Eph receptors inactivate R-Ras through different mechanisms to achieve cell repulsion

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Summary
Eph receptor tyrosine kinases regulate the spatial organization of cells within tissues. Central to this function is their ability to modulate cell shape and movement in response to stimulation by the ephrin ligands. The EphB2 receptor was reported to inhibit cell-matrix adhesion by phosphorylating tyrosine 66 in the effector domain of R-Ras, a Ras family protein known to regulate cell adhesion and motility. Here, we further characterize the role of R-Ras downstream of both EphA and EphB receptors. Our data show that besides inhibiting R-Ras function through phosphorylation, Eph receptors can reduce R-Ras activity through the GTPase-activating protein, p120RasGAP. By using R-Ras mutants that cannot be inactivated by p120RasGAP and/or cannot be phosphorylated at tyrosine 66, we show that the two forms of R-Ras negative regulation – through increased GTP hydrolysis and phosphorylation – differentially contribute to various ephrin-mediated responses. Retraction of the COS cell periphery depends only on R-Ras inactivation through p120RasGAP. By contrast, both reduced R-Ras GTP levels and tyrosine 66 phosphorylation contribute to the ephrin inhibitory effects on COS cell migration and to ephrin-dependent growth cone collapse in primary neurons. Therefore, Eph receptors can regulate R-Ras in two different ways to achieve cell repulsion.

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Key words: Ephrin, GTPase-activating protein, P120RasGAP, Cell retraction, Cell motility, Growth cone collapse

Introduction
The Eph receptor tyrosine kinases play important roles in both embryonic development and adult tissue homeostasis by regulating diverse biological functions, including the establishment of neuronal connectivity, modulation of synaptic plasticity, formation of tissue boundaries, and remodeling of vascular and lymphatic vessels (Palmer and Klein, 2003; Pasquale, 2005). In addition, recent evidence has linked Eph receptors to pathologic processes such as tumor growth and tumor angiogenesis (Brantley-Sieders et al., 2004) and to genetic mutations involving defects in tissue patterning that lead to skeletal malformations (Twigg et al., 2004; Wieland et al., 2004). The Eph receptors can initiate signals that result in cell repulsion, which involve reorganization of the actin cytoskeleton and changes in cell adhesion leading to retraction of cellular processes. For example, EphA receptor activation by ephrin ligands triggers the collapse of the enlarged tips (growth cones) of growing neuronal processes, while in the dendrites of mature hippocampal pyramidal neurons it causes retraction of the small protrusions – called dendritic spines – where excitatory synapses are located (Drescher et al., 1995; Murai et al., 2003; Pasquale, 2005). Additionally, the repulsive effects of Eph receptors restrict cell positioning in various tissues, such as the rhombomeres of the developing hindbrain, streams of migrating embryonic neural crest cells, and differentiating epithelial cells in adult intestinal microvilli (Batlle et al., 2002; Poliakov et al., 2004).

The Eph receptors are grouped into two classes (EphA and EphB) that have binding preference for ephrin ligands of the corresponding A or B class (Pasquale, 2004). Both the glycosylphosphatidylinositol-anchored ephrin-A ligands and the transmembrane ephrin-B ligands are on the cell surface. Thus, the Eph receptors transduce signals as a result of direct cell-cell contact, which enables receptor-ligand interaction (Pasquale, 2005). Despite growing knowledge of Eph receptor signal transduction, very little is known regarding the similarities and differences in the signaling pathways downstream of the EphA and EphB receptors.

Several pathways downstream of Eph receptors have been implicated in regulation of the cytoskeleton, a majority of which converge on small GTPases of the Ras and Rho families (Murai and Pasquale, 2005; Noren and Pasquale, 2004). GTPases cycle between GTP-bound ‘on’ and GDP-bound ‘off’ conformations and are regulated by guanine nucleotide exchange factors, which activate Ras proteins by promoting the exchange GDP for GTP, and GTPase-activating proteins, which inactivate Ras proteins by stimulating GTP hydrolysis. R-Ras is a member of the Ras family that positively affects integrin-mediated adhesion in diverse cell types and has been implicated in Eph receptor signaling pathways (Hughes et al., 2001; Keely et al., 1999; Kwong et al., 2003; Sethi et al., 1999;
Eph receptors inactivate R-Ras

**Results**

Ephrin-dependent activation of the EphA2 and EphB2 receptors induces COS cell retraction and rounding

