. In Rac1A(V12)$_{FRET}$ and Rac1A(N17)$_{FRET}$ constructs, wild-type Rac1A is replaced by the constitutively active and constitutively inactive mutants of Rac1A, respectively. (**B**) Triple construct Neg$_{FRET}$ [DYFP–PAK1(GBD)–mRFPmars] reports the suppression of constitutive FRET in the cell cortex upon interaction between endogenous Rac1A–GTP and PAK1 GBD. (**C**) An example of raw data images where three cells, expressing the Rac1A(V12)$_{FRET}$ construct (f), a pure donor species (d) and a pure acceptor species (a) were sequentially recorded in donor, fret and acceptor channels. (**D**) Colour-coded maps of the FRET efficiency as determined for a cell expressing Rac1A(V12)$_{FRET}$ (left) and a cell expressing Neg$_{FRET}$ (right). (**E**) Apparent FRET efficiencies measured for the five designated constructs (mean ± s.d.).

Apparent FRET efficiencies of the positive control, $E(Pos_{FRET})=20.5\pm6.0$ $(n=12)$, and $E[Rac1A(V12)_{FRET}]=17.0\pm4.6$ $(n=10)$, are statistically indistinguishable $(P>0.15)$, which suggests an interaction between PAK1 GBD and activated

Rac1A in vivo (Fig. 3E). We next compared FRET efficiency of the Rac1A(V12)$_{FRET}$ construct with a tripartite construct DYFP–PAK1(GBD)–mRFPmars that served as a negative control, Neg$_{FRET}$ (Fig. 3B). Although this construct is designed to enable constitutive FRET, interaction of the PAK1 GBD with endogenous Rac1A–GTP precludes close contact between DYFP and mRFPmars, and thereby diminishes FRET (Itoh et al., 2002). $E[Rac1A(WT)_{FRET}]=11.1\pm4.2$ $(n=10)$ was significantly higher than $E(Neg_{FRET})=7.1\pm4.3$ $(n=13; P<0.05)$, indicating a GEF activity in the cell cortex that activates both endogenous and ectopically expressed Rac1A (Fig. 3E). Owing to a low signal-to-noise ratio, it was not possible to reliably measure FRET efficiency of the Rac1A(N17)$_{FRET}$ probe.

GBD probe is transiently recruited to the cell cortex upon stimulation with folic acid and cAMP

We next expressed PAK1(GBD)–DYFP (abbreviated hereafter to 'GBD probe', see Table 1) in wild-type cells and in cells that overexpress mRFPmars–Rac1A(WT) fusion protein and performed a series of uniform stimulation experiments using folic acid and cAMP as chemoattractants. The primary goal was to test whether activation of Rac1 GTPases is triggered by these stimuli, which would indicate that Rac1 is participating in signalling pathways downstream of heterotrimeric G proteins in *Dictyostelium* (Van Haastert and Devreotes, 2004). Additionally, an enhanced response to chemoattractants in cells that overexpress Rac1A would be an independent verification of an interaction between GBD probe and the active form of Rac1A in living cells, shown by FRET probes. Stimulation of vegetative cells by a pulse of folic acid induced a transient recruitment of GBD probe to the cell cortex lasting approximately 12 seconds (Fig. 4A,B). Cortical enrichment of the probe was stronger in cells overexpressing mRFPmars–Rac1A(WT) than in wild-type cells, whereas pulse-induced enrichment of mRFPmars–Rac1A(WT) was barely visible. Similar results were obtained when early stage aggregation-competent cells were stimulated by a pulse of cAMP (Fig. 4C,D), albeit with a lower cortical contrast. An overall lower ratio between intensities of cortical versus cytoplasmic GBD probe in aggregation competent cells is due to a gradually diminishing level of the probe in the course of starvation (western analysis, data not shown).

Results of the uniform stimulation experiments corroborated that the GBD probe interacts with activated Rac1 in living cells, and that Rac1 activation can be triggered by G-protein-mediated signal transduction pathways. Kinetics of Rac1 activation in the cortex, as visualized by cortical enrichment of the GBD probe, is comparable to the kinetics of cortical enrichment of Ras and phosphatidylinositol-3,4,5-trisphosphate probes upon stimulation with folic acid and cAMP (Sasaki et al., 2007).

GBD probe is transiently recruited to leading edges, dorsal ruffles and endocytotic cups and localizes to sites of active actin assembly

On the basis of a specific interaction between PAK1 GBD and activated Rac1A that was demonstrated in vitro and in vivo, we decided to use the GBD probe as a reporter of active Rac1 in living cells. In contrast to Rac1A(WT)$_{FRET}$, which was localized to the cortical layer of motile cells uniformly and continuously, localization of GBD probe to the cell cortex was spatially confined and transient. In migrating vegetative cells, GBD probe
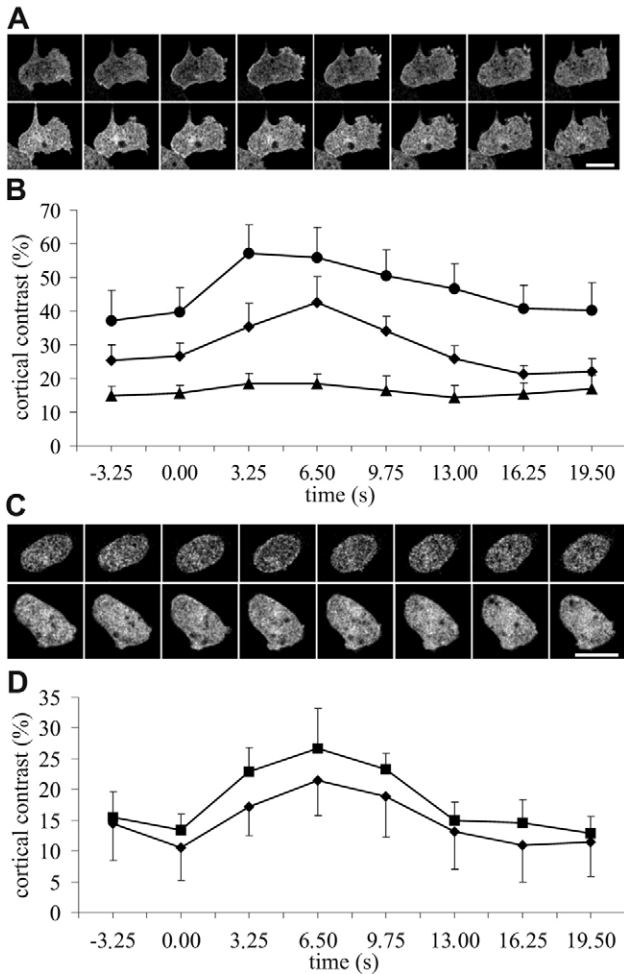
Fig. 4. Translocation of fluorescent probes to the cell cortex upon pulse stimulation by folic acid and cAMP. (**A**) Typical response of a cell that co-expresses the GBD probe (top row) and mRFPmars–Rac1A (bottom row) upon stimulation with folic acid. (**B**) Time courses of the cortical enrichment of GBD probe in wild-type cells (♦), GBD probe in Rac1A-overexpressing cells (●), and mRFPmars-Rac1A in Rac1A-overexpressing cells (▲) upon application of 50 μM folic acid. (**C**) Typical dynamics of the cortical enrichment of GBD probe in a Rac1A-overexpressing cell (top row) and in a wild-type cell (bottom row) upon stimulation with cAMP. (**D**) Time courses of the cortical enrichment of GBD probe in wild-type cells (♦), and in Rac1A-overexpressing cells (■) upon application of 30 μM cAMP. Cortical contrast designates average enrichment of the fluorescent signal in the cell cortex as compared with the cytoplasm (B,D; see the Materials and Methods). Shown results are averaged over six cells from at least three independent experiments (mean ± s.e.m.). Scale bars: 10 μm. Times in seconds relate to the time-point of application of the pulse (B,D). Acquisition times of images in A and C correspond to measurement points in B and D.

