

Localized suppression of RhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration

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RhoA activity is transiently inhibited at the initial phase of integrin engagement, when Cdc42- and/or Rac1-mediated membrane spreading and ruffling predominantly occur. Paxillin, an integrin-assembly protein, has four major tyrosine phosphorylation sites, and the phosphorylation of Tyr31 and Tyr118 correlates with cell adhesion and migration. We found that mutation of Tyr31/118 caused enhanced activation of RhoA and premature formation of stress fibers with substantial loss of efficient membrane spreading and ruffling in adhesion and migration of NMuMG cells. These phenotypes were similar to those induced by RhoA(G14V) in parental cells, and could be abolished by expression of RhoA(T19N), Rac1(G12V), or p190RhoGAP in the mutant-expressing cells. Phosphorylated

Tyr31/118 was found to bind to two src homology (SH)2 domains of p120RasGAP, with coprecipitation of endogenous paxillin with p120RasGAP. p190RhoGAP is known to be a major intracellular binding partner for the p120RasGAP SH2 domains. We found that Tyr31/118-phosphorylated paxillin competes with p190RhoGAP for binding to p120RasGAP, and provides evidence that p190RhoGAP freed from p120RasGAP efficiently suppresses RhoA activity during cell adhesion. We conclude that Tyr31/118-phosphorylated paxillin serves as a template for the localized suppression of RhoA activity and is necessary for efficient membrane spreading and ruffling in adhesion and migration of NMuMG cells.

Introduction

The Rho-family GTPases, RhoA, Rac1, and Cdc42, play essential roles in the formation and remodeling of intracellular actin-based structures (for review see Hall, 1998). These Rho-family GTPases can be activated by integrin signaling (Barry et al., 1997; Clark et al., 1998; Price et al., 1998; Ren and Schwartz, 1998; del Pozo et al., 2000; Cox et al., 2001). RhoA activity is primarily responsible for the formation of actin stress fibers (Ridley et al., 1992), which potentially exert contractile force and are necessary for the maintenance of sufficient adhesion to substrates. However, RhoA activity is transiently down-regulated at the initial phase of integrin activation during cell adhesion to the extracellular matrix (Ren et al., 1999; Arthur et al., 2000), when Cdc42- and/or Rac1-mediated active membrane spreading and ruffling predominantly occur (Price et al., 1998; Arthur and Burridge, 2001; Cox et al., 2001). Thus, the precise regulation of

RhoA activity, both temporally and spatially within a single cell, probably in concert with activities of other small GTPases, such as Rac1 and Cdc42, is crucial for efficient cell adhesion and migration. Although factors regulating RhoA activity have been well documented, molecular mechanisms for such localized regulation of RhoA activity still remain largely unknown.

Paxillin, an integrin assembly adaptor protein (for review see Turner, 2000), is recruited to the leading cell edges promptly upon the initiation of migration (Nakamura et al., 2000; Laukaitis et al., 2001). Paxillin has four major tyrosine phosphorylation sites—Tyr31, Tyr40, Tyr118, and Tyr181—and phosphorylation of Tyr31 and Tyr118 is highly augmented during cell adhesion and migration, and present at the leading cell edges of migrating NMuMG normal epithelial cells (Nakamura et al., 2000). We made two different paxillin mutants, 2X and 2Y, in which Tyr31/118 and Tyr40/181 were mutated to phenylalanine, respectively. Their expression caused altered F-actin organization and loss of cell polarity, respectively, in motile NMuMG cells, indicating the possible crosstalk between downstream events of paxillin tyrosine phosphorylation and intracellular regulatory

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mechanism for the activities of Rho-family GTPases (Nakamura et al., 2000). Several proteins bearing the src homology (SH)*2 domain, including Crk-II and Csk, have been shown to bind to tyrosine phosphorylated paxillin (for review see Turner, 1998). In Nara Bladder Tumor-II cells, Crk-II has been shown to function downstream of Tyr31/118 phosphorylation to promote cell migration (Petit et al., 2000). On the other hand, Crk-II is unlikely to act as a downstream factor of paxillin in the case of migration of NMuMG cells, where Crk-II tightly binds to tyrosine phosphorylated p130Cas (Yano et al., 2000). We have also shown that paxillin and p130Cas, through their tyrosine phosphorylation, can potentially exert opposing effects on the haptotactic cell migration and transcellular invasion activities of several types of cells, such as NMuMG, COS7, and MM-1 cells, when these proteins were overexpressed (Yano et al., 2000). Therefore, the downstream events of tyrosine phosphorylation of paxillin may be cell-type specific, and still remain largely elusive.

