Quantitative imaging of Rac1 activity in *Dictyostelium* cells with a fluorescently labelled GTPase-binding domain from DPAKa kinase

Maja Marinović, Marko Šoštar, Vedrana Filić, Vlatka Antolović, and Igor Weber*

Ruđer Bošković Institute, Division of Molecular Biology, Bijenička 54, HR-10000 Zagreb, Croatia

*Author for correspondence

e-mail: iweber@irb.hr;

phone: +385-1-4571219;

fax: +385-1-4561177.

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ABSTRACT

Small Rho GTPases are major regulators of the actin cytoskeleton dynamics in eukaryotic cells. Sophisticated tools used to investigate their activity in living cells include probes based on fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation, and photoactivation. However, such methods are of limited use in quickly migrating cells due to a short time available for image acquisition leading to a low signal-to-noise ratio. Attempts to remedy this effect by increasing the intensity of illumination are restricted by photobleaching of probes and the cell's photosensitivity. Here we present design and characterization of a new fluorescent probe that selectively binds to active form of Rac1 GTPases, and demonstrate its superior properties for imaging in highly motile *Dictyostelium* cells. The probe is based on the GTPase-binding domain (GBD) from DPAKa kinase and was selected on the basis of yeast two-hybrid screen, GST pull-down assay and FRET measurements by fluorescence lifetime imaging microscopy. DPAKa(GBD) probe binds specifically to GTP-bound Rac1 at the cell membrane and features a low cytoplasmic background. The main advantage of DPAKa(GBD) in comparison with similar probes is its finely graded intensity distribution along the entire plasma membrane, which enables quantitative measurements of the Rac1 activity in different parts of the membrane. Finally, expression of DPAKa(GBD) induces no adverse effects on cell growth, motility and cytokinesis.

Keywords: Cell migration, Live cell imaging, Fluorescent probe, FLIM, Rho GTPases, *Dictyostelium*

INTRODUCTION

Rho family small GTPases are key regulators of the actin cytoskeleton dynamics (Nobes and Hall 1995; Sit and Manser 2011; Murali and Rajalingam 2013). Since these molecular switches are present in the cell both in active, GTP-loaded and inactive, GDP-loaded forms, in most instances it is not sufficient to simply fuse them with a fluorescent protein in order to monitor their activity. Several sophisticated strategies have therefore been devised to specifically detect GTPases from the Rho family, and the Ras superfamily in general, in their GTP-bound, active state in living cells. Approaches that utilize fluorescence resonance energy transfer (FRET) between a fluorescently labelled GTPase and a domain that binds only to its activated form proved to be particularly fruitful when applied to large and slowly moving cells (Kraynov et al. 2000; Nakamura et al. 2005; Hodgson et al. 2008). An alternative approach based on bimolecular fluorescence complementation (BiFC) probes is not best suited for detection of Rho activity since the interactions of small GTPases with their effectors are mostly transient (He et al. 2015; Sun et al. 2015). Photoactivatable or caged Rho GTPases have also been successfully used, in order to produce bursts of Rho activity and thereby stimulate local actin polymerization leading to induction of lamellipodia on arbitrary positions along the cell edge (Wu et al. 2009).

Notably, however, applications of such advanced probe designs in highly motile cells such as *Dictyostelium* have been scarce (Janetopoulos et al. 2001; Han et al. 2002; Xu et al. 2005; Elzie et al. 2009). The main reason for this is that FRET measurements based on (1) intensity-based approaches such as acceptor photobleaching and sensitized emission of the donor, and (2) fluorescence lifetime imaging microscopy (FLIM), both depend on good photon statistics and require extensive post-processing of images to obtain reliable data (Sun et al. 2013). Time available for collection of photons from the regions of cells that are moving at a high speed is, however, limited. For instance, active Rac1 GTPases are localized mainly in the membrane of the leading pseudopods, which can protrude at speeds over 1 μm/s in *Dictyostelium* (Choi et al. 2013).

Consequently, total pixel-dwell-time during the diffraction-limited imaging of the cell edge is limited to only a few microseconds under conditions typically attained in confocal scanning microscopy (Pietraszewska-Bogiel and Gadella 2011; Zeug et al. 2012). The resulting signal-to-noise ratio of fluorescence in the plasma membrane is not sufficient to conduct reliable FRET analysis in quickly moving cells. Also, FRET probes and other probes based on GTPase effector domains are usually overexpressed and can induce dominant-negative effects based on saturation of the monitored protein (Pertz and Hahn 2004). It is therefore recommendable to check for possible adverse effects of the expression of fluorescent probes on cell migration and other cellular functions that depend on the activity of investigated Rho GTPases.

