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Cyclic AMP signalling in *Dictyostelium*: G-proteins activate separate Ras pathways using specific RasGEFs

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In general, mammalian Ras guanine nucleotide exchange factors (RasGEFs) show little substrate specificity, although they are often thought to regulate specific pathways. Here, we provide *in vitro* and *in vivo* evidence that two RasGEFs can each act on specific Ras proteins. During *Dictyostelium* development, RasC and RasG are activated in response to cyclic AMP, with each regulating different downstream functions: RasG regulates chemotaxis and RasC is responsible for adenylyl cyclase activation. RasC activation was abolished in a *gefA*$^-$ mutant, whereas RasG activation was normal in this strain, indicating that RasGEFA activates RasC but not RasG. Conversely, RasC activation was normal in a *gefR*$^-$ mutant, whereas RasG activation was greatly reduced, indicating that RasGEFR activates RasG. These results were confirmed by the finding that RasGEFA and RasGEFR specifically released GDP from RasC and RasG, respectively, *in vitro*. This RasGEF target specificity provides a mechanism for one upstream signal to regulate two downstream processes using independent pathways.

Keywords: Ras; RasGEF; signal transduction; GDP release; *Dictyostelium*

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INTRODUCTION

Ras proteins are small, monomeric GTPases that act as crucial regulators of a number of cellular signalling pathways, including those involved in proliferation, differentiation and apoptosis. Ras is inactive when bound to GDP but activated when bound to GTP; the exchange of GDP for GTP is catalysed by a group of proteins called Ras guanine nucleotide exchange factors (RasGEFs). Therefore, RasGEFs are crucial regulatory molecules for all Ras-linked signal-transduction pathways. Inactivation of Ras is catalysed by Ras GTPase-activating proteins (RasGAPs), which stimulate the hydrolysis of GTP by Ras.

Ras signalling in mammalian cells is extremely complex, involving 36 Ras subfamily proteins (Wennerberg *et al*, 2005) and an even greater number of RasGEFs (Quilliam *et al*, 2002). In addition, mammalian RasGEFs show little specificity for a particular Ras *in vitro*, making it difficult to define signalling networks. By contrast, the social amoeba *Dictyostelium discoideum* provides a relatively simple system in which to investigate Ras function in that there are fewer Ras proteins and each Ras protein seems to regulate a unique subset of functions (Lim *et al*, 2002). There are 25 sequences in the *Dictyostelium* genome with homology to the RasGEF catalytic domain, but little is known about which of these regulates the individual Ras proteins. Only three RasGEFs have been linked to possible Ras substrates, and in each case the evidence is merely based on phenotypic similarity of *ras* and *gef* gene knockout mutants (Lim *et al*, 2001; King & Insall, 2003; Wilkins *et al*, 2005). In this report, we provide *in vivo* and *in vitro* evidence for highly specific RasGEF–Ras interactions.

RESULTS

RasGEFA mediates cAMP induced RasC activation

In a previous report, we described a Ras-binding domain (RBD) binding assay that allowed the measurement of the level of activated Ras in *Dictyostelium* cell lysates. This assay uses the RBD of the *Schizosaccharomyces pombe* Ras effector protein Byr2, which preferentially binds to activated Ras, to separate Ras-GTP from the pool of total cellular Ras. We showed that cyclic

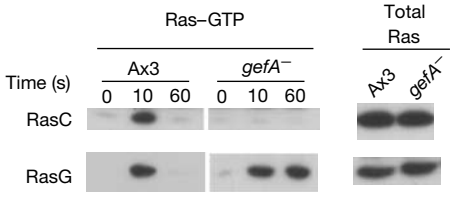

Fig 1 | *gefA*$^{-}$ mutants are deficient in RasC activation. Six-hour-pulsed A×3 and *gefA*$^{-}$ cells were stimulated by the addition of 200 nM cyclic AMP, samples were collected at the indicated time points after stimulation and the lysates from the cells were subjected to the RBD-binding assay. Samples were fractionated by SDS–PAGE, transferred onto polyvinylidine fluoride (PVDF) membrane and probed with RasC-specific, RasG-specific or a pan-specific Ras antibody, as indicated. This figure is representative of three independent experiments. *gef*, guanine-nucleotide exchange factor; RBD, Ras-binding domain; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