Soluble forms of the ephrin ligands fused to the Fc portion of human IgG1 can be used to activate Eph receptor signaling. Stimulation of COS cells with either ephrin-A1 Fc or ephrin-B1 Fc (which promiscuously activate multiple EphA or EphB receptors, respectively) revealed that these cells undergo dramatic morphological changes in response to either ephrin. These changes begin with retraction of the cell periphery and the appearance of actin-rich retraction fibers within 5 to 10 minutes, as shown by time-lapse microscopy (Fig. 1A) and phalloidin staining (Fig. 1B). By 30 to 45 minutes the cells occupy a much smaller area and have become rounded. The COS cell retraction and rounding probably involve both RhoA-dependent contraction of the actin cytoskeleton and decreased cell-substrate adhesion (Kaibuchi et al., 1999; Noren and Pasquale, 2004). Indeed, we found that treatment of cells with Y-27632 (an inhibitor of the RhoA-Rho kinase pathway) or with manganese [which activates several integrins (Bazzoni et al., 1995; Ivins et al., 2000)] prevents the morphological effects of both ephrin-A1 and ephrin-B1 (see supplementary material Figs S1 and S2).

EphB2 is present in COS cells and becomes phosphorylated on tyrosine residues in response to ephrin-B1 stimulation (Fig. 1C). In addition, we recently demonstrated that a peptide that selectively antagonizes ephrin binding to EphB2 and not other EphB receptors abrogates ephrin-B1-induced COS cell retraction (Koolpe et al., 2005). Therefore, the EphB receptor that mediates the repulsive effects of ephrin-B1 is EphB2. Among the EphA receptors, EphA2 is highly expressed in COS cells and becomes phosphorylated on tyrosine residues in response to ephrin-A1 stimulation (Fig. 1C,D). To determine whether EphA2 activation is sufficient to induce cell retraction, we treated COS cells with the YSA ephrin-mimic peptide (YSAYPDSVMMS), which selectively activates EphA2 and not other EphA receptors (Koolpe et al., 2002). The YSA peptide, but not a control peptide, induces cell retraction concomitant with EphA2 tyrosine phosphorylation (Fig. 1D-F). This result identifies EphA2 as an EphA receptor that causes COS cell retraction. However, we cannot exclude that other EphA receptors may also contribute to the effects of ephrin-A1. We detected EphA4 in COS cells, but at very low levels (data not shown), indicating that the role of EphA4 is probably small.

GTP-bound R-Ras inhibits ephrin-induced COS cell retraction

Given the known ability of activated R-Ras to regulate adhesion and counteract certain effects of repulsive molecules (Oinuma et al., 2004a; Zou et al., 1999), we examined whether R-Ras can inhibit ephrin-induced COS cell retraction. We focused on the earlier stages of the response, when retraction fibers are readily detectable, because at later stages some of the rounded cells may detach making analysis less reliable. We found that COS cells transfected with wild-type R-Ras undergo retraction of the cell periphery upon ephrin treatment, similarly to control-transfected cells (Fig. 2A,C). We then transfected the R-Ras38VY66F mutant, which is constitutively active because it can neither hydrolyze GTP nor become phosphorylated on tyrosine 66 (Zou et al., 1999). R-Ras38VY66F-transfected
cells are somewhat more spread than control-transfected cells in the absence of ephrins (data not shown) and do not retract following ephrin-A1 or ephrin-B1 treatment (Fig. 2B,D; Table 1). Surprisingly, R-Ras38V also completely blocks cell retraction (Fig. 2B,D; Table 1), suggesting that R-Ras inactivation by phosphorylation at tyrosine 66 does not significantly contribute to retraction of the cell periphery. These effects of GTP-bound R-Ras38V are selective, because GTP-bound H-Ras does not inhibit ephrin-induced COS cell retraction (Fig. 2E,F). By contrast, R-Ras 38V does not block cell retraction if it contains a tyrosine 66 to glutamic acid mutation, which introduces a negative charge similar to that of a phosphate group (see supplementary material Fig. S3; Table 1). It should be noted that this R-Ras38VY66E mutant was well expressed in the COS cells used in this experiment (not shown), in contrast to previous reports in CHO cells (Oertli et al., 2000). The lack of inhibition of cell retraction by R-Ras38VY66E indicates that modification of tyrosine 66 could impair the effects of GTP-bound R-Ras on cell retraction, for example by preventing binding to an effector (Table 1), thus confirming that R-Ras38V is not sufficiently inactivated by phosphorylation in this assay.

Finally, R-RasY66F, in which tyrosine 66 cannot be phosphorylated but the GTPase activity of R-Ras is normal, does not inhibit ephrin-induced cell retraction (Fig. 2B,D; Table 1). These data indicate that efficient COS cell retraction following ephrin-A1 or ephrin-B1 stimulation requires low levels of GTP-bound active R-Ras and suggest that endogenous Eph receptor signals can decrease the levels of R-RasGTP.

