Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism

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The Rho family GTPases Cdc42, Rac1 and RhoA control many of the changes in the actin cytoskeleton that are triggered when growth factor receptors and integrins bind their ligands [1,2]. Rac1 and Cdc42 stimulate the formation of protrusive structures such as membrane ruffles, lamellipodia and filopodia. RhoA regulates contractility and assembly of actin stress fibers and focal adhesions. Although prolonged integrin engagement can stimulate RhoA [3–5], regulation of this GTPase by early integrin-mediated signals is poorly understood. Here we show that integrin engagement initially inactivates RhoA, in a c-Src-dependent manner, but has no effect on Cdc42 or Rac1 activity. Additionally, early integrin signaling induces activation and tyrosine phosphorylation of p190RhoGAP via a mechanism that requires c-Src. Dynamic modulation of RhoA activity appears to have a role in motility, as both inhibition and activation of RhoA hinder migration [6–8]. Transient suppression of RhoA by integrins may alleviate contractile forces that would otherwise impede protrusion at the leading edge of migrating cells.

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Results and discussion

In response to ligation by extracellular matrix (ECM) proteins, integrins transmit signals that initiate rearrangements in the actin cytoskeleton [2]. Numerous studies have shown that these cytoskeletal changes are mediated by Rho family proteins [1]. The signaling pathways by which integrins regulate these GTPases are not well characterized, however. To study how early integrin-mediated signals regulate RhoA, we measured RhoA activity in cells stimulated with GRGDSP peptides (RGD, single-letter amino-acid notation) which mimic the integrin-binding region found in the ECM proteins vitronectin and fibronectin. RhoA activity was measured by affinity precipitation of active (GTP-bound) RhoA from cell lysates using a GST (glutathione-*S*-transferase) fusion protein containing the Rho-binding domain of its effector, Rhotekin [9]. Ren *et al*. showed RhoA is transiently inhibited prior to its activation during integrin-mediated adhesion to fibronectin [9]. We obtained equivalent results by stimulating suspended BALB/c3T3 fibroblasts and Rat1 cells with RGD (data not shown). Here, we examine the signaling events responsible for this inhibition of RhoA.

Several integrin-stimulated signals are transmitted by members of the Src family tyrosine kinases (SFKs) [10]. SFKs are transiently activated during integrin-mediated adhesion [11] and have an important role in cell spreading and migration [10]. Cells lacking one or more SFKs exhibit reduced motility [10,12,13], which may be attributed to their inability to turn over focal adhesions or to misregulation of Rho family proteins [14,15]. Constitutively active RhoA reverts v-Src-induced disruption of stress fibers [15], supporting a role for SFKs in the regulation of Rho proteins. These findings suggest that SFKs may mediate the inhibition of RhoA by integrins.

We investigated the role of SFKs in integrin-triggered inhibition of RhoA by examining the response to RGD stimulation in cells lacking c-Src, Yes and Fyn (SYF cells), and in SYF cells re-expressing c-Src (c-Src-expressing cells) [13]. Upon treatment with RGD, RhoA activity in c-Src-expressing cells decreased from basal to nearly undetectable levels by 15 minutes (Figure 1) and returned to baseline by 30 minutes (data not shown). In SYF cells, however, RGD failed to suppress RhoA activity. Given that fibronectin- or vitronectin-coated beads bind equally to c-Src-null and control cells [16], the inability of SYF cells to respond to RGD is unlikely to be due to a defect in integrin binding. To further explore the role of SFKs in early integrin signaling, we inhibited SFKs by treating cells with PP1, an inhibitor of SFKs [17], or by transiently overexpressing c-Src kinase (Csk), a negative regulator of SFKs [10]. Inhibition of SFKs by either of these methods blocked the transient inhibition of RhoA that results from the addition of RGD to Rat1 cells (data not shown). Taken together, these data suggest that c-Src is essential for transmitting an early inhibitory signal from engaged integrins to RhoA.