AMP (cAMP) induced activation of two Ras proteins: RasC, RasG (Kae *et al*, 2004). However, it was necessary to overexpress RasC to assay its activation. We have now increased the sensitivity of the assay by modifying the lysis buffer and by using an enhanced chemiluminescent detection system. With this improved assay, it has been possible to measure RasC activation in wild-type Ax3 cells. Both RasC and RasG showed the same rapid and transient activation in response to cAMP, and maximum activation was reached 10 s after stimulation (data not shown), which is consistent with the previous results.

Insall *et al* (1996) identified RasGEFA, formerly known as AleA, as a component involved in G-protein-mediated cAMP signalling events. Strains with a disrupted *rasC* gene had a phenotype similar to that of the *gefA*$^{-}$ mutant; therefore it was speculated that RasGEFA might mediate RasC activation (Lim *et al*, 2001). However, it was not possible to test this idea genetically by the expression of activated RasC in the *gefA*$^{-}$ strain because such expression had deleterious effects on early development (Lim, 2002). The modifications to the RBD binding assay allowed for an alternative strategy. Six-hour cAMP-pulsed Ax3 and *gefA*$^{-}$ cells were stimulated with cAMP, and the levels of activated RasC and RasG were measured at various time points after stimulation. No activation of RasC was observed in cells lacking RasGEFA (Fig 1), indicating that RasGEFA is required for all of the cAMP-stimulated RasC activation. By contrast, there was no reduction in RasG activation on cAMP stimulation in *gefA*$^{-}$ cells (Fig 1), indicating that RasGEFA is not involved in RasG activation. There was an additional effect on activated RasG in the *gefA*$^{-}$ cells because normal return to basal level at 60 s did not occur (see Discussion).

RasGEFR is required for maximal activation of RasG

The availability of other *gef* gene null strains (Wilkins *et al*, 2005) provided an opportunity to search for the RasGEF protein responsible for activation of RasG. When pulsed *gefR*$^{-}$ cells were stimulated with cAMP there was a partial reduction in RasG activation, although there was no effect on RasC activation (Fig 2A). These results indicate a role for RasGEFR in the activation of RasG, but not in the activation of RasC. However, as the reduction in RasG activation was only partial, it seems that there are at least two RasGEFs that mediate cAMP-stimulated RasG

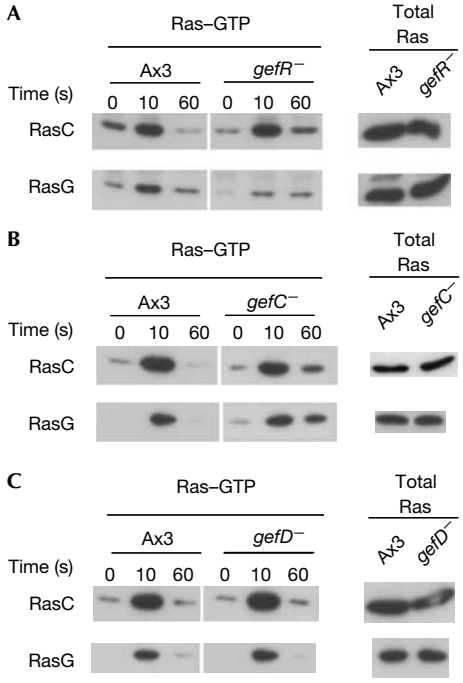

Fig 2 | Loss of RasGEFR reduces activation of RasG during development. Six-hour-pulsed (**A**) *gefR*$^{-}$, (**B**) *gefC*$^{-}$ and (**C**) *gefD*$^{-}$ cells were stimulated by the addition of 200 nM cyclic AMP and assayed for activated Ras as described in Fig 1. This figure is representative of two independent experiments. *gef*, guanine-nucleotide exchange factor.