was enriched in the cortical protruding regions and dorsal ruffles of polarized cells (Fig. 5A). During chemotaxis of aggregation-competent cells, however, cortical or polarized enrichment of the probe could no longer be detected (Fig. 5B). GBD probe also localized to nascent macropinosomes (Fig. 5A, frames 4 and 5; Fig. 5C, frame 4) and phagosomes (Fig. 5C), but was not enriched in the cortex during cytokinesis, either at the poles or in the cleavage furrow (Fig. 5D).

Because GBD probe transiently accumulated at sites of active actin assembly in migration and endocytosis, we correlated

spatial distribution of active Rac1 detected by our probe with localization of polymerized actin by co-expressing GBD probe with mRFPmars–LimEΔcoil, an established probe of filamentous actin in *Dictyostelium* cells (Gerisch et al., 2004). The two markers colocalized in leading edges and endocytotic cups, indicating a close association between Rac1 activity and treadmilling actin filaments in these structures (Fig. 5E; supplementary material Movie 1). Kinetics of the cortical recruitment of the two probes upon stimulation by folic acid also indicated temporal coincidence of Rac1 activation and actin polymerization in the cell cortex within the limits of our time resolution (Fig. 5F,G).

GBD probe specifically interacts with Rac1, and not RacC, in living cells

Results of the yeast two-hybrid and GST pull-down assays indicated that, besides its interaction with activated Rac1 GTPases, PAK1 GBD is also capable of interacting with activated RacC in vitro (Fig. 2). To determine whether this interaction occurs in the cortex of living cells, we performed two control experiments. First, we expressed a GFP–RacC(WT) fusion protein in *Dictyostelium* and monitored its localization in motile cells. GFP–RacC(WT) was accumulated in cytoplasmatic granules but was not enriched in the cell cortex (supplementary material Fig. S1). From the localization data, it therefore appears that Rac1 and RacC localize to non-overlapping compartments within the cell (cf. Fig. 1 and supplementary material Fig. S1). Our GBD probe also never displayed localization that resembled that of GFP–RacC(WT), which suggests that PAK1 GBD and RacC do not interact in vivo.

To strengthen this conclusion, we also constructed a unimolecular FRET probe where Rac1A in the Rac1A(WT)$_{FRET}$ construct was replaced with RacC, RacC(WT)$_{FRET}$. FRET experiments performed with RacC(WT)$_{FRET}$ resulted in a low FRET efficiency, E[RacC(WT)$_{FRET}$]=5.2±3.8 (n=10), which was significantly lower than the value for Rac1A(WT)$_{FRET}$ ($P<0.01$) (Fig. 3E). Taken together, these results indicate that RacC is generally not present in the cell cortex, and when its cortical localization is enforced, it still does not interact with the PAK1 GBD. We therefore conclude that the GBD probe specifically labels cortical sites of Rac1 activation.

GBD probe and DGAP1 both interact with activated Rac1, but show distinct localization in migrating cells