Here we report that paxillin phosphorylation at Tyr31/118 is necessary for efficient membrane spreading and ruffling of NMuMG cells during adhesion and migration, by suppressing RhoA activity. We found that mutation of Tyr31/118 of paxillin causes aberrant activation of RhoA and premature formation of actin stress fibers at the cell periphery. We show that Tyr31/118-phosphorylated paxillin binds to the two tandem SH2 domains of p120RasGAP (Trahey et al., 1988). The p120RasGAP SH2 domains have been shown to bind to tyrosine phosphorylated p190RhoGAP, as its major intracellular binding partner (Moran et al., 1991; Settleman et al., 1992b; Bryant et al., 1995; Hu and Settleman, 1997). We show that binding of Tyr31/118-phosphorylated paxillin to p120RasGAP blocked binding of p190RhoGAP to p120RasGAP in NMuMG cells, and propose a mechanism by which Tyr31/118-phosphorylated paxillin serves as a template for the suppression of RhoA activity. We also discuss that both paxillin and p130Cas, via their tyrosine phosphorylation, contribute to efficient membrane spreading and ruffling with the different downstream signalings.

Results

Mutation of Tyr31/118 of paxillin causes aberrant activation of RhoA

In migrating NMuMG cells, membrane ruffles prevail at the leading edges, whereas stress fibers are formed at least several μm away from the cell periphery (Nakamura et al., 2000; Fig. 1 B, a–c). We previously showed that expression of the 2X mutant of paxillin in migrating NMuMG cells causes premature formation of stress fibers at the leading cell edges with the concomitant loss of efficient membrane ruffles (Fig. 1 B, d–f), whereas expression of the 2Y mutant instead causes apparent loss of cell polarity in migration (Nakamura et al., 2000). We have also shown that the 2X mutant induces aberrant formation of robust focal complexes at the leading edges, which are apparently connected to stress fi-

bers, though the overall amounts of stress fibers were almost unchanged compared to those in the parental cells (Fig. 1 B). Both 2X mutant and endogenous paxillin localizes to the same aberrant focal complexes (Nakamura et al., 2000). Immunoblotting using phosphorylation site-specific antibodies revealed that the Tyr31/118 phosphorylation of endogenous paxillin was significantly reduced in the 2X cells (Fig. 1 A).

Because the actin cytoskeletons was altered by expression of the 2X mutant, we expressed mutants of Rho-family GTPases in parental NMuMG cells and examined cellular phenotypes after inducing cells to migrate by scratching the confluent culture. Similar to those induced by the 2X mutant, expression of RhoA(G14V) induced premature formation of robust stress fibers and focal adhesions at the leading edges, which accompanied substantial loss of lamellipodia structures (Fig. 1 C, a–d). The constitutive active forms of Rac1 and Cdc42 did not induce such phenotypes (Fig. 1 C, e–l). To verify the involvement of integrin signaling, cells were also examined at an early stage of adhesion onto collagen. Unlike wild-type paxillin-expressing NMuMG cells (Fig. 1 D, a–c), cells expressing the 2X mutant (Fig. 1 D, d–f) or RhoA(G14V) (Fig. 1 D, g–j) were again found to exhibit premature formation of stress fibers and formation of robust aberrant focal complexes at the cell periphery, and did not spread well. We then found that expression of RhoA(T19N) in the 2X cells could restore the phenotypes to almost normal: these cells exhibited efficient membrane ruffles and do not form stress fibers directly at the leading cell edges (Fig. 1 E, a–d). Expression of Rac1(G12V) also caused similar restoration (Fig. 1 E, e–h). These restorations were also confirmed at an early phase of cell adhesion (unpublished data). On the other hand, the GTP binding-deficient forms of Rac1 and Cdc42 did not cause such restorations: 2X cells expressing these mutants exhibited complicated phenotypes of poorly formed lamellipodia and/or disorganized stress fibers (Fig. 1 E, i–p). Expression levels of each of the exogenous GTPases were confirmed to be similar by immunoblotting for the tag sequence (unpublished data). We then measured RhoA activity and found that it became higher in the 2X cells as compared to those in control cells expressing wild-type enhanced GFP (EGFP)-paxillin during adhesion to collagen (Fig. 1 F). Therefore, normal regulation of Tyr31/118 phosphorylation of paxillin appears to be necessary for efficient membrane spreading and ruffling, and participates in the suppression of RhoA activity in cell adhesion, and probably also in cell migration.

Identification of proteins binding to phosphorylated Tyr31/118 peptides

Several SH2-containing proteins have been shown to bind to tyrosine phosphorylated paxillin, as mentioned earlier. However, none of them seemed to interpret the mechanism for the possible suppression of RhoA activity. Using synthetic peptides, we reexamined the proteins that bind to tyrosine phosphorylated paxillin (Fig. 2). Biotinylated peptides, each corresponding to the four major tyrosine phosphorylation sites of paxillin, were incubated with NMuMG cell lysates, and proteins bound to the peptides were precipitated using streptavidin-Sepharose beads. The

*Abbreviations used in this paper: EGFP, enhanced GFP; SH, src homology.