Ephrin stimulation decreases the levels of GTP-bound R-Ras

To investigate the effects of ephrin treatment on the levels of GTP-bound R-Ras, we performed a pull-down assay using a GST fusion protein of the Ras-binding domain of Raf1, which binds only to the activated form of R-Ras (de Rooij and Bos, 1997). This assay was performed without treating the cell lysates with the phosphatase inhibitor vanadate, to reduce any
tyrosine 66 phosphorylation that may be present and inhibit Raf1 binding (Zou et al., 1999). Immunoblotting of the proteins bound to the GST-Raf1 fusion protein with anti-R-Ras antibody showed that ephrin-A1 and ephrin-B1 both decrease the levels of R-RasGTP (Fig. 3). Furthermore, the time course of R-Ras inactivation correlates with that of cell retraction. The findings that R-RasGTP inhibits COS cell retraction and that Eph receptors decrease R-RasGTP levels suggest that Eph receptors reduce R-RasGTP levels to induce cell retraction.

Eph receptors regulate R-Ras through p120RasGAP
The Eph receptors could decrease the levels of GTP-bound R-Ras in a RhoA-dependent manner, as knockdown of p120RasGAP induced cell retraction (Fig. 4A). Furthermore, Eph receptors decrease R-RasGTP levels in a p120RasGAP-dependent manner (Fig. 4B), suggesting that Eph receptors regulate R-Ras through p120RasGAP.

**Table 1. Raf1 binding and effects of R-Ras mutants on ephrin-mediated repulsive responses**

<table>
<thead>
<tr>
<th>R-Ras construct</th>
<th>RBD binding*</th>
<th>Inhibits cell retraction</th>
<th>Inhibits cell migration</th>
<th>Inhibits growth-cone collapse</th>
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<td>WT</td>
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<tr>
<td>38V</td>
<td>++</td>
<td>Yes</td>
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</tr>
<tr>
<td>38VY66F</td>
<td>+</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>38VY66E</td>
<td>–</td>
<td>No</td>
<td>No</td>
<td>n.d.</td>
</tr>
<tr>
<td>Y66E</td>
<td>–</td>
<td>No††</td>
<td>No††</td>
<td>n.d.</td>
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</table>

*Independent of phosphorylation on tyrosine 66 because orthovanadate was not included in the buffers; RBD, Ras-binding domain of Raf1.
†Induced by ephrin stimulation.
‡The R-Ras Y66E and 43N mutants do not cause cell retraction in the absence of ephrin, suggesting that R-Ras inactivation is not sufficient to cause cell retraction.
§See supplementary material Fig. S5.
¶Zou et al., 1999.
**Based on the GTP-binding deficiency of this mutant.
††Data not shown.
†n.d., not determined.
Ras either by inhibiting the activity of an exchange factor for R-Ras or by enhancing the activity of a GTPase-activating protein. A R-Ras exchange factor that could conceivably be regulated by Eph receptors is C3G. C3G is activated through its association with Crk (Ichiba et al., 1997), an adaptor protein that binds to activated EphA and EphB receptors and has been implicated in their effects on cell morphology (Hock et al., 1998; Lawrenson et al., 2002; Nagashima et al., 2002; Smith et al., 2004). Binding of Crk to C3G is inhibited by Crk phosphorylation at tyrosine 221 (Feller et al., 1994) and we found that treatment of COS cells with ephrin-B1 causes an increase in Crk tyrosine phosphorylation (see supplementary material Fig. S4A). However, ephrin-B1 treatment still decreases R-RasGTP levels and causes retraction in COS cells expressing CrkY221F, a mutant of Crk that cannot be inactivated by phosphorylation (see supplementary material Fig. S4B and C). Thus, negative regulation of C3G through Crk phosphorylation does not appear to play a major role in R-Ras inactivation by Eph receptors in COS cells.

p120RasGAP is a GTPase-activating protein that has preferential activity toward R-Ras and functions downstream of both EphA2 (Tong et al., 2003) and EphB2 (Holland et al., 1997; Li et al., 1997; Tong et al., 2003). The activity of p120RasGAP can be inhibited using GAP-N, a truncated form that contains the SH2 domains of p120RasGAP (which mediate binding to activated receptor tyrosine kinases) but lacks the GAP domain (Elowe et al., 2001). Importantly, GAP-N is known to function as a dominant negative in EphB2 signaling (Elowe et al., 2001). We found that expression of GAP-N prevents both the decrease in R-RasGTP levels (Fig. 4A-C) and the cell retraction (Fig. 4D,E) that occurs after ephrin stimulation. These data are consistent with a model whereby

![Fig. 3.](image)

**Fig. 3.** Ephrin stimulation decreases the level of GTP-bound R-Ras. COS cells stably transfected with R-Ras were stimulated with ephrin-A1 Fc (A), ephrin-B1 Fc (B) or Fc as a control. GTP-bound R-Ras was isolated with GST-Raf1 RBD and detected with anti-R-Ras antibodies. The histograms show the mean levels of GTP-bound R-Ras relative to control from three experiments; the bars represent standard errors. Levels of GTP-bound R-Ras in ephrin Fc-treated cells were compared with those in Fc-treated cells by one-way ANOVA and Tukey’s post-hoc test. *P<0.05; **P<0.01.