activation. This conclusion is consistent with the observation that *gefR*$^{-}$ cells show no apparent defect in early development (Secko *et al*, 2004). The decrease in RasG activation in *gefR*$^{-}$ cells coincided with observed prolonged activation of RasC compared with wild-type cells (see Discussion). cAMP-stimulated RasC and RasG activation was normal in *gefC*$^{-}$ and *gefD*$^{-}$ strains (Fig 2B,C), indicating that RasGEFC and RasGEFD were not involved in the activation of either RasC or RasG during early development.

RasGEFA and RasGEFR are highly specific *in vitro*

RasGEFs catalyse the release of GDP from Ras-GDP and this can be measured by using an *in vitro* fluorescence nucleotide exchange assay. To determine the *in vitro* activity and substrate specificities of RasGEFA and RasGEFR, the CDC25 domains of RasGEFA (GefA^{CDC25}) and RasGEFR (GefR^{CDC25}) were expressed in bacteria as glutathione-*S*-transferase (GST) fusion proteins, purified and enzymatically cleaved from the GST tag. To provide substrates for these *in vitro* assays, full-length RasD and RasG, and carboxy-terminal truncations of RasB (1–169; RasC (1–168) and Rap1 (1–169; RasB^{CT}, RasC^{CT} and Rap1^{CT}) were also expressed in bacteria and purified. The C-terminal Ras truncations were used because they are similar to full-length proteins in their physical and biochemical properties (John *et al*, 1990); however their greater stability facilitated their expression and purification. The Ras proteins were loaded with a fluorescent GDP analogue, mGDP (2′-(or 3′)-*O*-(*N*-methylanthraniloyl)-guanosine 5′-diphosphate), incubated in the presence of an excess of GDP and either GefA^{CDC25} or GefR^{CDC25}. Nucleotide release was followed as the