One possible mechanism by which c-Src-mediated integrin signaling may modulate RhoA activity is by cross-talk between members of the Rho family. Given that v-Src induces a Rac1- and Cdc42-like protrusive phenotype [10] and that Rac1 can inhibit RhoA [18], a plausible mechanism is that stimulation of c-Src by integrin engagement activates

Figure 1
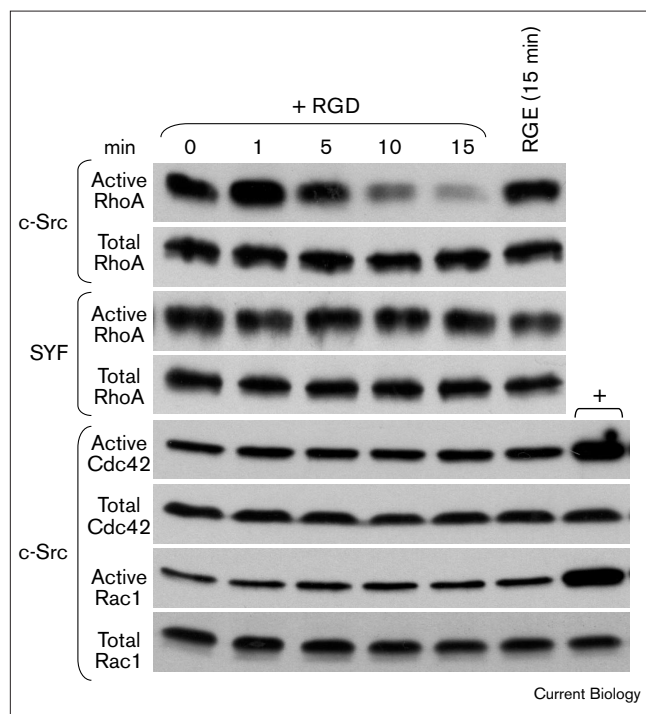
Integrin engagement inhibits the activity of RhoA, but not Cdc42 or Rac1, in a c-Src-dependent manner. c-Src-expressing cells (c-Src) or SYF cells (SYF) were suspended for 2 h in serum-free media. Integrins were engaged by the addition of GRGDSP peptides (RGD) (Life Technologies) to 250 µg/ml for 0, 1, 5, 10 or 15 min or treated with GRGESP (RGE) for 15 min (a control for nonspecific binding). Cells were also plated on 50 µg/ml fibronectin for 30 min or treated with 100 ng/ml bradykinin for 7 min as positive controls (+) for Rac1 and Cdc42 activity, respectively. Active GTP-bound RhoA, Cdc42 and Rac1 were precipitated from cell lysates as described previously [9,19]. Precipitated proteins (active) and cell lysates (total) were immunoblotted with monoclonal antibodies (mAb) recognizing RhoA, Cdc42 or Rac1 (all antibodies were purchased from Transductions Laboratories, unless otherwise noted).

activity by p190RhoGAP immunoprecipitates from c-Src-expressing cells (Figure 2a). In contrast, RGD failed to significantly ($p > 0.4$) alter the activity of p190RhoGAP recovered from SYF cells. These findings suggest that integrin engagement stimulates p190RhoGAP activity through a mechanism that requires c-Src.

Although the regulation of p190RhoGAP is not well understood, its tyrosine phosphorylation and specific localization correlate with its presumed activation. p190RhoGAP is thought to mediate stress fiber disassembly in response to stimulation with epidermal growth factor or expression of v-Src or constitutively active c-Src [15,21,22]. This disassembly is accompanied by p190RhoGAP tyrosine phosphorylation ([21,22] and V.J. Fincham, A. Chudleigh, M.C. Frame, personal communication) and localization to sites lacking stress fibers [22]. Nakahara and colleagues demonstrated that, like growth factor receptors, integrins also stimulate tyrosine phosphorylation of p190RhoGAP and its localization to membrane protrusions devoid of stress fibers [23]. A reasonable explanation for the requirement of c-Src in p190RhoGAP activation by integrins is that c-Src or a c-Src-activated tyrosine kinase phosphorylates, and thereby activates, p190RhoGAP. To test this hypothesis, we examined levels of p190RhoGAP tyrosine phosphorylation in response to integrin engagement. We found that treatment with RGD resulted in increased tyrosine phosphorylation of p190RhoGAP in c-Src-expressing cells but not in SYF cells (Figure 2b). These data, along with our GAP assay results, show that p190RhoGAP activation in response to integrin engagement correlates with its c-Src-dependent tyrosine phosphorylation.