![Fig. 4.](image)

**Fig. 4.** A dominant negative form of p120RasGAP prevents the decrease in R-RasGTP levels and COS cell retraction induced by ephrins. (A,B) A COS cell line stably transfected with R-Ras was transiently transfected with a dominant negative form of p120RasGAP (GAP-N) or pcDNA3 vector control and stimulated with ephrin-A1 Fc, ephrin-B1 Fc or Fc as a control. GTP-bound R-Ras was isolated with GST-Raf1 RBD and detected with anti-R-Ras antibodies in duplicate samples for the GAP-N transfected cells. (C) The histogram shows the relative levels of R-Ras GTP quantified from the experiment in A and B and normalized to the R-Ras levels in the lysates. (D) COS cells were transiently transfected with EGFP-tagged GAP-N or EGFP vector as a control. Cells were stimulated with ephrin-A1 Fc, ephrin-B1 Fc or Fc as a control and stained with rhodamine-phalloidin (red). Bar, 20 μm. (E) Histogram showing the mean percentage of cells that have spikes at the periphery; bars represent standard errors from three experiments. GAP-N-transfected cells treated with ephrin-A1 Fc or ephrin-B1 Fc were compared with similarly treated control-transfected cells by one-way ANOVA and Tukey’s post-hoc test, **P<0.01 and ***P<0.001.
p120RasGAP mediates the morphological effects of activated EphA and EphB receptors by reducing R-RasGTP, which enables COS cell retraction and rounding. Indeed, co-transfection of the dominant negative R-Ras43N with GAP-N appears to restore cell retraction in ephrin-treated cells (data not shown). A caveat with the interpretation of this experiment, however, is that although transfection of R-Ras43N alone or GAP-N alone does not affect cell morphology, many of the R-Ras43N and GAP-N co-transfected cells had to be excluded from analysis because of abnormal morphology both in the presence and in the absence of ephrin treatment.

It should be noted that although p120RasGAP has been reported to mediate neurite retraction downstream of EphB2 in a neuronal cell line through inactivation of H-Ras (Elowe et al., 2001), the constitutively active H-RasV12 mutant does not inhibit ephrin-dependent retraction of the COS cell periphery (Fig. 2E,F). Thus, inactivation of R-Ras and not H-Ras plays a role in the repulsive effects of Eph receptors in COS cells.

GTP-bound R-Ras inhibits the repulsive effects of ephrin-B1 on COS cell migration only if it cannot be phosphorylated at tyrosine 66

In addition to modulating cell shape, the Eph receptors regulate cell motility (Pasquale, 2005). For example, ephrin-B1 inhibits COS cell haptotactic migration towards fibronectin (Fig. 5A). Similar to its effect on ephrin-induced cell retraction, the R-Ras38VY66F mutant also counteracts the repulsive effect of ephrin-B1 on cell migration (Fig. 5A; Table 1). However, both R-Ras38V and R-RasY66F fail to do so (Fig. 5B; Table 1). These drastically different activities of R-Ras38V and R-Ras38VY66F suggest that phosphorylation of R-Ras at tyrosine 66 contributes to the negative effects of ephrin-B1 on cell migration. To confirm the importance of tyrosine 66, we used the R-Ras38VY66E mutant. Cells expressing R-Ras38VY66E display reduced migration in the presence of ephrin-B1, similar to control-transfected cells (Fig. 5C; Table 1), confirming that a negative charge at position 66 impairs the ability of R-Ras to counteract the effects of Eph receptors. Supporting a role for R-Ras phosphorylation, we detected an increase of tyrosine-phosphorylated R-Ras in ephrin-B1-stimulated COS cells (Fig. 5D). We also confirmed that the R-Ras38V mutant is susceptible to tyrosine phosphorylation (data not shown). Therefore, these data suggest that either decreased R-RasGTP or phosphorylation of tyrosine 66 is sufficient to inhibit COS cell migration.