Highly motile cells of *Dictyostelium discoideum* represent one of the major models in which to investigate regulation of the actin cytoskeleton dynamics by Rho GTPases (Noegel and Schleicher 2000; Rivero et al. 2001; Rivero and Somesh 2002; Rivero and Xiong 2016). Probes are therefore needed that can keep track of rapid re-localization of the Rho GTPase activity, which occurs during fast re-polarization of these cells (Faix and Weber 2013). With the aforementioned requirements in mind, we introduce a probe consisting of the GTPase-binding domain (GBD) from DPAKa kinase fused to a yellow fluorescent protein (DYFP), which binds specifically to active forms of Rac1 GTPases in *Dictyostelium*. DPAKa(GBD)-DYFP localizes almost exclusively to the cell plasma membrane and reflects variability of the Rac1 activity along the cell periphery. Importantly, growth, migration and division of cells that express this probe are not altered in comparison with their wild-type counterparts. We therefore propose to use DPAKa(GBD)-DYFP for quantitative measurements of Rac1 activity in *Dictyostelium* cells.

MATERIALS AND METHODS

Expression vectors and construction of *D. discoideum* cell lines

The cDNA fragment encoding GDB domain of DPAKa (aa 731-890) was amplified by PCR from an auxiliary plasmid and cloned into ClaI and HindIII sites of the pDEXRH-DYFP-MCS plasmid. The plasmid pDEXRH-DYFP-MCS was derived from pDEXRH (Faix et al. 1992) by insertion of a sequence encoding DYFP (Müller-Taubenberger 2006) and a multiple cloning site into EcoRI and HindIII sites. Plasmids for FRET measurements are based on the FRET plasmids for PAK1(GBD) (Filić et al. 2012), with two modifications: (1) the GBD domain from PAK1 (aa 57-200) was substituted with the GBD domain from DPAKa (aa 731-890) and (2) cDNAs of the components of FRET constructs were amplified by PCR and cloned into BglII and SpeI sites of the extrachromosomal expression vector pDM304 (Veltman et al. 2009). DA_{FRET} consists of DYFP, a spacer (Gly-Ser-Gly-Gly-Thr-Gly-Gly-Gly-Gly-Thr), mRFPmars (aa 1-225) and Rac1A C-terminal motif responsible for membrane anchorage (aa 177-194). WT_{FRET} consists of DYFP (aa 1–238), a spacer (Ile-Asp), DPAKa(GBD), a spacer (Ser-Gly-Gly-Thr-Gly-Gly-Gly-Gly-Ser), the wild-type form of Rac1A (aa 1–176), a spacer (Gly-Gly-Arg-Val-Asp), mRFPmars, a spacer (Gly-Arg-Ser-Arg) and C-terminal Rac1A motif (aa 177–194). In $V12_{FRET}$ and $RacC_{FRET}$ constructs, Rac1A (aa 1-176) is substituted with the constitutively activated mutant form of Rac1A (aa 1-176, G12V) or with the wild-type form of RacC (aa 1-179), respectively. All FRET constructs are listed in Table 1. Cell lines used in this work express the following constructs: PAK1(GBD)-DYFP, DPAKa(GBD)-DYFP (Filić et al. 2012), DYFP-DPAKa(GBD), FRET constructs (Table 1) and pDM304.

Cells of wild-type AX2 strain and all cell lines derived from this parental strain were cultivated in polystyrene culture dishes at 22 °C in axenic HL5 medium (Formedium, Ref No HLF), with addition of maltose, 50 μg/ml ampicillin and 40 μg/ml streptomycin, except for measuring growth in suspension, when ampicillin and streptomycin were omitted from the medium. Transfection of

AX2 cells was done by electroporation and 10 μg/ml G418 (Gibco) was added to the medium for the selection of transformants. When vectors based on pDEXRH were used, clonal selection was done as previously described (Kimmel and Faix 2006). For the assessment of growth in suspension and on bacterial lawns, cells were grown in liquid nutrient medium with shaking at 150 rpm or on SM agar with K. aerogenes, respectively. For growth in suspension, 20 ml of fresh medium was inoculated with 2×10^5 cells/ml from the exponential growth phase in a 100 ml 100-ml flask and cell concentration determined every 12 h. 12 h. For growth on bacteria, 50 cells in the exponential growth phase were plated with bacteria and diameters of developed plaques, *d*, measured after 5 days. Duration of cytokinesis, T_C, was measured using phase-contrast microscopy from the initial cell elongation stage in the late anaphase to the separation of daughter cells (Weber et al. 1999).

GST pull-down assay

Rac1A was expressed as a GST fusion protein (Faix et al. 1998) and purified from *E.coli* strain Rosetta 2 following a published procedure (Filić et al. 2012). Purified GST-Rac1A was bound to glutathione Sepharose beads (GE Healthcare) and loaded with either GDP or GTPγS (Faix et al. 1998). GST-bound beads were used as a negative control. Loaded GTPase was incubated for 2 h at 4°C with the lysates of *D. discoideum* cells expressing DPAKa(GBD)-DYFP. The cells were lysed in a lysis buffer containing 30 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1 mM EGTA, 2 mM DTT, 5 mM $MgCl_2$, 0.5 % *n*-octylpolyoxyethylene, 1 mM PMSF, 5 mM benzamidine and a protease inhibitor cocktail. After incubation, beads were extensively washed in the lysis buffer and bound proteins were eluted with the SDS sample buffer. The bound proteins were detected by Western blot using monoclonal anti-GFP antibody 264-449-2 (Schirenbeck et al. 2005).