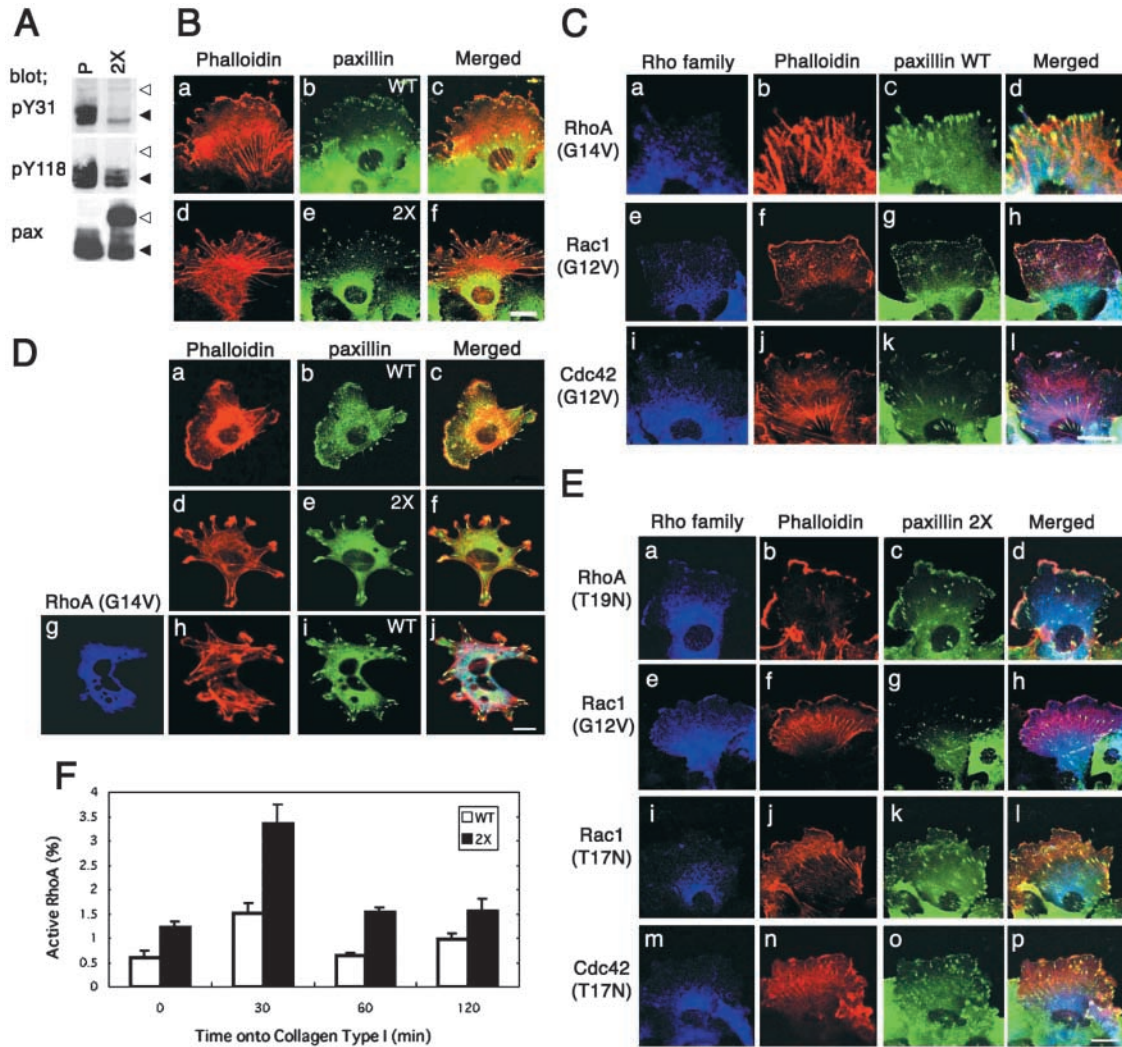


Figure 1. The 2X mutant of paxillin induces phenotypes similar to those by RhoA(G14V) in NMuMG cells. (A) Phosphorylation of Tyr31 and Tyr118 of endogenous paxillin (\blacktriangle) and the EGFP-tagged 2X mutant (\triangle) in parental NMuMG cells (P) or the 2X-expressing cells (2X) were visualized using the phosphorylation site-specific antibodies (pY 31 and pY118). Paxillin was detected using an anti-paxillin antibody (pax). (B–E) Formation of stress fibers and membrane ruffles in migration (B, C, and E) or adhesion onto collagen type I (D). NMuMG cells expressing EGFP-tagged wild-type paxillin or the 2X mutant, or HA-tagged RhoA, Rac1, or Cdc42 mutants are shown, as indicated. F-actin was visualized by phalloidin, EGFP-tagged paxillin by autofluorescence of the tag, and Rho GTPases by an anti-HA antibody. Bars, 20 μ m. (F) NMuMG cells expressing EGFP-tagged wild-type paxillin (WT) or the 2X mutant (2X) were replated onto collagen coated dishes in the presence of 1% serum, and cell lysates prepared from each time point were incubated with GST-Rhotekin-RBD to measure the activities of RhoA. RhoA activity is indicated as percentages of the amount of RBD-bound RhoA normalized to the amount of RhoA in total cell lysates. Results are means \pm SEM from three independent experiments.