DGAP1 is an IQGAP-related protein from *Dictyostelium* that preferentially binds the activated form of Rac1 (Dumontier et al., 2000), and is part of a quaternary complex also containing a heterodimer of actin-bundling proteins cortexillin I and cortexillin II (Faix et al., 2001). Charging of Rac1 with GTP is mandatory for the assembly of this quaternary complex in vitro and in vivo. This Rac1-containing complex plays a crucial role in driving cytokinesis in *Dictyostelium*, and accumulates in the cleavage furrow of dividing cells. We first examined localization of a component of the complex, cortexillin I, in migrating cells. We used GFP-labelled C-terminal region of cortexillin I (residues 352–444), which is sufficient for targeting cortexillin I to the cell cortex, for the rescue of cytokinesis (Weber et al., 1999a), and for the interaction with DGAP1 (Faix et al., 2001). GFP–cortexillin-I(352–444) localized to the cortex of unipolar and multipolar cells, but was excluded from protruding regions (Fig. 6A), which is the opposite to the localization of the GBD probe.
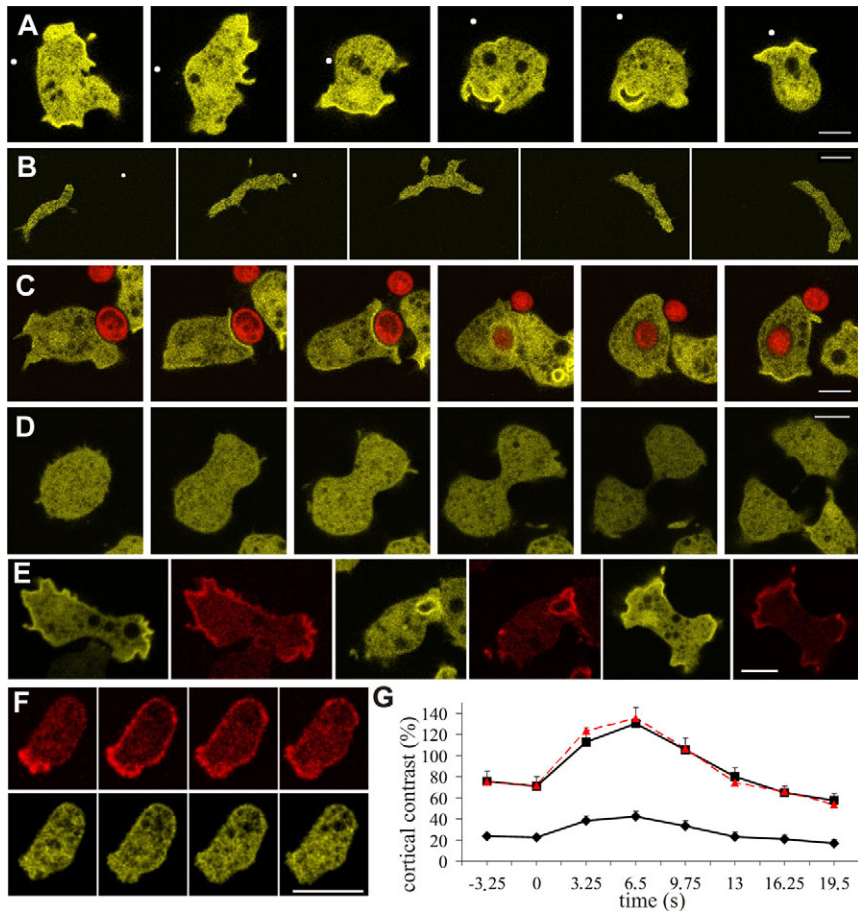
Fig. 5. Localization of GBD probe in wild-type
***Dictyostelium* cells.** (**A**) Random movement. (**B**) Directed
movement of aggregation competent cells.
(**C**) Phagocytosis. (**D**) Cytokinesis. (A) White dots
designate a fixed reference point on the substratum. Note
that the cell moved significantly downward between the
third and the fourth frame. (B) White dots mark the
position of the micropipette tip, i.e. the source of
chemoattractant, in the first two frames. Afterwards, the
micropipette was relocated 20 μm underneath the lower
left corner of the displayed frame. (C) Phagocytosis was
induced using fluorescent, TRITC-labelled yeast particles.
(**E**) Colocalization of co-expressed GBD probe (yellow)
and mRFPmars-LimEΔcoil (red), a marker of F-actin.
(**F**) Typical dynamics of the cortical enrichment of the F-
actin marker mRFPmars-LimEΔcoil (top row, red) and
GBD probe (bottom row, yellow), in a cell that co-
expresses the two probes, upon stimulation with folic
acid. (**G**) Time courses of the cortical enrichment of
mRFPmars-LimEΔcoil (■), and GBD probe (♦), in cells
that co-express the two probes upon application of 50 μM
folic acid. The red dashed curve superimposed with the
mRFPmars-LimEΔcoil curve corresponds to the cortical
contrast values for GBD probe (lower curve) multiplied
by the ratio between the cortical contrast for mRFPmars–
LimEΔcoil and the cortical contrast for GBD probe
avaraged over all time points. Shown results are averaged
over six cells from at least three independent experiments
(mean ± s.e.m.). Acquisition times of images in F
correspond to every other measurement points in G,
starting at the 0 second time point. Scale bars: 5 μm
(A,C,D); 10 μm (B,E,F). Time spans: 325 seconds (A);
120 seconds (B); 210 seconds (C); 200 seconds (D).

This result prompted us to construct a cell line that
simultaneously expresses two direct interaction partners of
Rac1–GTP: mRFPmars–DGAP1 (referred to as DGAP1 probe
hereafter) and GBD probe. Notably, during migration, when
these cells adopted a polarized shape, the two fluorescent markers
segregated into two non-overlapping cortical compartments.
Whereas the GBD probe was clearly enriched at the leading
edge, the DGAP1 probe labelled the lateral and posterior cell
cortex (Fig. 6B; supplementary material Movie 2). During
random motility, *Dictyostelium* cells occasionally acquire a
non-polarized, rounded shape. Cells recorded in such stages
showed enrichment of DGAP1 probe throughout the cortex and
no cortical enrichment of the GBD probe, thus indicating that the
two probes detect two distinct Rac1 populations (Fig. 6C).

GBD probe and DGAP1 probe show opposite localization and dynamics in actin-driven processes

To further characterize the inter-relationship between the GBD
and DGAP1 probes, we monitored cells that express both probes
during cytokinesis and phagocytosis. In both processes, the two
probes were mutually exclusive. At the onset of cytokinesis, the
DGAP1 probe occupied the entire cortex and was gradually
restricted to the furrow region, whereas the GBD probe
occasionally appeared at the leading edges of incipient
daughter cells only at the time of scission (Fig. 7A). In
phagocytosis, the DGAP1 probe was excluded from the
phagocytotic cups demarcated by GBD probe (Fig. 7B;
supplementary material Movie 3). Stimulation with folic acid

revealed an opposite dynamics of the cortical enrichment of the
two probes. Whereas the GBD probe was recruited to the cortex
of the DGAP1-overexpressing cells with a similar dynamics as
observed in wild-type cells, DGAP1 probe was, on the contrary,
displaced from the cortex with an analogous dynamics
(Fig. 7C,D). Because this result indicated that the two probes
compete for the same population of activated Rac1, we expressed
the GBD probe in cells devoid of DGAP1. Compared with
wild-type cells, cortical enrichment of the GBD probe upon
chemoattractant stimulation was indeed remarkably stronger in
DGAP1-null cells, whereas in DGAP1-overexpressing cells it
was weaker than in the wild type (Fig. 7C,D). We also checked
localization of GBD probe in DGAP1-null cells during random
movement, endocytosis and cytokinesis, and found its spatial
distribution not altered compared with distribution in wild-type
cells (data not shown).