Eph receptors inactivate R-Ras to induce growth cone collapse in hippocampal and retinal neurons

The retraction of the COS cell periphery that occurs following ephrin stimulation is reminiscent of the retraction of growth cones that occurs in neurons during growth cone collapse, suggesting that activated R-Ras may also inhibit ephrin-induced growth cone collapse. GTP-bound R-Ras has indeed been recently shown to inhibit growth cone collapse induced by semaphorins in cultured rat hippocampal neurons, while R-Ras knockdown by treatment with siRNA increases the number of growth cones with collapsed morphology under basal conditions (Oinuma et al., 2004a). Neurons cultured from the embryonic rat hippocampus express endogenous R-Ras (Fig. 6A) and are responsive to both ephrin-A1 and ephrin-B1 (Fig. 6B,C). Expression of wild-type R-Ras increases the fraction of well spread growth cones (Fig. 6B,C), which still collapse following ephrin-A1 stimulation and, to a lesser extent, following ephrin-B1 stimulation (Fig. 6C). Interestingly, R-Ras38VY66F, R-Ras38V and R-RasY66F all significantly inhibit growth cone collapse, although R-Ras38V does not completely block ephrin-A1-induced growth cone collapse (Fig. 6C; Table 1). The increased spreading of growth cones in neurons transfected with wild-type R-Ras and the ability of both the R-Ras38V and the R-RasY66F mutants to inhibit ephrin-induced growth cone collapse suggest that R-Ras is highly activated in hippocampal neurons. Therefore, Eph receptor signaling may inactivate R-Ras sufficiently to cause growth cone collapse in hippocampal neurons only through the combined effects of tyrosine phosphorylation and increased GTP hydrolysis.

In temporal retinal explants, where growth cones are well known to collapse in response to ephrin-A5 treatment (Drescher et al., 1995), R-Ras38VY66F also inhibits collapse...
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Discussion

We found that EphA as well as EphB receptor stimulation by ephrins decreases the levels of R-RasGTP, in addition to inhibiting the activity of GTP-bound R-Ras through phosphorylation. Expression of a dominant negative form of the GTPase-activating protein, p120RasGAP, prevented the decrease in R-Ras GTP levels and COS cell retraction, downstream of Eph receptors, implicating p120RasGAP in these effects. Indeed, p120RasGAP has been shown to interact with activated Eph receptors through its two SH2 domains (Holland et al., 1997). Although p120RasGAP is an established regulator of H-Ras, this GTPase-activating protein has been shown to preferentially regulate R-Ras in vitro (Li et al., 1997) and, here, we demonstrate that it regulates R-RasGTP levels in COS cells. Experiments with the same dominant-negative mutant have linked p120RasGAP to the ephrin-dependent
increase in H-Ras activity and neurite retraction in neuronal NG108 cells stably expressing EphB2 (Elowe et al., 2001). Therefore, p120RasGAP enables the characteristic repulsive signals of Eph receptors, whereas its role downstream of growth factor receptor tyrosine kinases probably is to terminate activation of the H-Ras-MAP kinase pathway induced by these receptors (Ekman et al., 1999; Tong et al., 2003).

We have not found evidence that inhibition of an exchange factor for R-Ras, such as C3G, plays a role in COS cell retraction downstream of Eph receptors. We also have not found evidence for a role of SHEP1, a signaling intermediate that binds to Eph receptors through its SH2 domain and to R-Ras through an exchange factor-like domain (Dodelet et al., 1999). A truncated form of SHEP1 containing the SH2 domain but not the exchange factor-like domain, which, like the truncated form of p120RasGAP, should act as a dominant negative, did not inhibit COS cell retraction (data not shown).

R-Ras is one of several Ras family proteins that have been implicated in repulsive responses initiated by EphB2 in different cell types. In the EphB2-NG108 cells, transfection with the constitutively active H-RasV12 mutant prevents neurite retraction induced by ephrin-B1 stimulation, consistent with a role for inactivation of H-Ras in this repulsive response (Elowe et al., 2001). We also found that H-RasGTP levels were decreased in COS cells stimulated with ephrin-B1 (data not shown). However, expression of H-RasV12 did not prevent COS cell retraction, suggesting that H-Ras is not involved in this repulsive response in COS cells. Another Ras protein, Rap1, has also been recently implicated in Eph-mediated repulsion. The constitutively active Rap1V12 mutant was shown to prevent ephrin-B1-induced retraction and rounding of DLD1 colon cells (Riedl et al., 2005). The contribution of specific Ras family proteins to Eph receptor-mediated repulsive signals probably depends on cell type-specific differences in the function or relative abundance of the Ras proteins. For example, we only detected low levels of Rap1 in the COS cells used in our experiments (data not shown). Alternatively, R-Ras and Rap1 may also function in a common pathway (Self et al., 2001).