Fluorescence microscopy

Imaging of random motility, cytokinesis and endocytosis of *D. discoideum* cells was preformed essentially as previously described (Filić et al. 2012). Chemotaxis experiments were performed with aggregation-competent cells in a Dunn chamber assembled as previously described (Annesley

et al. 2011). For counting nuclei, cells were fixed with 2 % paraformaldehyde and stained with 1 μg/ml DAPI for half an hour. Fluorescence microscopy was performed on Leica TCS SP8 X microscope equipped with a supercontinuum excitation laser (Leica Microsystems). The excitation wavelengths and detection ranges used for imaging were: (1) 511 nm and 520-565 nm for DYFP, and (2) 543 nm and 570-700 nm for TRITC-labelled yeast particles during phagocytosis and 430–550 nm for DAPI. The hybrid HyD detectors were operated in the gated mode in order to supress parasite reflection from the coverslip surface, with the delay time between excitation and detection set to 0.3 ns.

Random migration assay

For cell tracking experiments, vegetative cells in the nutrient medium or aggregation-competent cells starved for 6 hours in Sørensen phosphate buffer were adjusted to an appropriate density, resulting in approximately 30 cells per field of view (1.3×0.9 mm), placed into a glass-bottom 35-mm-diameter dish (MatTek), and allowed 60 min to settle before starting with measurements. Cells were recorded by dark-field microscopy using a 5× objective (Antolović et al. 2014), and images were acquired in 30 s intervals. Image processing was performed with the ImageJ plug-in module MOSAIC ParticleTracker (Sbalzarini and Koumoutsakos 2005). Individual cell speed has been defined as displacement of its geometrical centre divided by the time interval between successive images. Trajectory analysis was performed using *Mathematica* programming package.

Fluorescence lifetime imaging microscopy (FLIM)

FLIM data acquisition was performed at TCS SP8 X confocal microscope (Leica Microsystems), paired with the time–correlated single photon counting (TCSPC) module (PicoQuant). For photon detection, spectral (SP) FLIM photomultipliers were used. Data analysis was done with SymPhoTime64 software (PicoQuant). Fluorescence from single cells was recorded for approximately 1 min, with the peak count rate adjusted between 200 and 500 kcounts/s. Laser pulse frequency, i.e. the repetition rate, was set to 40 MHz. To determine lifetime of the donor

fluorescent species DYFP, we used cells expressing DPAKa(GBD)-DYFP. Lifetime of the donor fluorescence, τ_0, was obtained by fitting a mono-exponential model of fluorescence decay ($N(t) = Ae^{-t/\tau_0}$) to experimental TCSPC curve. The obtained value, τ_0, was used as a fixed parameter in further fitting procedures. For data obtained with cells expressing FRET probes (see Table 1), a double-exponential model of fluorescence decay ($N(t) = A_1 e^{-t/\tau_{FRET}} + A_2 e^{-t/\tau_0}$) was used, with A_1, A_2 and τ_{FRET} as free parameters. FRET efficiency for each probe was calculated from the ratio of the donor lifetime in a FRET sample over the unquenched donor lifetime as $E = 1 - \tau_{FRET}/\tau_0$. Amplitudes A_1 and A_2 were used to determine the fraction of molecules that exhibit FRET. Ratio $F = A_2/(A_1 + A_2)$ represents the fraction of molecules that undergo FRET, whereas $A_1/(A_1 + A_2)$ represents the fraction that does not. Parameters A_1, A_2 and E were calculated for 10 cells from each strain and subsequently averaged (mean ± s.d.). Statistical comparison of the data for E and F between the four FRET probes was performed by one-way ANOVA, followed by the Tukey post-hoc test (Table 2).

RESULTS

Selection of DPAKa(GBD) for construction of a probe for active Rac1 GTPases

In our previous screen for effector domains that interact specifically with the constitutively active form of Rac1A, we had employed yeast two-hybrid (Y2H) assay using 11 different *Dictyostelium* Rac GTPases as preys (Filić et al. 2012). In that screen we had found that the GTPase-binding domain from *Dictyostelium* kinase DPAKa, DPAKa(GBD), interacts specifically with the constitutively active mutants of highly homologous GTPases Rac1A and Rac1C. In order to demonstrate the specificity of the interaction of DPAKa(GBD) with the GTP-loaded form of Rac1A *in vitro*, in the present study we used a pull-down assay. Recombinant Rac1A was immobilized to the matrix via a GST tag and loaded either with GDP or a non-hydrolysable form of GTP (GTPγS). DPAKa(GBD) was pulled down from the cell lysates only when the constitutively active form of Rac1A was bound to the matrix, thereby corroborating results of the Y2H assay (Fig. 1a).