SH2 domains of Crk-II, Crk-L, and Csk have been shown to bind to tyrosine phosphorylated paxillin. Immunoblotting revealed that Crk-II, together with Crk-I and Crk-L, efficiently and specifically bind to the Tyr31 and Tyr118 peptides in their phosphorylated form. Csk was also recovered efficiently by the Tyr118 phosphopeptide, but not by other phosphopeptides or nonphosphorylated peptides. We extended the identification by using antibodies against other SH2-containing signaling molecules. We found that p120RasGAP was efficiently precipitated by the Tyr31 and Tyr118 phosphopeptides, but not by other peptides (Fig. 2). Marginal levels of binding were also detected with several proteins, such as Nck and the p85 α subunit of phosphatidylinositol-3-kinase, and binding was almost undetectable

with c-Src, Vav-2, Shc, and Shp-2 (Fig. 2; unpublished data). In these assays, relative binding affinity of each protein was assessed by comparing amounts of proteins present in total cell lysates to those precipitated.

Tyr31/118-phosphorylated paxillin binds to p120RasGAP via the two tandem SH2 domains

We then examined whether binding of paxillin and p120RasGAP occurs intracellularly with endogenous proteins. As shown in Fig. 3 A, immunoprecipitation of paxillin from NMuMG cells using an anti-paxillin antibody coprecipitated p120RasGAP. Consistent with the above result of the peptide binding assay, the amount of p120RasGAP coprecipitating with paxillin became much higher when cells

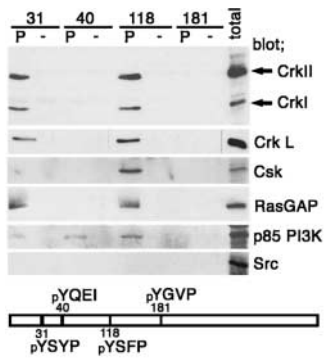


Figure 2. Protein binding to specific paxillin peptides. 500 μ g of TGF β -treated NMuMG cell lysate was incubated with 250 pmol of the phosphorylated (P) and unphosphorylated (–) forms of paxillin peptides immobilized on streptavidin-Sepharose beads, in a total volume of 500 μ l for 1 h at 4°C. Proteins precipitated were analyzed by immunoblotting using antibodies as indicated. The numbers indicate the peptides corresponding to the tyrosine phosphorylation sites of paxillin. 10 μ g of total cell lysate was included as controls (Total).

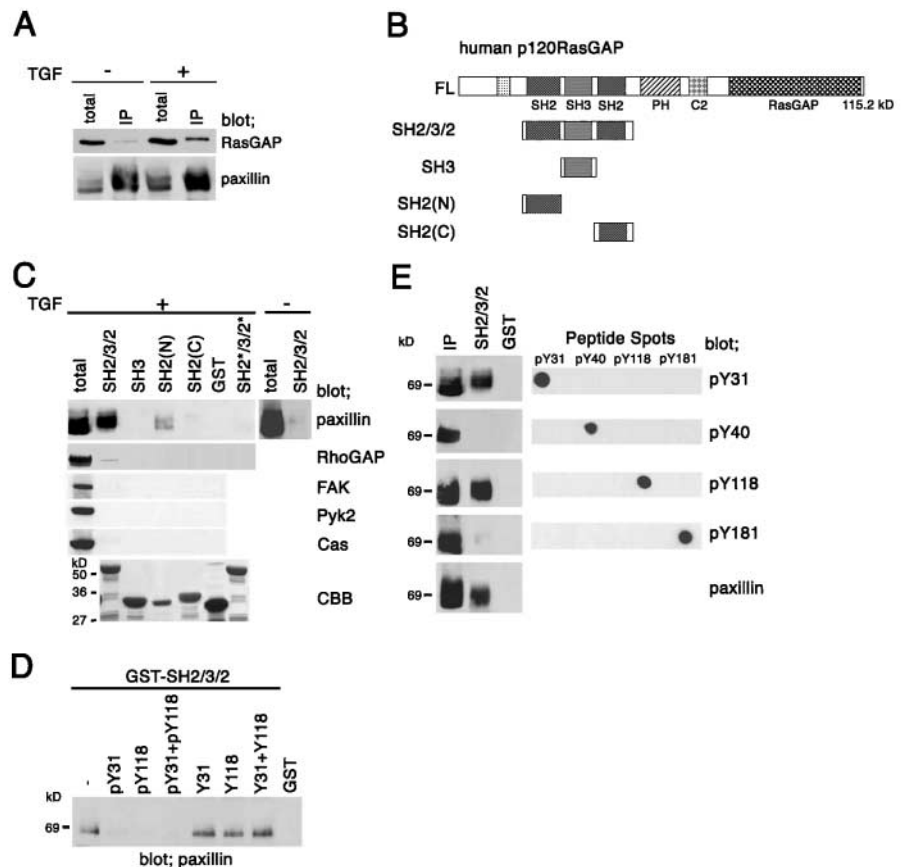
were pretreated with TGF β , in which paxillin phosphorylation at Tyr31/118, but not Tyr40/181, become highly augmented (Nakamura et al., 2000). Several anti-p120RasGAP antibodies have been shown to coprecipitate p120RasGAP-binding proteins, such as p190RhoGAP (Ellis et al., 1990;