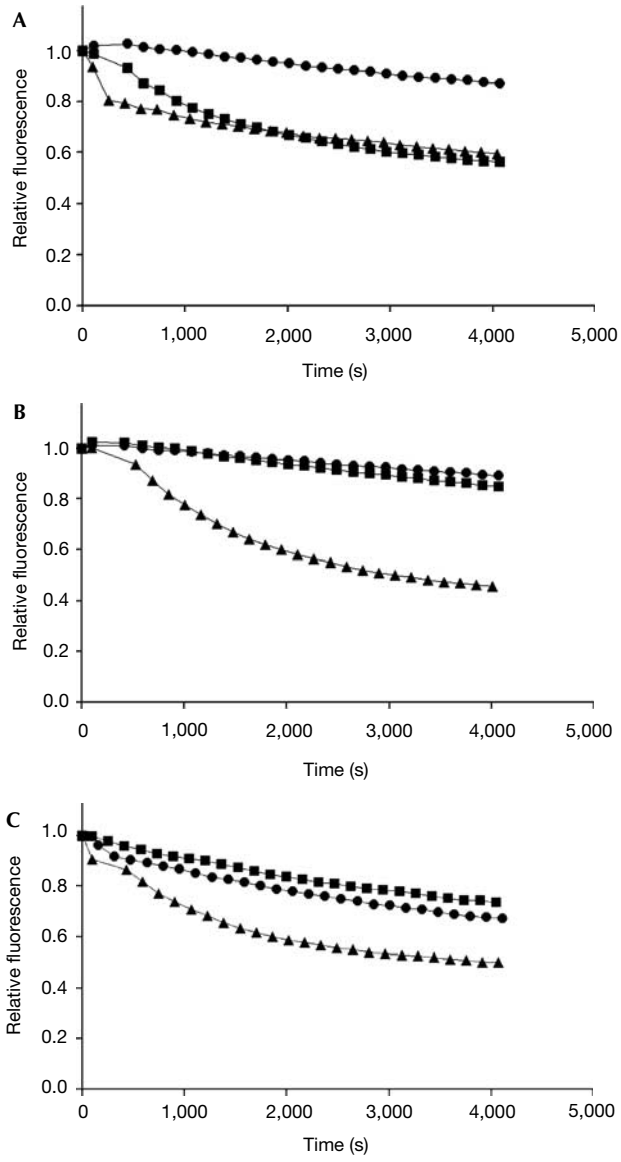

Fig 3 | RasGEFA stimulates GDP release from RasC but not RasD or RasG. Ras proteins were loaded with mouse GDP, incubated with (filled squares) or without (filled circles) 1 μM $GefA^{CDC25}$, or with (filled triangles) 1 μM CDC25, and GDP release was plotted as a decrease in relative fluorescence with time. The curves shown are (**A**) $RasC^{CT}$, (**B**) RasG and (**C**) RasD. *gef*, guanine-nucleotide exchange factor.

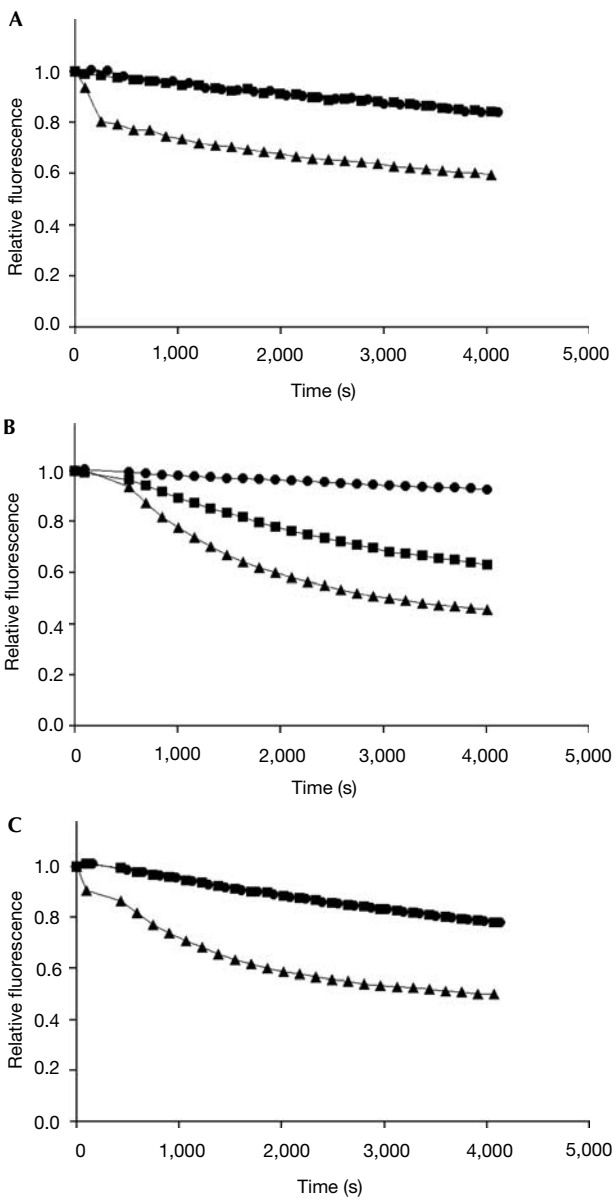

Fig 4 | RasGEFR stimulates the release of GDP from RasG but not RasC or RasD. Ras proteins were loaded with mouse GDP, incubated with (filled squares) or without (filled circles) 1 μM $GefR^{CDC25}$, or with (filled triangles) 1 μM CDC25, and GDP release was plotted as a decrease in relative fluorescence with time. The curves shown are (**A**) $RasC^{CT}$, (**B**) RasG and (**C**) RasD. *gef*, guanine-nucleotide exchange factor.

decrease of fluorescence with time. To control for the stability and folding of the purified *Dictyostelium* Ras and Rap proteins, the human RasGEF CDC25 (also known as RasGRF1) and the RapGEF C3G were used as controls (Figs 3,4; data not shown). mGDP release was also measured in the absence of a RasGEF to monitor the intrinsic GDP release (Figs 3,4).