The FRET effect is an established diagnostic tool for validation of binary interactions between fluorescently labelled molecules in living cells (Kaminski et al. 2014). In order to verify the interaction between DPAKa(GBD) and Rac1A in the context of living *Dictyostelium* cells, we used a panel of unimolecular FRET probes where DYFP was used as donor and mRFPmars as acceptor. The FRET probes expressed in AX2 cells are listed in Table 1 and described in detail in Materials and methods. DA_{FRET} consists of DYFP and mRFPmars connected via a flexible linker and serves as a control for the occurrence of FRET between the two fluorescent proteins. WT_{FRET} and $V12_{FRET}$ probes encompass DPAKa(GBD) and wild-type or constitutively activated form of Rac1A, respectively, nested between the donor and the acceptor. $RacC_{FRET}$ contains RacC instead of Rac1A, and is used to measure the residual FRET for this donor-acceptor pair separated by non-interacting polypeptides, since RacC does not interact with DPAKa(GBD) in Y2H assay. All

probes contain a C-terminal motif responsible for the insertion of the probes into the plasma membrane, thus mimicking the anchorage mechanism of endogenous Rac1 proteins.

We used the FLIM method to measure changes in fluorescence lifetime of the donor (DYFP) induced by the presence of the acceptor (mRFPmars) in FRET probes. Both the fraction of interacting fluorescent proteins and the distance between them, influence the measured lifetime distribution function, which is standardly approximated by a double-exponential model (Becker 2012). In this model, a slow lifetime component corresponds to the non-interacting (unquenched) and a fast component to the interacting (quenched) donor molecules (Wlodarczyk et al. 2008). We, therefore, analysed the data obtained by FLIM using a model with two independent parameters: FRET efficiency, *E*, and fraction of the probe population that exhibits FRET, *F* (for details, see Materials and methods). Results obtained by fitting the data to this model show that the three probes which have similar structures, WT_{FRET}, $V12_{FRET}$ and $RacC_{FRET}$, have indistinguishable FRET efficiencies, suggesting that they can be either in a FRET-positive or a FRET-negative conformation (Fig. 1b, upper panel; Table 2). DA_{FRET}, which lacks the bulky GTPase molecule and the DPAKa(GBD) domain, has a significantly higher FRET efficiency due to a closer apposition of the donor and acceptor fluorescent proteins in the FRET-positive conformation (Table 2). The fraction of DA_{FRET} probe molecules that on average contribute to the FRET signal is higher than 40%, clearly the highest among the four probes (Fig. 1b, lower panel, Table 2). On the other end of the spectrum, a baseline fraction of $F = 9.7 \pm 2.2$ % has been determined for $RacC_{FRET}$ in which RacC substitutes Rac1A. The fraction of FRET-positive molecules was significantly increased for $V12_{FRET}$ ($F = 18.5 \pm 2.2$ %), whereas an intermediate value was determined for WT_{FRET} ($F = 14.6 \pm 1.6$ %) (Fig. 1b, lower panel; Table 2). These results additionally corroborate results obtained from genetic and biochemical assays, indicating a specific interaction between DPAKa(GBD) and the active form of Rac1A in living cells.

DPAKa(GBD)-DYFP exhibits superior properties for live cell imaging

After demonstrating the specific interaction of DPAKa(GBD) with activated Rac1A *in vitro* and *in cellulo*, we tested DPAKa(GBD) fused to DYFP in both orientations for imaging by confocal microscopy in cells of the wild-type axenic *Dictyostelium* strain AX2. We designate these fluorescently labelled probes as DPAKa(GBD)-DYFP and DYFP-DPAKa(GBD). When imaged with a confocal microscope, both probes showed distinct localization to the cell membrane, with a remarkably lower cytoplasmic background compared to the PAK1(GBD)-DYFP probe (Fig. 2a). Such improved contrast between the membrane and the cytoplasm revealed the presence of a weak signal in the parts of the membrane not discernible with the previously used probes for active Rac1 GTPases (cf. tripolar cells in Fig. 2a, third column). Cortical localization of DPAKa(GBD)-DYFP and DYFP-DPAKa(GBD) was also clearly visible in round cells, whereas no enrichment of PAK1(GBD)-DYFP in the cortex of round cells could be detected (cf. Fig. 2a, second column). The improved contrast, visible with the use of new probes, was confirmed during the recording of extended time series of randomly moving cells, where DPAKa(GBD)-DYFP showed a particularly strong signal throughout the cortical layer (Fig. 2b). Importantly, it was possible to continuously record cells expressing DPAKa(GBD)-DYFP over ten minutes, with a frame rate of 0.3 Hz and the image pixel size of 230×230 nm^2, practically without any loss of fluorescence and no evidence of phototoxicity (Fig. 2b, bottom row).

In order to quantitatively compare contrast of the three fluorescent probes for active Rac1 between the cell cortex and the cytoplasm, we measured the ratio of fluorescence intensity in a thin layer at the cell border and the average intensity in the cytoplasm. For image analysis we used QuimP (Quantitative Image analysis of Membrane associated Proteins), a software package which allows accumulating data of cortical fluorescence from many cells to perform detailed statistical analysis (Dormann et al. 2002). The results obtained by pooling measurements from 20 randomly chosen cells clearly shows that the DPAKa(GBD)-DYFP probe displays a contrast superior to

other two probes (Fig. 3). It is important to note that the average ratio of approximately 4 between the cortical signal and the cytoplasmic background includes also the weakly labelled membrane regions, whereas the local contrast can be as high as 15 (cf. also Fig. 4a).