Moran et al., 1991; Settleman et al., 1992b). However, none of these antibodies, including clone B4F8 (Settleman et al., 1992a), could coprecipitate detectable amounts of paxillin from NMuMG cell lysates, while coprecipitation of p190RhoGAP could be detected (see Fig. 5).

p120RasGAP has two tandem SH2 domains, spaced by a single SH3 domain (Trahey et al., 1988; Fig. 3 B). It has been reported that the two SH2 domains of p120RasGAP synergistically participate in binding to two sites of tyrosine phosphorylation of p190RhoGAP, Tyr1087, and Tyr1105, whereas each single SH2 domain of p120RasGAP exhibits only weak binding to p190RhoGAP (Bryant et al., 1995; Hu and Settleman, 1997). However, it should be noted that others have suggested that the single major phosphorylation at Tyr1105 is sufficient for binding (Roof et al., 1998). In the case of paxillin binding to p120RasGAP, phosphorylated Tyr31 and Tyr118 of paxillin, and the two SH2 domains of p120RasGAP appeared to be necessary. We found that the glutathione *S*-transferase (GST) fusion form of the SH2-SH3-SH2 domain of p120RasGAP pulled down paxillin from lysates of NMuMG cells, where again much higher amounts were pulled down from TGF β -treated cells than untreated cells (Fig. 3 C). On the other hand, the GST fusion protein of either the NH₂-terminal SH2 domain or the COOH-terminal domain alone showed very weak or almost undetectable binding (Fig. 3 C). Loss of function mutations in the SH2 domains abolished its binding to paxillin, and

Figure 3. Tyr31/118-phosphorylated paxillin binds to p120RasGAP, via the two SH2 domains.

(A) TGF β -treated (+) or untreated (–) NMuMG cell lysates were immunoprecipitated with a mouse monoclonal anti-paxillin antibody coupled with anti-mouse IgG-Sepharose beads, and subjected to immunoblotting, as indicated. (B and C) TGF β -treated (+) or untreated (–) NMuMG cell lysates were incubated with GST fusion proteins, each corresponding to various parts of the SH2-SH3-SH2 domain of p120RasGAP (B), and proteins bound to the beads were analyzed by immunoblotting, as indicated (C). Asterisks indicate mutations in the SH2 domains. Coomassie blue staining of the GST proteins used are also shown (CBB). (D) The GST-SH2-SH3-SH2 protein was preincubated with 100 μ M each of the phosphorylated or unphosphorylated paxillin peptides (or combinations of peptides), as indicated, before being incubated with TGF β -treated NMuMG cell lysate, and bound proteins were subjected to anti-paxillin blotting. (E) TGF β -treated NMuMG cell lysates were incubated with a mouse monoclonal anti-paxillin antibody (IP) or the GST-SH2-SH3-SH2 protein, and bound proteins were analyzed using phosphorylation site-specific antibodies for paxillin (pY31, pY40, pY118 and pY181) or an anti-paxillin antibody, as indicated. 20 pmol each of the paxillin peptides used in Fig. 2 was spotted onto filters and used as controls (right). Total cell lysate (Total) or cell lysate incubated with GST were included as controls, and the molecular sizes in kD are shown, where necessary.



(A) TGF β -treated (+) or untreated (–) NMuMG cell lysates were immunoprecipitated with a mouse monoclonal anti-paxillin antibody coupled with anti-mouse IgG-Sepharose beads, and subjected to immunoblotting, as indicated. (B and C) TGF β -treated (+) or untreated (–) NMuMG cell lysates were incubated with GST fusion proteins, each corresponding to various parts of the SH2-SH3-SH2 domain of p120RasGAP (B), and proteins bound to the beads were analyzed by immunoblotting, as indicated (C). Asterisks indicate mutations in the SH2 domains. Coomassie blue staining of the GST proteins used are also shown (CBB). (D) The GST-SH2-SH3-SH2 protein was preincubated with 100 μ M each of the phosphorylated or unphosphorylated paxillin peptides (or combinations of peptides), as indicated, before being incubated with TGF β -treated NMuMG cell lysate, and bound proteins were subjected to anti-paxillin blotting. (E) TGF β -treated NMuMG cell lysates were incubated with a mouse monoclonal anti-paxillin antibody (IP) or the GST-SH2-SH3-SH2 protein, and bound proteins were analyzed using phosphorylation site-specific antibodies for paxillin (pY31, pY40, pY118 and pY181) or an anti-paxillin antibody, as indicated. 20 pmol each of the paxillin peptides used in Fig. 2 was spotted onto filters and used as controls (right). Total cell lysate (Total) or cell lysate incubated with GST were included as controls, and the molecular sizes in kD are shown, where necessary.