When $RasC^{CT}$-mGDP was incubated with $GefA^{CDC25}$, there was a significantly enhanced decrease in fluorescence compared with the basal intrinsic decrease in the absence of a GEF indicating that $GefA^{CDC25}$ was able to catalyse GDP release from $RasC^{CT}$ (Fig 3A). By contrast, there was no increase in the loss of fluorescence when RasG-mGDP was used as a substrate (Fig 3B). Incubation of $GefR^{CDC25}$ with RasG-mGDP enhanced the rate of decrease of fluorescence relative to the basal intrinsic activity (Fig 4B), whereas a similar decrease in fluorescence did not occur when $RasC^{CT}$-mGDP was used as a substrate (Fig 4A). Neither $GefA^{CDC25}$ nor $GefR^{CDC25}$ stimulated the release of GDP from RasD (Figs 3C,4C), $RasB^{CT}$ or $Rap1^{CT}$ (data not shown). These results support the conclusion that RasGEFA and RasGEFR specifically mediate GDP release from RasC and RasG, respectively.

DISCUSSION

The *Dictyostelium* genome encodes 14 Ras subfamily proteins (Weeks *et al*, 2005), a number that is considerably greater than that found in yeast and other fungi. In addition, there are more *gef* genes than *ras* genes encoded by the *Dictyostelium* genome (Wilkins *et al*, 2005), indicating that at least some Ras proteins are activated by more than one RasGEF. Consistent with this idea, of the ten *gef* gene knockouts that have been described, several have no distinct phenotype (Wilkins *et al*, 2005).

The isolation of a *gefA*$^-$ strain that failed to aggregate was the first indication of a Ras protein being involved in cAMP signalling during *Dictyostelium* development (Insall *et al*, 1996). The subsequent isolation of a *rasC*$^-$ null mutant with a similar phenotype indicated the possibility that RasC functioned downstream of RasGEFA (Lim *et al*, 2001). The pattern of Ras activation in the *gefA*$^-$ mutant provided convincing evidence that RasGEFA is necessary for the activation of RasC in response to cAMP, but not required for RasG activation in response to cAMP (Fig 1). This has been verified by the use of an *in vitro* nucleotide exchange assay. RasGEFA catalysed the removal of GDP from RasC but not from other Ras subfamily proteins, confirming that RasGEFA is specific for RasC (Figs 3,4).

A proteomic study had shown that RasGEFR was the only RasGEF that showed an alteration in phosphorylation in response to the expression of activated RasG, indicating a possible connection (Secko *et al*, 2004). However, there was no direct evidence to show that RasGEFR mediated RasG activation. The finding of a significant reduction in the magnitude of RasG activation in response to cAMP in a *gefR*$^-$ mutant (Fig 2C) provided direct evidence that RasGEFR was responsible for mediating at least some of the RasG activation. This conclusion was confirmed by the *in vitro* nucleotide exchange assay, which showed that RasGEFR was specific for RasG (Fig 4; data not shown). As *gefR*$^-$ strains show no phenotype, and as RasG activation is not completely abolished in this strain, there might be at least one other RasGEF responsible for the activation of RasG upon cAMP stimulation. At present, we do not know the identity of the other RasG-specific RasGEF(s), although, as far as cAMP signalling is concerned, we can eliminate RasGEFC and RasGEFD because *gefC*$^-$ and *gefD*$^-$ (Fig 2) null mutants are not defective in RasG activation.