To demonstrate potential of the DPAKa(GBD)-DYFP probe for mapping the spatio-temporal activity of Rac1, we used QuimP to analyse the fluorescence intensity along the cell outline over time. Examples of such measurements are shown in Fig. 4, where the intensity profiles along the cell contour are plotted for an irregularly shaped bipolar cell (Fig. 4a) and a rounded unipolar cell (Fig. 4b). The cell shown in Fig. 4a has been recorded over ten minutes at the rate of 1 frame per 3 s, and the temporal evolution of the intensity profile is displayed in the form of a kymograph for the first 6 min in Fig. 4c. From these results, it is obvious that DPAKa(GBD)-DYFP can be imaged with a good temporal resolution resulting in a wide dynamic range, which makes it suitable for precise measurements of the Rac1 activity in living cells.

Intracellular distribution of DPAKa(GBD)-DYFP in chemotaxis, endocytosis and cytokinesis

When deprived of nutrients, *Dictyostelium* cells enter a developmental programme that sets off with cAMP-mediated aggregation (Chisholm and Firtel 2004). After approximately 6 h of starvation, the cells become aggregation competent and move in a directed fashion when exposed to gradients of cAMP. We monitored localization of DPAKa(GBD)-DYFP during this process of chemotaxis and found that, analogous to its localization during random migration, the probe is enriched in the leading edge of cells moving up-gradient (Fig. 5a). In comparison with the PAK1(GBD)-DYFP probe, whose expression markedly declined during the aggregation phase, DPAKa(GBD)-DYFP remained expressed at a sufficient level to support imaging in most aggregation-competent cells.

Localization of DPAKa(GBD)-DYFP was also monitored during the actin-dependent processes of phagocytosis and macropinocytosis, which are the major mechanisms of nutrient uptake in *Dictyostelium*. Both phagocytosis and macropinocytosis are mediated by the formation and closure of cup-shaped protrusions that are driven by actin polymerization (May and Machesky 2001; Kerr and Teasdale 2009). In both types of endocytosis, DPAKa(GBD)-DYFP was strongly enriched in endocytotic cups during their formation and dispersed away soon after their closure (Fig. 5b, c). This dynamics resembles the transient localization of F-actin probes to endocytotic cups (Lemieux et al. 2014) and thus shows a close correlation between the Rac1 activity and the actin polymerization in endocytosis.

The previously developed probe for active Rac1 GTPases in *Dictyostelium*, PAK1(GBD)-DYFP, was unexpectedly absent from the cell membrane during cytokinesis (Filić et al. 2012). By using DPAKa(GBD)-DYFP, we now show that active Rac1 GTPases are present in the cortex of dividing cells and are notably enriched at the distal poles of incipient daughter cells (Fig. 5d). This result is consistent with the previous findings that proteins and protein complexes involved in the regulation of actin polymerization, such as DdVASP, Arp2/3 and SCAR/WAVE complexes, are enriched both at the leading edges of moving cells and at the poles of dividing cells, thus indicating functional equivalence of these two locations of intense actin polymerization (Insall et al. 2001; Han et al. 2002; King et al. 2010; Veltman et al. 2012). The failure of the previously used Rac1 probe PAK1(GBD)-DYFP to localize to the membrane of dividing cells can be explained by a lower affinity of PAK1(GBD)-DYFP for the active Rac1 GTPases compared to DPAKa(GBD)-DYFP. This finding also suggests a lower accessibility/affinity of the PAK family GBDs for active Rac1 in mitosis compared to the interphase.

Cell growth, migration and cytokinesis are not altered in cells expressing DPAKa(GBD)-DYFP

Overexpression of probes for small GTPases based on GTPase-binding domains can interfere with the innate interaction of a GTPase with its effectors. Although it is difficult to entirely exclude such effects, in our case as well, we proceeded to check whether the expression of DPAKa(GBD)-DYFP influences processes which are highly dependent on actin polymerization, the cell migration, cell growth and cytokinesis. We utilized a recently developed migration assay to analyse random migration of cells expressing DPAKa(GBD)-DYFP and compared their migration to wild-type AX2 cells (Antolović et al. 2014). Based on the comparison of more than 50 cells of each cell line (Fig. 6a, b), we conclude that no changes in the average speed of migration have been induced by the expression of DPAKa(GBD)-DYFP, neither in vegetative nor in aggregation-competent cells. We also compared the duration of cytokinesis and the proportion of multinucleated cells between the two strains and found no significant differences (Fig. 6c, d). Finally, we show that cell growth on bacterial lawns and in suspension was not slowed down by the expression of DPAKa(GBD)-DYFP (Fig. 6e, f).

DISCUSSION

Dictyostelium cells feature a distinctive repertoire of small GTPases involved in regulation of the actin cytoskeleton dynamics, where the roles of Rho and Cdc42 proteins present in vertebrates are apparently taken over by Rac and Ras GTPases (Wilkins and Insall 2001; Vlahou and Rivero 2006). This unique situation hints at robustness of signalling modules that regulate cell motility and provides a fruitful platform to investigate the universal principles involved. Phenotypic characterization of mutant *Dictyostelium* cells is increasingly complemented in recent years by monitoring intracellular localization and activity of small GTPases (Rivero and Xiong 2016).