To further define the role of p190RhoGAP in RhoA inhibition by integrins, we expressed a GAP-deficient p190RhoGAP mutant (p190RhoGAP^{R1283A}) tagged with the hemagglutinin (HA) epitope [24] in c-Src expressing cells. We found that expression of this mutant perturbed RhoA inactivation in response to RGD stimulation (Figure 3). These data indicate that p190RhoGAP^{R1283A}–HA interferes with the function of the endogenous protein, and that p190RhoGAP is necessary for RhoA inhibition in response to early integrin signaling.

In this study, we investigated the regulation of Rho family proteins by integrin signaling events that precede integrin clustering and focal adhesion formation. Using RGD-containing peptides that mimic the integrin-binding region of fibronectin and vitronectin, we found that integrin engagement initially inhibited the activity of RhoA through a c-Src-dependent mechanism. Integrin engagement stimulated c-Src-dependent tyrosine phosphorylation and activation of p190RhoGAP. In addition, RGD-induced inhibition of RhoA was antagonized by expression of a GAP-deficient p190RhoGAP mutant. We propose a signaling pathway in which, following engagement, integrins

Cdc42 or Rac1 and that effectors of these GTPases inhibit RhoA. To test this hypothesis, we measured the levels of active Cdc42 and Rac1 [19] in c-Src-expressing cells treated with RGD. We found that, in contrast to its effect on RhoA activity, RGD treatment did not alter the activity of either Cdc42 or Rac1 over a similar time course (Figure 1). These data suggest that neither Cdc42 nor Rac1 have a role in integrin-mediated inhibition of RhoA.

A second pathway by which c-Src-dependent integrin signaling could inhibit RhoA is through activation of p190RhoGAP [1]. *In vivo*, this GAP (GTPase activating protein) potentiates the intrinsic GTPase activity of RhoA rendering it inactive [20]. To explore the role of p190RhoGAP in signaling by early integrin engagement, we first measured its activity using an *in vitro* GAP assay [15]. Relative to untreated cells, RGD stimulated a significant ($p < 0.003$) increase in RhoA-directed GAP