In three cases (Figs 1,2) loss of a RasGEF was associated with prolonged activation of those Ras proteins that were still activated. In particular, *gefA*$^-$ cells showed prolonged RasG activation (Fig 1), *gefR*$^-$ cells showed prolonged RasC activation (Fig 2A), and *gefC*$^-$ cells showed prolonged activation of both RasC and RasG (Fig 2B). Given the general model of the GTP-dependent activation cycle of Ras proteins, this extended peak of Ras-GTP should reflect reduced activity of a negative regulator of Ras, such as a RasGAP. Nine genes have been annotated in the *Dictyostelium* genome that encode for proteins with putative RasGAP domains (Chisholm *et al*, 2006), but none has been characterized so far. Two other proteins with RasGAP domains—DGAP1 and GAPA—have been identified as IQGAPs but the evidence shows that these two proteins act on Rho family GTPases and not Ras (Adachi *et al*, 1997; Faix *et al*, 1998). Whether the nine putative RasGAPs show specificity towards individual Ras proteins is unknown. It is possible that RasGAPs are activated by the same signal that activates the Ras proteins, thereby entraining the adaptation response with the activation response; however, explaining such a regulation will require detailed investigation of the RasGAP proteins.

The findings that RasGEFA is specific for RasC and that RasGEFR is specific for RasG are consistent with the idea that RasC and RasG are involved in different responses generated from the cAMP stimulus: RasC is predominantly involved in the activation of the adenylyl cyclase expressed during aggregation (ACA), whereas RasG predominantly involved in chemotaxis (Bolourani *et al*, 2006). In chemotaxing cells it has been shown that activated Ras appears at the tip of the pseudopod within 10 s of an applied cAMP stimulus (Sasaki *et al*, 2004). We believe that the activated Ras detected by Sasaki *et al* (2004) is RasG because the Raf1–RBD used for the assay has a higher affinity for RasG than RasC (Kae *et al*, 2004). RasC and RasG each have their own specific RasGEFs. As ACA is localized at the rear of the cell (Kriebel *et al*, 2003), it is tempting to speculate that RasGEFA is also localized at the rear of the cell where RasC activation would be responsible for ACA activation. A precedent for Ras signalling occurring in two distinct subcellular domains was proposed for T cells, in which Ras is activated at the plasma membrane by SOS1 and at the Golgi membrane by RasGRP1 (Bivona *et al*, 2003). At present, a reagent that will specifically detect activated RasC in the cell is not available; therefore, it is not possible to determine whether the activation of RasC and RasG have different localizations.

The high degree of RasGEF–Ras specificity in *Dictyostelium* is in contrast to the situation in mammalian cells, in which overlapping specificities are common (Quilliam *et al*, 2002). For example, SOS1 shows *in vitro* exchange activity on one group of Ras proteins (H-Ras, K-Ras, N-Ras, M-Ras and R-Ras; Porfiri *et al*, 1994; Ohba *et al*, 2000; Nielsen *et al*, 2001), and RasGRF1 has been shown to activate a second group of Ras proteins (H-Ras, K-Ras, N-Ras, M-Ras, R-Ras1 and R-Ras2; Ohba *et al*, 2000; Nielsen *et al*, 2001). Little is known about the structural determinants of Ras that might be involved in conferring RasGEF specificity. Analysis of a SOS1–Ras crystal structure has shown that three regions are involved in the RasGEF–Ras interaction: Switch 1 region (residues 21–40), Switch 2 region (57–75) and helix α3 region (95–111; Boriack-Sjodin *et al*, 1998). RasC and RasG show a number of differences in the Switch 1 (4 out of 19 residues), Switch 2 (2 out of 19) and helix α3 regions (6 out of 17), providing various possible determinants of specificity. However, RasGEFR is able to activate RasG but not RasD, despite the fact that RasD and RasG are highly related (89% identity). In fact, RasD and RasG have identical Switch 1 and 2 regions and differ by only one residue, at position 111 in the helix α3 region, with most of the differences between these two proteins confined to the C-terminal residues. Therefore, the high degree of identity in the Switch 1 and Switch 2 regions, and even the helix α3 regions, between RasD and RasG indicates that the RasGEFR–RasG specificity might involve other residues. A detailed mutational analysis is required to determine which Ras residues are the important determinants that confer specificity for RasGEFA as opposed to RasGEFR.