Several GTPases from the Rac and Ras families were fused to green fluorescent protein (GFP) in order to monitor their localization in living *Dictyostelium* cells. Whereas some GTPases were expressed in their wild-type form only, e.g. RacC (Larochelle et al. 1997; Filić et al. 2012) and RacF1 (Rivero et al. 1999), others were also expressed in their constitutively active and inactive forms, e.g. RacE (Larochelle et al. 1997), RacG (Somesh et al. 2006b), RacH (Somesh et al. 2006a), RasB and RasG (Müller-Taubenberger et al. 2014). This approach has the disadvantage that overexpression of GTPases locked in a certain nucleotide state can have adverse, dominant-negative effects on the cell. Taken together, expression of fluorescently labelled Rac and Ras proteins could provide only limited information about sites of their activity in living cells.

In order to obtain spatial and temporal information about the activity of small GTPases in *Dictyostelium*, fluorescent probes based on GTP-binding domains (GBDs) from effector molecules were introduced. For example, GBDs from Dictyostelium WASP and PakB fused with fluorescent proteins have been used to monitor the dynamics of RacC and Rac1, respectively (Han et al. 2006; Chung et al. 2013; Veltman et al. 2012), but the specificity of these probes is possibly broader (Rivero and Xiong 2016). Within the Ras family, Ras-binding domain (RBD) of mammalian Raf1 or RalGDS fused to GFP was used as biosensors for activated RasG (Kae et al.

2004). However, RalGDS(RBD) also binds activated Rap1 (Kang et al. 2002). Further, it has been shown that the RasG biosensor Raf1(RBD) does not interact with RasC, but interaction with other Ras GTPases has not been tested, leaving open the possibility that Raf1(RBD) binds activated forms of other Ras proteins (Kae et al. 2004). Taken together, these examples stress the need to test biosensors designed for a specific GTPase for cross-reactivity with other GTPases.

Dictyostelium cells express three highly similar Rac1 isoforms, Rac1A, B and C, which share an identical effector-binding domain, and interact with a number of proteins associated with the actin cytoskeleton (Faix and Weber 2013; Filić et al. 2014). Two fluorescently labelled probes have been previously used to monitor Rac1 activity in *Dictyostelium* cells, which are based on GBD domains from PakB (Veltman et al. 2012), and the PAK1 kinase from rat (Filić et al. 2012). Although valuable data have been obtained by the use of these probes, their utility has been restricted by a high cytoplasmic background, which can be explained either by their insufficient affinity for Rac1 GTPases, or as an overexpression artefact. Moreover, their weak cortical contrast and a narrow dynamic range of the fluorescent signal in the cortex limited the information provided by these probes to an essentially binary response. It could only be estimated whether the probe wss enriched in a certain region of the cell contour or not, but no fine grading of the fluorescent intensity and, thereby, the Rac1 activity in the membrane could be assessed.

The new probe DPAKa(GBD)-DYFP introduced in the present paper represents a significant improvement in this respect and enables precise quantification of the Rac1 activity in the membrane over a wide dynamic range (Fig. 4). DPAKa(GBD) has been tested by a Y2H screen for interaction with a panel of 11 Rac GTPases from *Dictyostelium*, and it was shown that it interacts only with the activated form of Rac1 GTPases, as corroborated further by GST pull-down and FRET/FLIM assays. DPAKa(GBD)-DYFP has superior properties regarding its dominant membrane localization and therefore facilitates the use of automatic image processing algorithms

that rely on a precise discrimination between cytoplasmic and membrane domains (Dormann et al. 2002; Mosaliganti et al. 2012).

DPAKa belongs to the PAKA subfamily of STE20 kinases in *Dictyostelium* (Goldberg et al. 2006), where it participates in the control of myosin II filament assembly, and is regulated by PI3 kinase and protein kinase B (Chung and Firtel, 1999; Chung et al. 2001). Localization studies have suggested that the N-terminal part of DPAKa, harbouring a coiled-coil domain and proline-rich motifs, is chiefly responsible for its recruitment to the cell cortex and the posterior end of migrating cells (Chung and Firtel 1999; Müller-Taubenberger et al. 2002). The C-terminal part, which harbours incomplete GBD, a catalytic domain and a G_{β}–binding motif, did not show localization to the cortex when expressed as a GFP fusion in living cells (Müller-Taubenberger et al. 2002). Moreover, it appeared that during chemotaxis the C-terminal part restricts localization of the full-length protein to the rear end of migrating cells. Our present results, showing an enrichment of DPAKa(GBD)-DYFP in the cell front, suggest that DPAKa(GBD) does not play a significant role in localization of the full-length protein.