Finally, we monitored localization of the GBD and DGAP1
probes in chemotaxis. Owing to its stronger cortical enrichment
in aggregation-competent DGAP1-null cells, the GBD probe was
found to localize to the leading edge of these cells during
chemotaxis to cAMP (Fig. 7E; supplementary material Movie 4).
As in other actin-driven processes, localization of the DGAP1
probe in chemotaxis was just the reverse: it was enriched at the
trailing edge of aggregation-competent cells that express both
probes (Fig. 7F; supplementary material Movie 5). Taken
together, these results strengthened our conclusion that the
GBD and DGAP1 probes detect two distinct populations of
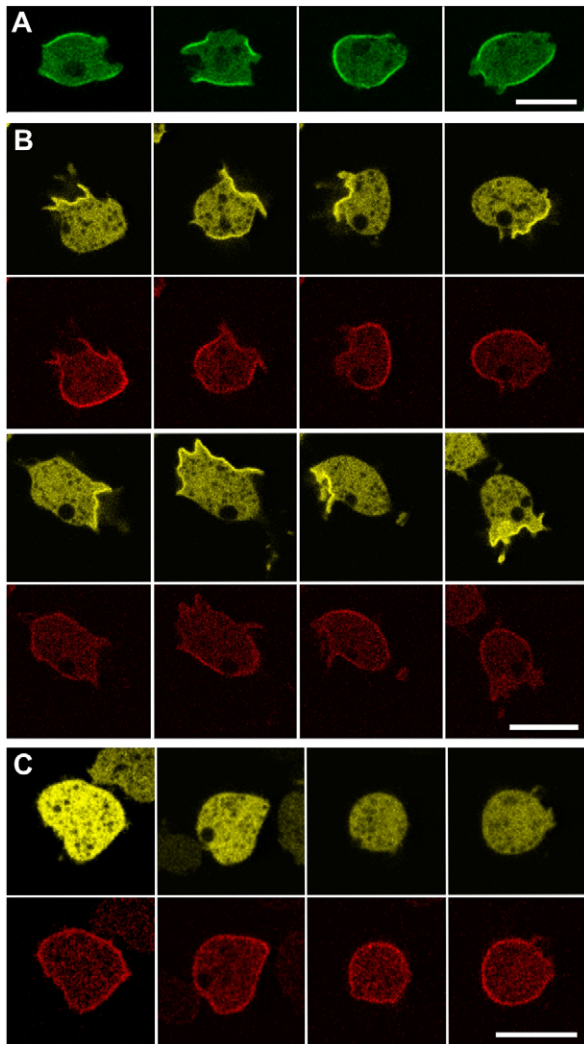activated Rac1 in living *Dictyostelium* cells.

Fig. 6. Localization of fusion proteins in polarized and non-polarized cells. (**A**) Localization of fusion protein GFP–CI(352–444) in cortexillin-I-null cells during random movement. (**B**) Opposite localization of the GBD probe (yellow) and the DGAP1 probe (red) in a polarized *Dictyostelium* cell that expresses both fusion proteins. (**C**) Four *Dictyostelium* cells that simultaneously express the GBD probe (yellow) and DGAP1 probe (red) were recorded in non-polarized phases during random movement. Scale bars: 10 μm. Time spans: 280 seconds (A); 250 seconds (B).

Cortical localization of GBD probe is abolished by treatment with latrunculin B

Prompted by the different behaviour of the two Rac1-GTP-binding probes in both polarized and non-polarized cells, we next examined the intracellular localization of the two probes upon disruption of the actin cytoskeleton with latrunculin B. Shortly after addition of 20 μM latrunculin B, the GBD probe was completely removed from the cortical layer of treated cells (Fig. 8A, left column). However, the DGAP1 probe was retained in the cortical layer and in the small spherical blebs that typically form after latrunculin treatment (Fig. 8A, middle column). Localization to these membranous remnants indicates that the DGAP1 probe stays firmly attached to the plasma membrane irrespective of the absence of cortical F-actin. By contrast, a fluorescently labelled probe for F-actin, mRFPmars–ABD120,

disappeared from the cortex upon treatment with latrunculin B, as expected, except from a few small remaining nodules (Fig. 8A, right column). All these changes were reversible after removal of latrunculin B. These results indicate that cortical localization of the DGAP1-containing quaternary complex is independent of its cortexillin-mediated binding to F-actin, suggesting that the complex binds to the cell plasma membrane. Cortical localization of the population of activated Rac1 that is recognized by GBD probe, however, critically depends on the integrity of the actin cytoskeleton.

Discussion

Similarly to motile animal cells such as fibroblasts and leukocytes, *Dictyostelium* cells harbour a rich repertoire of Rho family GTPases as well as their regulators and downstream effectors (Rivero and Somesh, 2002). Rac is considered to be the founder member of the Rho family (Boureux et al., 2007), which is exemplified in *Dictyostelium* cells that lack Rho GTPases from the other two groups: Rho and Cdc42. Functional analysis of *Dictyostelium* Rho GTPases thus offer a useful complement to studies conducted in other cells. Biological roles of some *Dictyostelium* Rho GTPases have been assessed by genetic knockout and overexpression experiments, as well as localization studies, and a number of their effectors have been identified (Vlahou and Rivero, 2006). RacE, for instance, plays a role in the regulation of cytokinesis (Larochelle et al., 1997), RacB is involved in the control of directional sensing (Park et al., 2004), whereas RacG is important for cell shape, motility and phagocytosis (Somesh et al., 2006). However, direct evidence for the sites of activity and dynamics of RhoGTPases in *Dictyostelium* has been lacking so far.

Here we investigated the dynamics of activated Rac1 GTPases in *Dictyostelium* cells using the biosensor approach. *Dictyostelium* Rac1A, Rac1B and Rac1C are most closely related to human Rac1, and all of them share an identical effector domain (Dumontier et al., 2000). Their general importance for regulation of the actin cytoskeleton was previously demonstrated by stable expression of constitutively active Rac1A and Rac1C, which resulted in dominant-negative effects on actin-based processes including cell migration, endocytosis and cytokinesis, as well as in atypical cell morphology (Dumontier et al., 2000). Similar effects have also been induced by overexpression of constitutively active Rac1B (Duleh et al., 2005). By monitoring the dynamics of two fluorescently labelled Rac1 effectors, PAK1 GBD and DGAP1, in live cells, we now obtained experimental evidence showing that active Rac1 GTPases are involved in the regulation of multiple processes. Most notably, active Rac1 GTPases are simultaneously involved in distinct processes at two opposite sides of migrating cells, protrusion at the front and retraction at the back.