the SH3 domain alone did not precipitate paxillin at detectable levels (Fig. 3 C). However, it should be noted that only a marginal level of p190RhoGAP was detected to be bound to the SH2-SH3-SH2 protein (Fig. 3 C), as reported previously (Ellis et al., 1991). In several previous reports, purified recombinant proteins at high concentrations or proteins intracellularly overexpressed by cDNA transfection were used to show binding of p190RhoGAP to the GST-SH2-SH3-SH2 domain (Bryant et al., 1995; Hu and Settleman, 1997). p130Cas, Pyk2, and Fak, all highly tyrosine phosphorylated in NMuMG cells, were not detected to bind to the SH2-SH3-SH2 domain (Fig. 3 C).

Phosphorylated Tyr31 and Tyr118 peptides, either alone or in combination, but not the nonphosphorylated peptides, blocked the binding of the GST-SH2-SH3-SH2 to tyrosine phosphorylated paxillin (Fig. 3 D). Moreover, blotting of paxillin with phosphorylation site-specific antibodies also supported the notion that Tyr31- and Tyr118-phosphorylated paxillin, but not the Tyr40- or Tyr181-phosphorylated forms, binds to the p120RasGAP SH2-SH3-SH2 domain (Fig. 3 E).

p120RasGAP colocalizes with Tyr31/118-phosphorylated paxillin at the cell periphery but not at focal adhesions

We then examined the subcellular colocalization of paxillin and p120RasGAP. Tyr31/118-phosphorylated paxillin localizes both to the leading cell edges and focal adhesions in migrating NMuMG cells (Nakamura et al., 2000). p120RasGAP molecules, tagged with HA, were also found to localize to the leading cell edges in migrating NMuMG cells, where it was well colocalized with Tyr31/118-phosphorylated paxillin (Fig. 4 A; unpublished data). On the other hand, p120RasGAP did not accumulate at the focal adhesions and was diffusely distributed throughout the cytoplasm (Fig. 4 A). Absence of p120RasGAP accumulation at focal adhesions was also confirmed using p120RasGAP tagged with EGFP, by detecting autofluorescence from the tag (unpublished data). On the other hand, in the migrating 2X cells, p120RasGAP could no longer be detected clearly at the cell periphery or at the aberrant focal complexes formed at the cell periphery (Fig. 4 B). Similar levels of expression of HA-p120RasGAP in these cells were confirmed (Fig. 4 C).

Because p120RasGAP binds to p190RhoGAP, the subcellular localization of p190RhoGAP was also investigated. Like p120RasGAP, this protein was also found at the cell periphery but not accumulated at focal adhesions in migrating NMuMG cells (Fig. 4 A). Again, expression of the 2X mutant abolished the peripheral localization of p190RhoGAP (Fig. 4 B).

Blockage of p190RhoGAP binding to p120RasGAP by Tyr31/118-phosphorylated paxillin

We then examined whether binding of Tyr31/118-phosphorylated paxillin to p120RasGAP affects intracellular binding of p190RhoGAP to p120RasGAP. For this, we first confirmed that the Tyr31- and Tyr118-phosphorylated peptides have the ability to block the binding of p190RhoGAP to the GST-SH2-SH3-SH2 protein of p120RasGAP in vitro (Fig. 5 A). To examine the possible competition