The convergence of ephrin and semaphorin repulsive signaling pathways on R-Ras suggests that both classes of molecules act in concert to guide axons in the developing nervous system. For example, ephrins and semaphorins could guide axonal projections at different points along their trajectory or enhance each other’s repulsive effects by signaling through a common effector, R-Ras. The Plexin semaphorin receptors can directly regulate R-RasGTP levels through their intrinsic R-Ras GAP activity, instead of binding a GTPase-activating protein such as p120RasGAP (Oinuma et al., 2004a). However, the R-Ras GAP activity of the Plexins requires coincident semaphorin engagement and intracellular association with Rnd1, a Rho family GTPase, suggesting a tightly controlled regulation of R-Ras activity (Oinuma et al., 2004a; Oinuma et al., 2004b). Whether the Plexin receptors, which do not have a kinase domain, may also cause R-Ras phosphorylation by recruiting a tyrosine kinase remains to be determined. The Src family kinase Fyn, for example, functions in semaphorin signaling, and Src has been reported to phosphorylate R-Ras on tyrosine 66 (Sasaki et al., 2002; Zou et al., 2002). Indeed, Src may also contribute to R-Ras phosphorylation downstream of Eph receptors (Zisch et al., 1998; Zou et al., 1999). We found that the Src inhibitor PP2 inhibits ephrin-induced cell retraction at a concentration of 0.5 \( \mu \text{M} \) (data not shown). However, these effects may also be explained by an inhibition of phosphorylation of Rho family exchange factors by Src family kinases (Sahin et al., 2005). In addition, we found that PP2 partially inhibits tyrosine phosphorylation of EphB2 (but not EphA2), and thus probably inhibits EphB2 kinase activity (data not shown).

The combined effects of increased GTP hydrolysis and phosphorylation may sometimes be necessary to achieve sufficient R-Ras inactivation to enable repulsive responses, as is the case for ephrin-dependent growth cone collapse in hippocampal neurons transfected with R-Ras. However, the two modes of R-Ras inactivation downstream of Eph receptors may also have different consequences. Reducing R-RasGTP levels turns off R-Ras signals completely. Phosphorylation of tyrosine 66, which is in the effector domain, could inhibit interaction of GTP-bound R-Ras with some effectors but not others, similar to previously described effector loop point mutations (Oertli et al., 2000; Osada et al., 1999; Zou et al., 1999). Inactivating modifications of tyrosine 66, however, seem to impair most of the activities of R-RasGTP investigated so far (Kinashi et al., 2000; Oertli et al., 2000; Osada et al., 1999) (this study). Phosphorylation of tyrosine 66 may also serve some other, as yet unknown, function, in addition to regulating R-Ras effector binding. For example, it could create a binding site for an SH2 domain or induce a conformational change affecting R-Ras function.

Whether the two different modes of R-Ras regulation may be spatially or temporally separate remains to be determined. We found that both R-Ras phosphorylation and increased GTP hydrolysis are important for inhibition of COS cell migration, whereas R-Ras phosphorylation does not seem to play a role in retraction of the COS cell periphery. A possible explanation for this result is that the pool of GTP-bound R-Ras that is concentrated in pseudopodia at the leading edge of migrating COS cells (Wozniak et al., 2005) may be particularly susceptible to phosphorylation by activated Eph receptors. This could result in a localized high concentration of phosphorylated R-Ras in pseudopodia, which probably plays a critical role in inhibiting directional migration.

Cell retraction and rounding as well as growth cone collapse typically involve contraction of the cytoskeleton, a process regulated by the Rho family GTPase, RhoA (Nobes and Hall, 1995; Ridley and Hall, 1992), and weakening of cell-substrate adhesion mediated by integrins. Consistent with this scenario, we found that inhibition of the RhoA-Rho kinase pathway with the Y-27632 compound and activation of integrins with manganese both prevent ephrin-induced COS cell retraction. Indeed, RhoA is known to be activated downstream of both EphA and EphB receptors (Cowan et al., 2005; Lawrenson et al., 2002; Ogita et al., 2003; Shamah et al., 2001; Tanaka et al., 2003; Wahl et al., 2000). For example, RhoA activity has been shown to be required for ephrin-A5-mediated growth cone collapse in retinal neurons and EphB2-mediated retraction and rounding of colon cells (Riedl et al., 2005; Wahl et al., 2000). Both R-Ras inactivation and RhoA activation have also been implicated in semaphorin-mediated repulsive responses (Oinuma et al., 2004a; Oinuma et al., 2004b; Pasterkamp, 2005) and in the retraction and rounding of fibroblasts and epithelial cells exposed to \textit{Clostridium difficile} toxin B variants.
(Chaves-Olarte et al., 2003). Interestingly, these toxins inactivate R-Ras through a third mechanism: glucosylation. The ability of both Eph receptors and Plexins to regulate RhoA activity supports a scenario where R-Ras and RhoA act in concert, downstream of repulsive molecules. Consistent with the requirement for other pathways in the ephrin repulsive effects, we found that expression of dominant negative R-Ras43N or of R-RasY66E is insufficient to induce cell retraction (data not shown).