A number of recent studies utilize microscopy and image analysis to correlate the local activity of signalling molecules, Rho GTPases in particular, with the shape changes in living cells (Tkachenko et al. 2011; Welch et al. 2011; Depry et al. 2013). For example, it has been shown that the activities of GTPases Rac1, RhoA and Cdc42 are coordinated to each other and to the local protrusions of the cell edge in mouse embryonic fibroblasts with well-defined phase relationships (Machacek et al. 2009). This approach complements biochemical studies of protein-protein interactions in constructing comprehensive molecular models of regulatory pathways that govern dynamics of the actin cytoskeleton (Ryan et al. 2012; Lockley et al. 2015; Bement et al. 2015). A vital prerequisite for obtaining high-quality imaging data, which are necessary to implement this approach, are fluorescent probes that: (1) report the localization and activity of signalling proteins with high specificity, (2) have an optimal affinity for their target proteins and (3) do not induce

adverse effects on the cell behaviour. DPAKa(GBD)-DYFP fulfils these criteria with respect to Rac1 GTPases in *Dictyostelium* and is therefore the prime candidate for conducting detailed studies of spatio-temporal correlations between Rac1 signalling and actin polymerization in this model organism.

Experiments in which motile cells expressing chimeric fluorescent proteins must be followed over periods of ten minutes or more with a high recording frequency Photobleaching and phototoxicity are hampering (Hoebe et al. 2008; Waters 2013). During imaging at the scanning speed of 1 frame per second, even the photobleaching rates on the order of 0.1 % per scan will render the signal indistinctive from noise after ca. 10 min. In addition, *Dictyostelium* cells are notoriously sensitive to low-wavelength light and tend to cringe and round up under conditions typically used for imaging with point-scanning and spinning-disc confocal microscopes. We used DYFP instead of GFP for the construction of our probe because its excitation spectrum is shifted 26 nm to higher wavelengths. Using the low-intensity illumination, we were able to successfully image motile *Dictyostelium* cells expressing DPAKa(GBD)-DYFP over 10 min without any visible sign of phototoxicity and a negligible photobleaching-induced loss of signal (Fig. 2b, bottom row; Fig. 4c).

Besides an optimal design of the fluorescent probe, overall efficiency of the confocal imaging system strongly contributed to the results presented in this report, in particular the use of hybrid detectors that have quantum efficiency over 40 % in the region of DYFP emission spectrum (Becker et al. 2011; Foltánková et al. 2013). Using the combination of improved fluorescent probes and innovative imaging systems, including the recently introduced techniques such as Bessel beam plane illumination microscopy and lattice light-sheet microscopy (Gao et al. 2014; Chen et al. 2014), three-dimensional maps of the small GTPases' dynamics in highly motile cells should soon be obtained.

FIGURE CAPTIONS

Fig. 1 GBD domain of the DPAKa kinase interacts specifically with the active form of *Dictyostelium* Rac1A. (**a**) Interaction of Rac1A and DPAKa(GBD) was tested *in vitro* using a GST pull-down assay. GST-Rac1A was immobilized on glutathione Sepharose beads and loaded either with GDP or GTPγS. After incubation with the lysate of cells expressing DPAKa(GBD)-DYFP, DPAKa(GBD)-DYFP was analysed by Western blotting using anti-GFP antibody. A band of 46 kDa was detected only when GTPγS-loaded GST-Rac1A was used. The first lane corresponds to the whole lysate of DPAKa(GBD)-DYFP expressing cells. (**b**) FLIM experiments corroborate the interaction of DPAKa(GBD) with active Rac1A. Calculated FRET efficiency *E* between the donor DYFP and the acceptor mRFPmars (*upper panel*), and the fractional population of the molecules that undergo FRET *F* (*lower panel*), for cell lines expressing DYFP-mRFPmars (Donor-Acceptor, DA_{FRET}), DYFP-DPAKa(GBD)-Rac1A(V12)-mRFPmars ($V12_{FRET}$), DYFP-DPAKa(GBD)-Rac1A(WT)-mRFPmars (WT_{FRET}) and DYFP- DPAKa(GBD)-RacC-mRFPmars ($RacC_{FRET}$). Average values for parameters *E* and *F* were calculated from the data for 10 cells of each investigated strain (mean ± SD). Results of the statistical comparison between constructs are displayed in Table 2.

Fig. 2 Comparison of localization between the three probes for active Rac1: PAK1(GBD)-DYFP, DYFP-DPAKa(GBD) and DPAKa(GBD)-DYFP. (**a**) Examples of different types of polarization in vegetative cells. From left to right: flattened, rounded, tripolar, unipolar and bipolar cells. Two nuclei are visible as faintly labelled by PAK1(GBD)-DYFP in the flattened, leftmost cell. (**b**) Selected frames from time sequences of randomly moving vegetative cells. The cell expressing DPAKa(GBD)-DYFP shown in the *bottom row* was recorded for 650 s with no signs of

phototoxicity and only slight photobleaching. Time after the start of a series is denoted in seconds in (**b**). Scale bar 10µm.

Fig. 3 Quantification of the average normalized cortical intensity (I_N) in cells expressing three probes for active Rac1. The normalized cortical intensity is defined as the signal intensity of a single point at the membrane divided by the average signal intensity in the cytoplasm. Shown are the median values of I_N obtained from a set of 1000 points (for each probe we randomly chose 20 cells sampled at 50 points along the cell membrane), and bars show interquartile ranges. I_N (PAK1(GBD)-DYFP) = 1.4 [1.1, 1.7]; I_N (DYFP-DPAKa(GBD)) = 2.9 [1.6, 4.2]; I_N (DPAKa(GBD)- DYFP) = 4.1 [2.6, 6.3] (median [Q1, Q2]). I_N is significantly stronger for DPAKa(GBD)-DYFP than for other two probes ($p < 10^{-5}$, Mann-Whitney test).