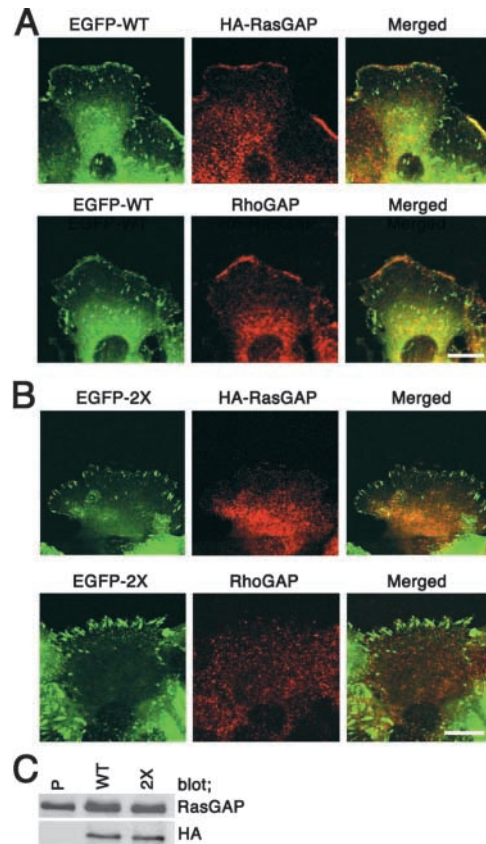


Figure 4. Colocalization of paxillin, p120RasGAP, and p190RhoGAP at the cell periphery, but not at focal adhesions. HA-p120RasGAP was stably expressed at a low level in NMuMG cells expressing EGFP-tagged wild-type paxillin (A) or the 2X mutant (B), and cells were analyzed during migration. EGFP-paxillin was visualized by autofluorescence from the tag. HA-p120RasGAP and endogenous p190RhoGAP were visualized using an anti-HA antibody and an anti-p190RhoGAP antibody, respectively, coupled with Cy5-conjugated anti-mouse IgG. Each right panel is the merged image of the left and middle panels. Bars, 20 μ m. Levels of HA-tagged and endogenous p120RasGAP expression in these cells (WT and 2X) are shown by immunoblotting, as indicated (C). Parental NMuMG cells were also included (P).

between paxillin and p190RhoGAP toward binding to p120RasGAP, transient protein overexpression in COS7 cells was used. We found that overexpression of EGFP-p190RhoGAP significantly reduced the amounts of p120RasGAP coprecipitating with an anti-paxillin antibody, with a concomitant increase in the amounts of p190RhoGAP and its EGFP fusion protein coprecipitating with an anti-p120RasGAP antibody (Fig. 5 B). Overexpression of wild type EGFP-paxillin was similarly found to decrease the amount of RhoGAP coprecipitating with an anti-RasGAP antibody (Fig. 5 C). The transient overexpression method was not applicable for NMuMG cells, as NMuMG cells overexpressing p190RhoGAP at high levels could not adequately adhere to the extracellular matrices and tyrosine phosphorylation of paxillin in these cells became much reduced (Fig. 6; unpublished data). Hence, we addressed this issue with NMuMG cells stably expressing EGFP-tagged wild type paxillin or the 2X mutant. In this regard, it should be noted that Tyr31/118-phosphorylation of endogenous

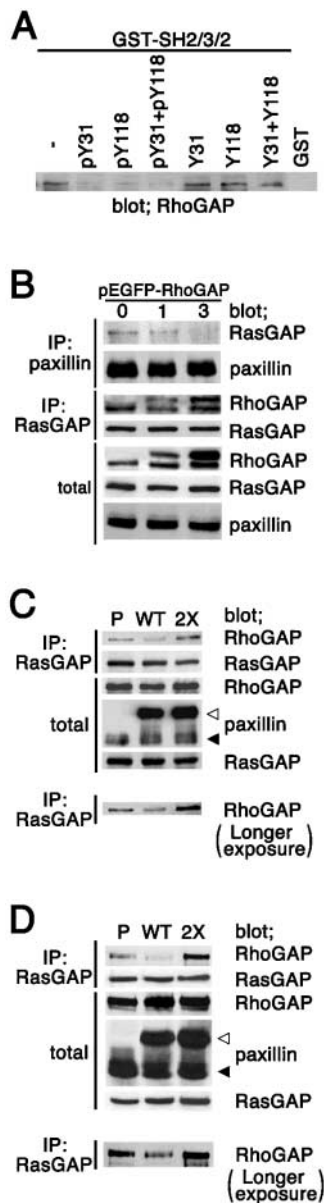


Figure 5. Competition of Tyr31/118-phosphorylated paxillin with p190RhoGAP for binding to p120RasGAP. (A) 3Y1/*v*-Src cell lysates were incubated with the p120RasGAP GST-SH2-SH3-SH2 protein, preincubated with a paxillin peptide (or combinations of peptides) as in Fig. 3 E. The control included the GST fusion protein without incubation with peptides (–). Proteins precipitated were analyzed by anti-p190RhoGAP immunoblotting. (B and C) COS7 cells were transiently transfected with 0, 1, or 3 μ g of pEGFP-RhoGAP, as indicated (B), or transfected with 3 μ g of pBabe/EGFP-paxillin (WT) or 3 μ g of pBabe/EGFP-paxillin 2X (2X), as indicate (C). Mock-transfected cells were also included (C, P). 24 h later, cells were detached from culture dishes by incubation with PBS containing 5 mM EDTA, washed, and replated onto collagen type I-coated dishes in the presence of 0.5% BSA for 1 h. Cells were then lysed, and protein coprecipitation was analyzed using an anti-paxillin antibody coupled with anti-mouse IgG-Sepharose beads (B), or an anti-RasGAP antibody (clone B4F8) coupled with protein A-Sepharose beads (C). Protein immunoblotting was performed as indicated, with immunoprecipitated proteins (IP) and total cell lysates (Total). (D) Amounts of p190RhoGAP coprecipitating with p120RasGAP using antibody B4F8 were analyzed, as above, with cell lysates from parental NMuMG cells (P), or NMuMG cells expressing EGFP-paxillin (WT) or cells expressing the 2X mutant (2X), all pretreated with TGF β .