Our results indicate that the posterior activity of Rac1 is mediated by the quaternary cortical complex consisting of the GTPase, IQGAP-related protein DGAP1 and the heterodimeric actin-bundling protein cortexillin. This complex supports cytokinesis and critically determines the mechanical properties and morphology of *Dictyostelium* cells (Simson et al., 1998; Weber et al., 1999a; Weber et al., 1999b). The most likely candidate for a Rac1 effector at the cell front is the hetero-pentameric Scar/WAVE complex, which regulates Arp2/3-driven actin polymerization at the leading edge (Insall et al., 2001; Linkner et al., 2011; Pollitt and Insall, 2009; Pollitt et al., 2006). Another possible candidate for a Rac1-GTP-interacting protein at
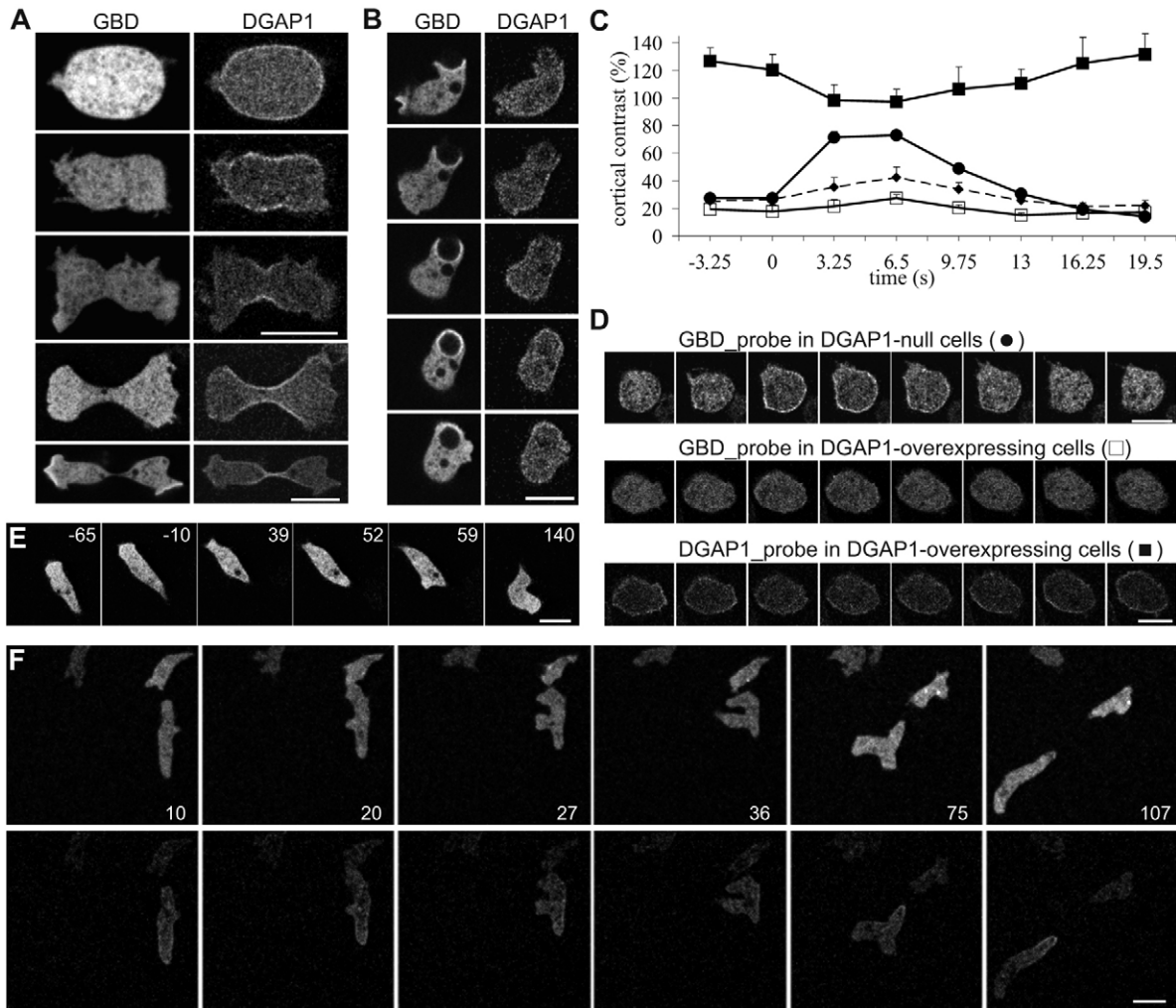
Fig. 7. Localization of the GBD and DGAP1 probes in *Dictyostelium*. (**A**) Localization of GBD probe (left column) and DGAP1 probe (right column) in successive phases of cytokinesis in five cells that co-express the two probes. (**B**) Localization of GBD probe (left column) and DGAP1 probe (right column) during phagocytosis in a cell that co-expresses the two probes. Time span: 50 seconds. (**C**) Time courses of the cortical enrichment of GBD probe in wild-type cells (♦, dashed line, reproduced from Fig. 4B as a reference), GBD probe in DGAP1-null cells (●, middle curve), GBD probe in DGAP1-overexpressing cells (□, bottom curve), and DGAP1 probe in DGAP1-overexpressing cells (■, top curve) upon application of 50 µM folic acid. Times in seconds relate to the time-point of application of the pulse. Results are averaged for six cells from at least three independent experiments (mean ± s.e.m.). (**D**) Typical dynamics of the cortical enrichment of GBD probe in DGAP1-null cells (top row), GBD probe in DGAP1-overexpressing cells (middle row), and DGAP1 probe in DGAP1-overexpressing cells, upon stimulation with folic acid. Acquisition times of images in D correspond to measurement points in C. (**E**) Re-distribution of GBD probe in an aggregation competent DGAP1-null cell exposed to a cAMP gradient by means of a micropipette. The micropipette tip was initially positioned 25 µm away from the top left corner of the displayed frame, in the 11 o'clock position, and than re-positioned 25 µm away from the bottom right corner of the displayed frame, at 5 o'clock. (**F**) Re-orientation of an aggregation competent cell that co-expresses GBD probe (top row) and DGAP1 probe (bottom row). The micropipette tip was positioned 60 µm away from the bottom left corner of the displayed frame, at 8 o'clock. Times in seconds relate to the time-point of replacement (E), or initial placement (F), of the micropipette. Scale bars: 10 µm.

the leading edge is formin C because it has been previously found to accumulate in macropinosomes and phagocytotic cups at the cell front (Kitayama and Uyeda, 2003). Yet another Rac1-GTP effector is DPAKa, but the full-length kinase has not been found to localize to the front of migrating *Dictyostelium* cells (Müller-Taubenberger et al., 2002).