R-Ras itself may also influence RhoA activity, but this regulation appears to be complex and, probably, cell-type specific. R-Ras has been found to increase RhoA activity in some cell types, such as T47D breast cancer cells and non-transformed MCF10A breast epithelial cells (Jeong et al., 2005; Wozniak et al., 2005). Furthermore, R-Ras has been reported to inhibit Rac activity in T47D cells (Wozniak et al., 2005), which may lead to increased RhoA activity (Burridge and Wennerberg, 2004). However, R-Ras activates Rac in 32D myeloid cells (Holly et al., 2005) whereas no effect of R-Ras on Rac activity was detected in another breast cancer cell line, MCF7, and in MCF10A breast epithelial cells (Felekakis et al., 2005; Jeong et al., 2005). Therefore, R-Ras inhibition downstream of repulsive molecules may in some cases enhance and in other cases suppress RhoA activity, directly or indirectly through Rac, thus modulating RhoA activation by the repulsive molecule.

R-Ras is also known to positively regulate integrin function in many cell types (Holly et al., 2005; Hughes et al., 2001; Keely et al., 1999; Oertli et al., 2000; Sethi et al., 1999; Zhang et al., 1996; Zou et al., 1999) and activated R-Ras reportedly localizes to focal adhesions through the hypervariable region at the carboxyl terminus, which is also the region important for integrin activation (Furuhjelm and Peranen, 2003; Hansen et al., 2002; Oertli et al., 2000). Furthermore, activated R-Ras enhances the formation of focal adhesions in some cells (Kwong et al., 2003). Although R-Ras has not been detected in the focal adhesions of COS cells (Furuhjelm and Peranen, 2003), we observed that activated R-Ras enhances COS cell spreading, similar to manganese treatment. Furthermore, R-Ras causes spreading and enlargement of neuronal growth cones. These data suggest that activated R-Ras may counteract the repulsive effects of Eph receptors at least in part by promoting integrin adhesive function.

Whereas the signals regulating R-Ras inactivation are beginning to be elucidated, those causing activation of R-Ras remain elusive. Nevertheless, activated forms of R-Ras have been linked to cell transformation (Cox et al., 1994; Jeong et al., 2005; Keely et al., 1999; Nishigaki et al., 2005; Osada et al., 1999; Rincon-Arano et al., 2003; Saez et al., 1994; Zou et al., 2002). Given recent data that EphB2 has tumor suppressor activity in prostate and colorectal cancer (Batlle et al., 2005; Keely et al., 1999; Nishigaki et al., 2005; Osada et al., 2002). Given recent data that EphB2 has tumor suppressor activity in prostate and colorectal cancer (Batlle et al., 2005; Keely et al., 1999; Nishigaki et al., 2005; Osada et al., 2002), we are tempted to speculate that Eph receptors may exert anti-tumorigenic effects at least in part by negatively regulating R-Ras.

Here we demonstrate that constitutively active R-Ras can counteract the repulsive effects of both EphA and EphB receptors. Activated R-Ras has also been recently shown to prevent the repulsive effects of the PlexinA and PlexinB semaphorin receptors (Oinuma et al., 2004a). Therefore, R-Ras38VY66F – the mutant that is insensitive to negative regulation by either phosphorylation or GTP hydrolysis – could be useful to promote the outgrowth of neuronal processes in non-permissive environments. For example, this mutant might be particularly effective in promoting axon regeneration after acute nervous system injury or in reversing the effects of neurodegeneration, by allowing axon growth even in the presence of different classes of repulsive molecules such as semaphorins and ephrins.

Materials and Methods

Constructs

The EGFP-R-Ras constructs were obtained by subcloning wild-type R-Ras into pEGFP-C2 (Clontech), and R-Ras38V, R-RasY66F and R-Ras38VY66F into pCAGGS-myc (Invitrogen). GAP-N, containing nucleotides 1-1326 of human p120RasGAP, was generated by PCR from a cDNA clone (GenBank™ accession number BC033015) and inserted into pcDNA3-myc and pEGFP-C2. pCAGGS-myc-CrkY221F and pEGFP-CrkY221F have been described previously (Abassi and Vuori, 2002). pGEX-Rat1 RBD has been described previously (de Rooij and Bos, 1997).

Antibodies

The anti-EphB2 antibody was obtained using a GST fusion protein comprising amino acids 897-995 of chicken EphB2 (Holash and Pasquaile, 1995) as the antigen; anti-EphA2 antibodies were from Upstate Inc. (monoclonal) and Zymed Laboratories Inc. (polyclonal); anti-Crk antibodies were from BD Pharmingen; the anti-RasGAP and anti-s-tubulin antibodies were from Santa Cruz Biotechnology; the TuJ1 anti-beta-III-tubulin was from BABCO; the anti-R-Ras antibody was from Cell Signaling Technologies; the anti-Myc 9E10 monoclonal antibody and anti-phytohemagglutinin were from Sigma; the anti-Fc antibody used for clustering Fc fusion proteins was from Jackson Laboratory; the anti-phosphotyrosine antibody conjugated to horseradish peroxidase was from BD Pharmingen or Upstate Inc. Secondary anti-mouse and anti-rabbit IgG peroxidase-conjugated antibodies were from Amersham Biosciences fluorescent Alexa Fluor 647-goat anti mouse was from Molecular Probes; and anti-protein A peroxidase-conjugated antibody was from Bio-Rad.