Figure 2

Integrin engagement stimulates c-Src-dependent activation and tyrosine phosphorylation of p190RhoGAP.
(a) p190RhoGAP activity in response to integrin engagement. p190RhoGAP was immunoprecipitated (IP) with p190RhoGAP mAb (Upstate Biotechnology) from lysates of c-Src-expressing cells (c-Src) or SYF cells (SYF) untreated (−) or stimulated with RGD for 15 min (+). Lysates were incubated with beads alone as a negative control (mock). Samples were subjected to a GTPase activity assay as described previously [15] or immunoblotted (IB) for p190RhoGAP. GTPase activity is reported as the mean percentage increase of $[\gamma^{32}\text{-P}]$GTP hydrolyzed by GST–RhoA in the presence of p190RhoGAP IPs relative to mock IPs. Data were considered statistically significant if the p-value was less than 0.05 as determined by a two-tailed t-test. **(b)** Tyrosine phosphorylation of p190RhoGAP in
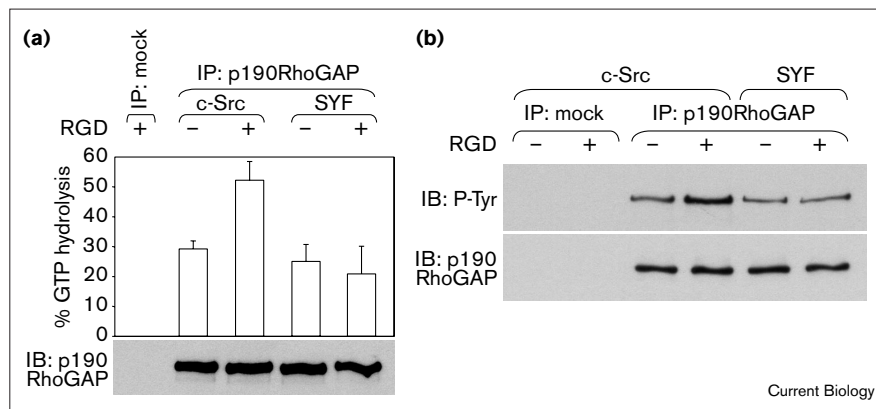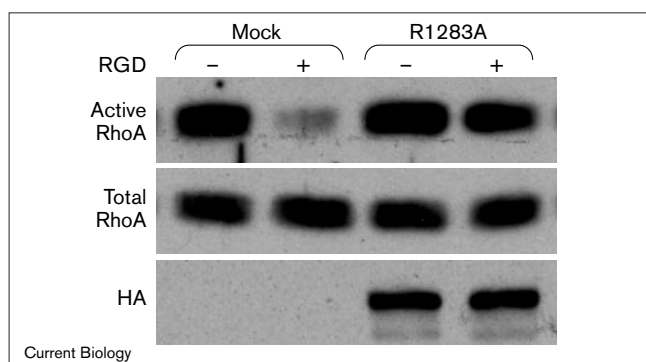
response to integrin engagement. c-Src-expressing cells or SYF cells were untreated (−) or stimulated for 15 min with RGD (+). p190RhoGAP was immunoprecipitated from cell lysates.

Separately, lysates were incubated with beads alone as a control for nonspecific binding (mock). IPs were divided and immunoblotted for phosphotyrosine (P-Tyr) with mAb PY20 or for p190RhoGAP.

act through c-Src to stimulate tyrosine phosphorylation and activation of p190RhoGAP. Activated p190RhoGAP then increases RhoA GTPase activity, rendering RhoA inactive by converting it to a GDP-bound state.

An initial localized suppression of RhoA activity by integrin engagement may be a critical step that allows cells to spread or migrate. RhoA-mediated contractile forces might otherwise impede protrusion, or collapse existing protrusive structures, at the leading edge of migrating cells. In fact, blocking RhoA signaling has been shown to promote protrusions in the form of membrane ruffles [25].

Figure 3

Expression of GAP-deficient p190RhoGAP antagonizes RhoA inactivation in response to integrin engagement. c-Src-expressing cells electrotransfected (~70% efficiency) with 40 µg/ml pEGFP-N3 (mock) (Clontech) or p190RhoGAP^R1283A–HA–pKH3 (R1283A) were untreated (−) or stimulated (+) for 15 min with RGD. Active RhoA was precipitated from cell lysates as in Figure 1. Precipitates were immunoblotted for RhoA (active) and cell lysates were immunoblotted for RhoA (total) or anti-HA mAb 12CA5 (Boehringer Mannheim).

Inhibition of RhoA by integrins via c-Src and p190RhoGAP may also contribute to focal adhesion disassembly at the rear of motile cells. The importance of positive and negative regulation of RhoA is underscored by the finding that both activation and inhibition of RhoA suppress migration [6–8]. These results, together with our findings that integrin engagement inhibits RhoA via c-Src, provide a plausible explanation for the defect in spreading and migration exhibited by cells lacking c-Src or c-Src, Yes and Fyn [11–13]. Future studies aim to determine the signaling events that activate RhoA following its transient inhibition by integrin engagement via c-Src and p190RhoGAP.

Acknowledgements

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