The GBD probe and DGAP1 probe, which both interact with activated Rac1 in vitro, apparently detect two separate populations of Rac1 GTPases in living cells. At the back of the migrating cells, a proportion of Rac1 is probably bound to a fairly stable complex with DGAP1 and cortexillin. Although formation of this complex depends on interaction between activated Rac1A and DGAP1 (Faix et al., 2001), the exact state of Rac1 inside the assembled complex is not known. It might be in a GTP-bound or a GDP-bound form, or might even assume a nucleotide-free form as shown for Cdc42 in a complex with IQGAP1 (Grohmanova et al., 2004). Either way, the complex sequesters Rac1 at the back of polarized cells and probably leaves a small number of activated Rac1 molecules accessible to GBD probe. A much faster Rac1 turnover at the front renders its GTP-bound form more accessible to a transient binding of GBD probe (Fig. 8B). Despite a mutually exclusive localization of the two probes, the extent of the free
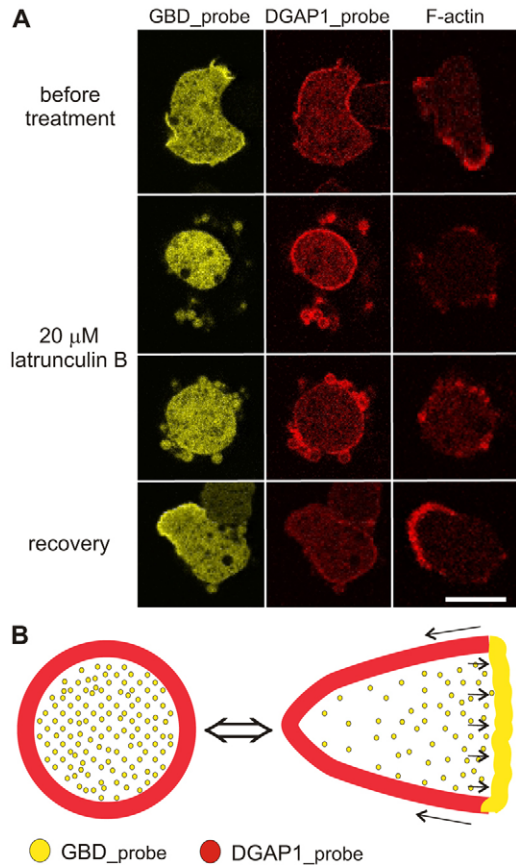
Fig. 8. Effect of latrunculin B on cortical localization of the GBD and DGAP1 probes. (**A**) Cells that co-express the GBD (yellow, left column) and DGAP1 probes (red, middle column) before treatment with latrunculin B (top row), 20 minutes after addition of 20 µM latrunculin B (two middle rows), and 30 minutes after the washout (bottom row). The right column shows cells that express an F-actin marker, the actin-binding domain ABD from ABP 120 kDa protein fused with mRFPmars. Scale bar: 10 µm. (**B**) Schematic presentation of the intracellular distribution of two Rac1 effectors, GBD probe (yellow) and DGAP1 probe (red), in non-polarized (left) and polarized (right) cells. In non-polarized cells, DGAP1 probe is localized to the entire cell cortex, whereas GBD probe remains in the cytoplasm. In polarized cells, GBD probe is recruited to the protruding leading edge, and DGAP1 probe is displaced from that cortical region.

Rac1-GTP zone appears not to be limited by the DGAP1 complex, because it is unchanged in DGAP1-null mutants. However, another IQGAP-related protein from *Dictyostelium*, GAPA, has partly overlapping functions with DGAP1 (Faix et al., 2001), and might play a role in restricting the Rac1-GTP zone labeled by the GBD probe in the absence of DGAP1.

The Rac1-sequestering function of the DGAP1–cortexillin complex is supported by its chemoattractant-induced disassembly and release from the cortex, coupled with the cortical recruitment of free Rac1A (Fig. 7). In polarized cells, the Rac1-sequestering activity of the complex appears to suppress membrane protrusions driven by actin polymerization in the rear of cells. The importance of the DGAP1-containing complex for regulation of cell migration and phagocytosis is manifested in phenotypes of DGAP1-null and DGAP1-overexpressing mutants (Faix et al., 1998). DGAP1-overexpressing cells are making fewer actin-based protrusions, move more slowly and phagocytose less efficiently than wild-type

cells, which is consistent with more Rac1 being trapped inside the DGAP1 complex and therefore not available to promote actin-based motility. DGAP1-null cells, on the contrary, are making more actin-based protrusions and move faster than wild-type cells. Consistently, it has also been shown that lack of DGAP1 leads to an increased cellular F-actin content (Faix et al., 1998).

Our finding of distinct and non-overlapping localizations of two Rac effector proteins is not without precedence. Distinct patterns of colocalization of Rac1 with its two effectors, IQGAP1 and Sra-1, at the inner side of the ventral membrane of Vero cells was determined by double immunogold labelling (Watanabe et al., 2008). Closely related to DGAP1, IQGAP1 is also involved in crosslinking of actin filaments, whereas Sra-1 is a component of the hetero-pentameric Scar/WAVE regulatory complex, which induces formation of lamellipodia (Chen et al., 2010). Similarly, distinct spatial association patterns between Cdc42 and its key effector proteins, PAK1 and N-WASP, were revealed by multiphoton fluorescence lifetime imaging microscopy in breast carcinoma cells (Parsons et al., 2005).

Evidence for additional roles of Rac beyond its involvement in protrusion of the leading edge was obtained using a FRET-based biosensor for Rac activity in living cells, where activated Rac was detected in the retracting tails of migrating neutrophils (Gardiner et al., 2002). Consistently, Rac1-deficient neutrophils were reported to display an inability to retract their tails (Sun et al., 2004). Ectopic expression of members of the Rac subfamily not only triggered the formation of lamellipodia but also induced the formation of bundles of actin filaments (Aspenström et al., 2004). Finally, Rac1 was shown not only to inhibit Rho signalling at the leading edge but also to positively regulate the function of RhoA–myosin-II at the back of chemotactic neutrophils (Pestonjamasp et al., 2006). In the present work, we provide yet another example of Rac activity at both ends of polarized cells, supporting multiple signalling roles of Rac GTPases in the control of cell migration.

Localization of DGAP1 probe at the cell boundary is independent of F-actin, which indicates that the quaternary posterior complex is probably bound to the plasma membrane by the phosphatidylinositol-4,5-bisphosphate-binding C-terminal domain of cortexillin I. This C-terminal nonapeptide has been shown to almost double the accumulation of cortexillin I in the cell cortical layer and to suppress the actin-bundling activity of cortexillin I when bound to phosphatidylinositol-4,5-bisphosphate (Stock et al., 1999). Posterior localization of the quaternary complex might be mediated by phosphatase and tensin homolog (PTEN), which converts phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-4,5-bisphosphate, because PTEN is also enriched at the lateral and rear regions of migrating cells (Arai et al., 2010). However, sensitivity of the cortical localization of GBD probe to latrunculin B treatment suggests that the anterior Rac1-GTP is not stably anchored to the membrane in the absence of an actin cortex, or that the cortical localization of a RacGEF responsible for Rac1 activation depends on F-actin. Interestingly, cortical localization of a member of the *Dictyostelium* Dock180 family of RacGEFs, DockD, indeed depends on actin polymerization (Para et al., 2009). A related example is provided by the WAVE complex in neutrophils, whose proper assembly and localization also rely on integrity of the actin cytoskeleton (Millius et al., 2009). Unlike the DGAP1 probe, the GBD probe is not enriched in the cortex of round, non-polarized cells that have an intact cortical actin layer

(Fig. 6C). It appears therefore that the default, symmetrical state of the cortex in *Dictyostelium* cells is determined by the activity of the posterior quaternary complex, whereas breaking of symmetry is brought about by an anterior activity, which leads to cell polarization.