Cell culture and transfections

COS cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. Transient transfections of COS cells were carried out using Superfect Transfection Reagent (Qiagen Inc.) or FuGENE 6 (Amersham Biosciences) and the cells were harvested 24-48 hours after transfection. Co-transfected EGFP (1:10) was used to identify the transfected cells. COS cells stably expressing R-Ras were generated using Superfect Transfection Reagent and screening G418 resistant clones by immunoblotting for R-Ras expression.

Primary hippocampal neurons were prepared from E17 time-pregnant Harlan-Sprague-Dawley rats and immediately transfected using Amaxa Nucleofector Technology (Amaxa Biosystems) according to the manufacturer’s protocol. Briefly, for each construct 5×10⁶ neurons were suspended in 100 µl of Nucleofector solution with 3 µg DNA and electroporated using program G-13. Transfected neurons were plated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; 50,000 cells/24-well) on coverslips coated with 50 µg/ml poly-D-lysine and 5 µg/ml laminin (Sigma). Two hours after plating, the medium was replaced with Neurobasal medium containing B-27 and pcDNA3-myc (Invitrogen). GAP-N, containing nucleotides 1-1326 of human p120RasGAP, was generated by PCR from a cDNA clone (GenBank™ accession number BC033015) and inserted into pcDNA3-myc and pEGFP-C2. pCAGGS-myc-CrkY221F and pEGFP-CrkY221F have been described previously (Abassi and Vuori, 2002). pGEX-Rat1 RBD has been described previously (de Rooij and Bos, 1997).

Cell culture retraction

For time-lapse microscopy, COS cells were seeded on 35 mm glass-bottom Petri dishes (MatTek Corporation) and 16 hours later, the cells were starved for 3 hours in DMEM with 0.5% FBS. The plates were then mounted on a plate heater and images were obtained with SPOT software.

For immunofluorescence microscopy with COS cells, untransfected COS cells or COS cells transiently transfected for 24 hours were plated onto glass coverslips. Sixteen hours later, cells were starved for 3 hours in DME with 0.5% FBS and stimulated with 150 µM YSA EphA2-binding peptide (YSAPYDPSVPMSM) or ephrin Fc fusion proteins. For ephrin stimulation experiments, 1 µg/ml ephrin-A1
Fc, 1-2 μg/ml ephrin-B1 Fc or 0.5-1 μg/ml Fc as a control. In all experiments, the Fc fusion proteins were preincubated with a 1/10 concentration of anti-Fc antibodies. All Fc proteins were from R & D Systems. The cells were then fixed with 4% formaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS and stained with rhodamine-conjugated phallolidin (Molecular Probes).

Growth cone collapse assays
Forty-eight hours after transfection and plating, hippocampal neurons were stimulated for 20 minutes with 1.5 μg/ml preincubated ephrin-A1 Fc, ephrin-B1 Fc or Fc, fixed in 4% paraformaldehyde and blocked/permeabilized for 2 hours in TBS containing 0.1% Triton X-100 and 5% goat serum (Sternberger Monoclonals, Lutherville, MD). Neurons were then stained with an anti-β-tubulin antibody followed by goat anti-primary and secondary antibodies. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with enhanced chemiluminescence detection systems from Amersham Biosciences or Pierce. Protein concentrations were determined using a BioRad DC protein assay.

Immunoprecipitation and immunoblotting
Transiently transfected cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 10 μM NaF, 1 μM sodium selenate and protease inhibitors. For immunoprecipitations, cell lysates were incubated with 4 μg anti-Crk antibody immobilized on GammaBind Plus Sepharose beads (Amersham Biosciences) or 15 μg anti-phosphotyrosine antibody conjugated to agarose. Primary cultures of rat hippocampal neurons and brain regions prepared from 10-day old C57/Bl6 mice were lysed in 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 10 μM sodium vanadate, 10 μM NaF and protease inhibitors and cleared by centrifugation. Protein concentrations were determined using a BioRad DC protein assay.

Cell migration
Transiently transfected COS cells (75,000 cells/well) were seeded in 0.5% FBS on Transwell® filters (Corning Inc.) that had been coated on the bottom with 10 μg/ml anti-EphB2 antibody immobilized on glutathione beads (de Rooij and Bos, 1997). Filter inserts were incubated with GST-Raf1 Ras-binding domain (RBD) immobilized on glutathione beads (de Rooij and Bos, 1997). Samples were separated by SDS-PAGE and probed with anti-R-Ras antibodies.

References