METHODS

Cell culture and development. *D. discoideum* Ax3, *gefA*$^-$, *gefC*$^-$, *gefD*$^-$ and *gefR*$^-$ cells were grown in HL5 medium (Watts & Ashworth, 1970) supplemented with 50 μg/ml streptomycin

(Sigma, St Louis, MO, USA). To prepare pulsed aggregation competent cells, vegetative cells were washed three times and resuspended at 5×10^6 cells/ml in KK_2 (20 mM potassium phosphate (pH 6.1)), shaken at 160 r.p.m. for 1 h and then pulsed with 50 nM cAMP every 6 min. After pulsing for 5 h, cells were collected by centrifugation, washed twice in KK_2 and resuspended in KK_2 at 5×10^7 cells/ml.

RBD-binding assay. The assay was carried out as described previously (Kae *et al*, 2004), with one modification. The buffer used for cell lysis (2 × RBD-LB buffer) contained 20 mM sodium phosphate (pH 7.2), 2% Triton X-100, 20% glycerol, 300 mM NaCl, 20 mM $MgCl_2$, 2 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF and two protease-inhibitor tablets (Roche, Basel, Switzerland) per 50 ml of buffer. Six-hour-pulsed aggregation-competent cells were stimulated by the addition of 200 nM cAMP. Aliquots (400 μl) were taken at selected time points and lysed by the addition of an equal volume of ice-cold 2 × RBD-LB and incubated on ice for 5 min. The lysates were cleared by centrifugation at 12,000*g* for 10 min and protein concentrations were determined by using the *DC* Protein Assay (Bio-Rad, Hercules, CA, USA). The same mass of protein (800 μg) from each lysate was incubated with 100 μg of purified recombinant GST–RBD bound to glutathione Sepharose beads (GE Healthcare, Baie d'Urfe, Canada) and the mixture was tumbled at 4 °C for 1 h. Beads were collected by centrifugation and washed three times in 1 × RBD-LB. A 50 μl portion of 1 × SDS gel loading buffer was added to the pelleted beads and the mixture was boiled for 5 min. Samples were fractionated by SDS–polyacrylamide gel electrophoresis, blotted onto nitrocellulose, blocked with non-fat milk and probed with antibodies specific for RasC or RasG. The bound antibody was detected by using an ECL-Plus (GE Healthcare) reaction.

Expression and purification of recombinant proteins. The catalytic domain of RasGEFA (aa 182–605) was amplified from complementary DNA using the primers 5′-GAGGATCCAAAAT GACCACAGAATACGATA-3′ and 5′-GAGCGGCCGCTTAAA GAACATCAGATCTTTGAGC-3′. The catalytic domain of RasGEFR (aa 1,298—1,676) was amplified from cDNA using the primers 5′-GAGGATCCATGGGTACATTAAATAAATTAATTTC-3′ and 5′-GAGCGGCCGCTTATCTTGGTTCACGAATTAAAGAG-3′. The resulting fragments were cloned into pGEX 4T-3 (Stratagene, La Jolla, CA, USA). The catalytic domain of CDC25 and the Ras proteins have been described previously (Lenzen *et al*, 1998; Kortholt *et al*, 2006). All of the recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) codonplus-RIL (Stratagene) and purified as described by Kae *et al* (2004) and Kortholt *et al* (2006).

***In vitro* guanine nucleotide release assays.** GEF-induced nucleotide release was measured as described previously (Lenzen *et al*, 1995). Briefly, the Ras proteins were loaded with the fluorescent GDP analogue mGDP (Jena Bioscience, Jena, Germany) by incubation in the presence of 10 mM EDTA and a 20-fold excess of mGDP for 2 h at 25 °C. The mGDP-loaded Ras proteins were incubated at 25 °C in assay buffer (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl and 5 mM DTE) containing a 200-fold excess of unlabelled GDP, with 1 μM RasGEF protein. Nucleotide exchange was measured in real time as decay in fluorescence using a Spex1 spectrofluorometer (Spex Industries, Meutchen, NJ, USA), with excitation and emission wavelengths of 366 and 450 nm, respectively.

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