By the use of Rac1A(WT$_{FRET}$) probe, RacGEF activity was detected throughout the cell cortex and in the perinuclear region of transfected cells (Fig. 3). FRET sensors used in this work are designed to indicate the presence of RacGEF proteins that facilitate the exchange of GDP by GTP on Rac1A, and thus drive interaction between activated GTPase and its effector PAK1 GBD within the probe. However, these sensors cannot discriminate between different RacGEFs and will, therefore, report Rac1A activation irrespective of a particular GEF activity and its specific interactions. Only by using two probes, a GBD probe and a DGAP1 probe, which are designed to interact with endogenous Rac1-GTP but based on different Rac1 effectors, did it become possible to pin down two non-overlapping dynamic populations of Rac1 that are probably activated by different GEFs. RacGEFs responsible for activation of Rac1 at the cell front probably belong to the *Dictyostelium* Dock180 family, because DockD was shown to form a complex with DdELMO1 and Rac1A (Para et al., 2009). A counterpart of Dock180 GEFs at the cell back remains to be identified.

Our findings lead us to propose a novel mechanism for spatial and temporal regulation of Rac activity by competing effector proteins. Our results imply that two spatially separated effector complexes allow for retention and selective recruitment of Rac to functionally distinct cortical regions. Thereby, a high abundance of an activated GTPase, as exemplified by Rac1 in *Dictyostelium*, can be maintained in the cortex and facilitates a fast and localized response to upstream signals (Postma et al., 2004). In conclusion, Rac1 GTPases appear to be key mediators in signalling cascades that lead to both protrusion at the front and retraction at the back of *Dictyostelium* cells. The tools described in this report will be instrumental to further explore spatial and temporal relationships between different signalling pathways and morphological changes in these small and fast-moving cells.

Materials and Methods
Cell culture, cell lines and transformation of *D. discoideum* cells
All *D. discoideum* cell lines used in this study were cultivated in axenic medium at 23°C on polystyrene culture dishes as previously described (Faix et al., 2001). Cell transformation by electroporation and clonal selection was performed as described (Kimmel and Faix, 2006). Cell lines were derived from AX2 parent strain and express the following constructs: GFP–Rac1A(WT), GFP–Rac1A(V12), GFP–Rac1A(N17) (Dumontier et al., 2000), GFP–RacC(WT) (this work), PAK1(GBD)–DYFP (this work), PAK1(GBD)–DYFP and mRFPmars–DGAP1 (this work), PAK1(GBD)–DYFP and mRFPmars–Rac1A(WT) (this work), PAK1(GBD)–DYFP and mRFPmars-LimEΔcoil (this work, derived from AX2/mRFPmars-LimEΔcoil) (Schneider et al., 2003), DPAKa(GBD)–DYFP (this work) and mRFPmars-ABD120 (Fischer et al., 2004). In this study, we used DYFP (Müller-Taubenberger, 2006) and mRFPmars (Fischer et al., 2004) fluorescent proteins that were adapted for the codon usage of *D. discoideum*. All unimolecular probes for FRET were also expressed in the AX2 parent strain (this work, see Table 1). Cortexillin-I-null mutants expressing GFP–CI(aa 352–444), CI/GFP–CI(352–444), were described previously (Weber et al., 1999a). PAK1(GBD)–DYFP was also expressed in a DGAP1-null strain (Faix et al., 1998).

D. discoideum transformation vectors
cDNAs encoding PAK1(GBD)–DYFP and GFP–RacC(WT) were cloned from auxiliary vectors into the *Hin*dIII site of pDEXH and into the *Eco*RI and *Hin*dIII sites of pDEXRH expression vectors, respectively (Faix et al., 1992). The orientation of the PAK1(GBD)–DYFP insert was confirmed by digestion with *Bgl*II and *Asp*718. For expression of a DPAKa(GBD)–DYFP construct, cDNA encoding the DPAKa GBD was amplified from auxiliary vector by PCR and

cloned into the *Bam*HI and *Sal*I sites of pDEXRH-MCS-DYFP, a vector derived from pDEXRH. The pDEXRH-MCS-DYFP was constructed by introduction of multiple cloning sites and DYFP into the *Eco*RI and *Hin*dIII sites of pDEXRH. Expression vectors for mRFPmars–DGAP1 and mRFPmars–Rac1A(WT) fusion proteins were generated by insertion of cDNA encoding DGAP1 and Rac1A(WT) into the *Bam*HI and *Eco*RI sites, and *Bam*HI and *Cla*I sites, of plasmid p339-3, respectively (Fischer et al., 2004). All vectors encoding unimolecular FRET probes are based on pDEXRH, and the cloning procedure closely followed construction of the Raichu-Rac probe described previously (Itoh et al., 2002). For the nomenclature of FRET probes, see Table 1. The Rac1A(WT)$_{FRET}$ fusion protein consists of DYFP (aa 1–238), a spacer (Ile-Asp), GBD from rat PAK1 (aa 57–200), a spacer (Ser-Gly-Gly-Thr-Gly-Gly-Gly-Gly-Ser), *D. discoideum* Rac1A (aa 1–176), a spacer (Gly-Gly-Arg-Val-Asp), mRFPmars (aa 1–225), a spacer (Gly-Arg-Ser-Arg) and *D. discoideum* Rac1A (aa 177–194) (Fig. 3A). In Rac1A(V12)$_{FRET}$, Rac1A(N17)$_{FRET}$ and RacC(WT)$_{FRET}$ unimolecular FRET probes, Rac1A (aa 1–176) was replaced with active Rac1A(V12) (aa 1–176), inactive Rac1A(N17) (aa 1–176), or the wild-type form of RacC (aa 1–179), respectively. Triple fusion protein for detection of endogenous activated Rac1 (Neg$_{FRET}$) consists of: DYFP (aa 1–238), a spacer (Ile-Asp-Met), GBD from rat PAK1 (aa 57–200), a spacer (Ser-Gly-Gly-Thr-Gly-Gly-Gly-Gly-Ser-Val-Asp), mRFPmars (aa 1–225), a spacer (Gly-Arg-Ser-Arg) and Rac1A (aa 177–194). Unimolecular probe Pos$_{FRET}$ was used as a positive control for intracellular FRET between DYFP and mRFPmars: DYFP (aa 1–238), a spacer (Gly-Ser-Gly-Gly-Thr-Gly-Gly-Gly-Gly-Thr), mRFPmars (aa 1–225) and Rac1A (aa 177–194).