Fig. 4 Quantitative analysis of the DPAKa(GBD)-DYFP probe signal in the cortex of motile cells. (**a**, **b**) *Left panels* cell contours extracted with the ImageJ plugin Quimp are represented with resulting nodes. A subset of nodes is shown for each cell, and the node labeled with a cross represents the starting point of segmentation (x = 0). *Right panels* corresponding plots of the normalized cortical intensity as a function of position along the contour in the clockwise direction. (**a**) Shows a cell in a bipolar state, and (**b**) shows a unipolar, rounded cell. (**c**) The spatio-temporal dynamics of the normalized cortical intensity in the cell shown in Fig. 4a is displayed as a kymograph. I_N along the cell contour (x-axis) has been sampled every 3 s and plotted against time (y-axis). During cell movement, local fluorescently labelled domains are transiently formed, as indicated by the colour look-up table for I_N. The cell contour length has been normalized to unity, and duration of the time series displayed is approximately 6 min.

Fig. 5 Localization of DPAKa(GBD)-DYFP probe during: (**a**) directed movement of aggregation-competent cells in chemotaxis; (**b**) phagocytosis; (**c**) macropinocytosis; (**d**) cytokinesis. In (**a**), white dot represents a fixed point on the substratum, and *arrow* designates the direction of cAMP gradient. Time after the start of each series is denoted in seconds. Scale bar 10 μm.

Fig. 6 Comparison of random migration, cell division and growth between wild-type cells and cells expressing the DPAKa(GBD)-DYFP probe. (**a**) Random migration of vegetative cells. *v*(AX2) = 3.85 [2.91, 4.39], n =141; *v* (DPAKa(GBD)- DYFP) = 3.63 [2.77, 4.70], n = 95. (**b**) Random migration of aggregation-competent cells. *v*(AX2) = 6.06 [4.52, 7.99], n = 82; *v*(DPAKa(GBD)- DYFP) = 6.55 [5.34, 8.31], n = 57. The median values of the cell migration speed (*v*) are displayed, with bars showing interquartile ranges. The speed of cell migration is not significantly different between the two measured strains in both developmental stages ($p > 0.2$, Mann-Whitney test). (**c**) Duration of cytokinesis (T_C). T_C(AX2) = (3.61 ± 0.21) min, n = 18; T_C(DPAKa(GBD)–DYFP) = (3.55 ± 0.34) min, n = 22 (mean ± S.D.). The duration of cytokinesis is not significantly different between the two measured cell lines ($p > 0.5$, two-tailed *t* test). (**d**) Distribution of the number of nuclei per cell. More than 550 cells were evaluated for each cell line. (**e**) Plaque diameter after 5 days of growth on bacterial lawns (*d*) . d(AX2) = (4.32 ± 1.11) mm, n = 68; *d*(DPAKa(GBD)–DYFP) = (4.48 ± 0.87) mm, n = 91 (mean ± SD). The plaque diameter is not significantly different between the two measured cell lines ($p > 0.2$, two-tailed t-test). (f) ($p > 0.2$, two-tailed *t* test). (**f**) Cell growth in suspension was measured in AX2 cells, cells expressing the DPAKa(GBD)-DYFP probe, and cells transfected with the empty vector pDM304.

Table 1 Composition and description of FRET constructs (for details, see "Materials and methods")

Composition of FRET constructs	Description of FRET constructs	Abbreviation
DYFP–mRFPmars	Donor–acceptor control	DA_{FRET}
DYFP–DPAKa(GBD)–Rac1A(WT)–mRFPmars	FRET probe for wild-type Rac1A	WT_{FRET}
DYFP–DPAKa(GBD)–Rac1A(V12)–mRFPmars	FRET probe for constitutively active Rac1A	$V12_{FRET}$
DYFP–DPAKa(GBD)–RacC(WT)–mRFPmars	FRET probe for wild-type RacC	$RacC_{FRET}$

Table 2 Tukey's post-hoc post hoc test for multiple comparison between FRET probes after one-way ANOVA for FRET efficiency, E ($F_{3,24} = 24.7$, $p < 0.0001$, $n = 10$), and for the fraction of the probe population that exhibits FRET, F ($F_{3,24} = 313.6$, $p < 0.0001$, $n = 10$). The values of p(E) are shown in the lower-left part of the table, and the values of p(F) in the upper-right part.

p(*E*)	p(*F*)			
	DA_{FRET}	$V12_{FRET}$	WT_{FRET}	$RacC_{FRET}$
DA_{FRET}		<0.0001	<0.0001	<0.0001
$V12_{FRET}$	<0.0001		0.0178	<0.0001
WT_{FRET}	<0.0001	0.6887		0.0021
$RacC_{FRET}$	<0.0001	0.4254	0.9722	

Conflict of Interest: The authors declare that they have no conflict of interest.

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