Yeast two-hybrid assay
For yeast two-hybrid (Y2H) assay, we used Matchmaker GAL4 Two-Hybrid System 3 (Clontech Laboratories). cDNAs encoding DGAP1 (aa 1–822), DGAP1 (aa 161–822), DPAKa (aa 731–890) and PAK1 (aa 57–200) were inserted into the *Eco*RI and *Bam*HI sites of pGADT7 vector. cDNAs encoding constitutively active forms of 11 *Dictyostelium* Rac GTPases [Rac1A(G12V), Rac1C(G12V), RacB(G12V), RacC(G15V), RacD(G17V), RacE(G20V), RacF1(G12V), RacG(G12V), RacH(M13V), RacI(S14V) and RacJ(D18V)] and constitutively inactive forms of three GTPases [Rac1A(T17N), Rac1C(T17N) and RacC(T20N)] were cloned into the *Eco*RI and *Sal*I sites of pGBKT7 vector. Constructs were introduced into yeast strain AH109 and interactions of expressed proteins estimated according to the growth on selective media at low and high stringency after 3 days at 30°C, as indicated in Fig. 2A. Control interactions were verified according to instructions of the manufacturer.

Protein expression and production of polyclonal anti-PAK1 GBD antibodies
The cDNA of the PAK1 GBD domain (aa 57–200) was fused, in one or two copies, to GST cDNA using pGEX-6P-1 plasmid (GE Healthcare), resulting in two expression plasmids that encode GST–PAK1(GBD) or GST–[PAK1(GBD)]$_2$. A plasmid encoding GST–Rac1A(WT) fusion protein has been described previously (Faix et al., 1998). All GST fusion proteins were expressed and purified from *E. coli* strain Rosetta by standard procedures and subsequently dialysed against PBS containing 140 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 10 mM Na$_2$HPO$_4$ and 1.8 mM KH$_2$PO$_4$, pH 7.3 (Faix et al., 1998). The GBD of PAK1 was expressed as a fusion with maltose-binding protein (MBP) using plasmid pMAL-c2X (New England Biolabs). MBP–PAK1(GBD) was expressed and purified from *E. coli* strain Rosetta by amylose agarose affinity chromatography according to the manufacturer's instructions. GST–PAK1(GBD) was used as an antigen for production of polyclonal anti-PAK1 GBD antibodies using female white New Zealand rabbits (Sigma). Antibodies against PAK1 GBD were affinity purified from serum using MBP–PAK1(GBD) covalently bound to cyanogen bromide-activated-Sepharose 4B (Sigma). The specificity of the purified antibodies was assessed by western blotting of total cellular proteins of AX2 cells expressing DYFP-tagged PAK1 GBD.

GST-fusion protein binding assays
All GST-binding assays were performed essentially as described previously (Faix et al., 1998). Briefly, GST–[PAK1(GBD)]$_2$ and GST–Rac1A(WT) fusion proteins were bound to glutathione–Sepharose beads. Beads with GST–[PAK1(GBD)]$_2$ were separately incubated with lysates of AX2 cells expressing GFP–Rac1A(WT), GFP–Rac1A(V12), GFP–Rac1A(N17) or GFP–RacC(WT). After elution of bound proteins with hot SDS sample buffer, interactions between PAK1 GBD and the assayed Rac proteins were determined by western blotting using polyclonal anti-GFP antibodies (Faix et al., 2001). GST–Rac1A(WT) bound to Sepharose was loaded with either GDP or non-hydrolysible GTP analogue GTPγS and then used to pull down PAK1(GBD)–DYFP from lysate of AX2/PAK1(GBD)–DYFP cells. The bound proteins were eluted with SDS sample buffer and probed in Western blots by polyclonal antibodies against PAK1 GBD.

Fluorescence microscopy and functional assays
Fluorescence microscopy of living *Dictyostelium* cells was carried out on a confocal microscope Leica TCS SP2 AOBS (Leica Microsystems) at 23°C, in

small chambers assembled on glass coverslips (Weber et al., 1995). Cells were washed twice in Sörensen phosphate buffer 30 minutes before scanning. For phagocytosis assays, cells were mixed with TRITC-labelled yeast particles, and for monitoring cytokinesis, *Klebsiella aerogenes* bacteria were added as described (Weber et al., 1999a). For disruption of the actin cytoskeleton, cells were exposed for 20 minutes to 20 μM latrunculin B (Sigma). For chemotaxis assays, cells were washed twice and incubated in phosphate buffer on Petri dishes for at least 6 hours at a near-confluent density, until cell stream formation commenced. Chemotaxis of single cells was induced by a micropipette (Femtotip II, Eppendorf) filled with 100 μM cAMP (Sigma) (Weber et al., 1995). Uniform stimulation of vegetative cells with folic acid was performed by rapidly adding 10 μl of 250 μM folic acid solution to 40 μl of cell suspension in phosphate buffer. Uniform stimulation with cAMP was performed by rapidly adding 10 μl of 150 μM cAMP solution to 40 μl of cell suspension in the phosphate buffer. For cAMP stimulation experiments, we used early-stage aggregation competent cells after 5 hours of starvation. Cortical contrast was defined as the following ratio of average fluorescence intensities (I) in the cortex and the cytoplasm: [I(cortex)–I(cytoplasm)]/I(cytoplasm). The fluorescence intensities were determined using the QuimP10 plug-in module for ImageJ (Dormann et al., 2002). In the module, thickness of the cortex was set to 2 μm, and cortical intensity determined at 200 node points per cell. The excitation wavelengths and detection ranges used for imaging were 488 nm and 500–560 nm for GFP, 514 nm and 525–565 nm for DYFP, and 594 nm and 605–670 nm for mRFPmars.

Fluorescence resonance energy transfer (FRET)

Sensitized emission of the acceptor (mRFPmars) was recorded upon excitation of the donor (DYFP) in live *D. discoideum* cells, using Leica Application Wizard for FRET Sensitized Emission (Leica Microsystems). Signals in three channels (donor, acceptor and FRET) were acquired by sequential scanning with 514 and 594 nm laser lines. Channel settings (excitation/detection) were as follows: donor (514 nm/525–560 nm); acceptor (594 nm/605–670 nm); FRET (514 nm/605–670 nm). For correction of bleed-through and cross-excitation effects, cells that express FRET probes were mixed with cells that express a pure donor species (AX2/DPAKa(GBD)–DYFP), and cells that express a pure acceptor species (AX2/mRFPmars-ABD120). At least one cell of each species was present in each recorded image (Fig. 3C). Image processing and quantification of FRET were performed using the PixFRET plug-in module for ImageJ (Feige et al., 2005). Apparent FRET efficiency was evaluated only for pixels belonging to the cell cortex using the Histogram Selection Tool of the PixFRET plug-in. Average cortical FRET efficiency was calculated for at least ten cells of each investigated strain and further averaged for each strain (mean ± s.d.). Pair-wise statistical comparison between strains was performed using the two-tailed *t*-test.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.089680/-/DC1

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