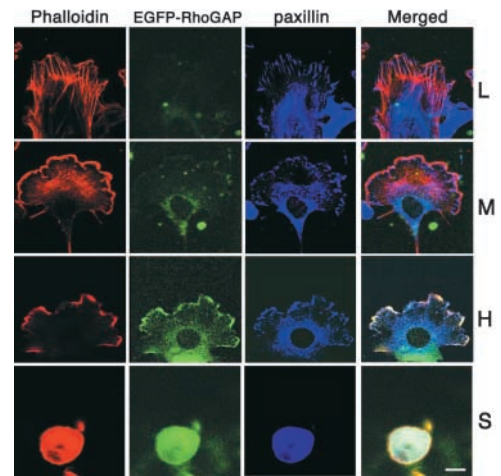


Figure 6. p190RhoGAP overexpression can restore the 2X-induced phenotypes. Confluent cultures of NMuMG cells expressing the 2X mutant were scratched. Cells at the wound edges were microinjected with the pEGFP-p190RhoGAP plasmid, and incubated for 12 h before fixation. Cloned cells stably expressing the paxillin 2X mutant not tagged with EGFP were used, in which expression of the 2X mutant was confirmed using an anti-paxillin and phosphorylation site-specific antibodies as in Fig. 1 A. Mutant and endogenous paxillin were visualized using an anti-paxillin antibody coupled with Cy5-conjugated anti-mouse IgG. F-actin and EGFP-p190RhoGAP were visualized using phalloidin and by the autofluorescence from the tag, respectively. The right panel is the merged image of the left three panels. Cells were classified into four groups, based on the levels of EGFP-RhoGAP expression, which was measured optically from the autofluorescence from the tag. Levels of EGFP-RhoGAP expression were also assessed by staining cells with an anti-RhoGAP antibody and compared with those of endogenous p190RhoGAP in untransfected cells. Levels of EGFP-RhoGAP in each cell of the low level expressors (L) correspond to about the same as that of endogenous p190RhoGAP, the medium (M) correspond to approximately two- to threefold higher, the high (H) correspond to approximately three- to fivefold higher, and super high (S) correspond to more than fivefold higher. Representative examples are shown from more than 100 cells examined. Bar, 20 μ m.

paxillin is greatly reduced in the 2X cells (Fig. 1 A). Compared with the parental cells, we found that the amount of p190RhoGAP coprecipitating with the anti-p120RasGAP antibody was notably reduced in cells expressing wild-type EGFP-paxillin, and was significantly increased in the 2X expressing cells (Fig. 5 D). The expression levels of wild-type EGFP-paxillin and the 2X mutant were very similar in these cells (Fig. 5 D). A similar effect was also seen when the 2X mutant was overexpressed in COS7 cells (Fig. 5 C). These results collectively support a notion that Tyr31/118-phosphorylated paxillin and p190RhoGAP act competitively in their binding to p120RasGAP, and changes in Tyr31/118 phosphorylation affects the intracellular amounts of p190RhoGAP in complex with p120RasGAP.

(C and D) To show the difference in the amounts of p190RhoGAP coprecipitating with p120RasGAP, a longer exposure of the immunoblots shown on the top of each panel, is also shown at the bottom. Exposure time was 15 s for the top, and 2 min for the bottom. (B–D) Total cell lysates were included as controls (Total). Molecular sizes are shown on the left. \blacktriangle , endogenous paxillin; \triangle , EGFP-tagged paxillin.

Suppression of the 2X phenotypes by p190RhoGAP

We next examined whether p190RhoGAP can suppress the phenotypes induced by expression of the 2X mutant. NMuMG cells stably expressing the 2X mutant were subjected to cloning, and cells with a clonal origin were microinjected with the EGFP-p190RhoGAP plasmid, and their cytoskeletal organization during migration were analyzed. As shown in Fig. 6, overexpression of EGFP-p190RhoGAP was able to suppress the 2X-induced phenotypes and restored efficient membrane ruffles and normal formation of stress fibers (Fig. 6 M). However, such restoration seemed to require the expression of appropriate levels of p190RhoGAP. In the 2X cells expressing EGFP-p190RhoGAP at relatively high levels, stress fiber formation was substantially blocked, and cell adhesion was hampered (Fig. 6, H and S). On the other hand, when EGFP-p190RhoGAP expression was too low, the 2X phenotypes were not significantly suppressed (Fig. 6 L). These observations suggest that the ratio between p190RhoGAP and the 2X mutant is important in determining the formation of membrane ruffles and appropriate stress fibers. Unlike p190RhoGAP, overexpression of p120RasGAP could not restore the 2X phenotype (unpublished data).

p120RasGAP is necessary for the efficient activation of RhoA in cell adhesion

The important question that then remained was whether p190RhoGAP not associated with p120RasGAP can efficiently suppress RhoA activity in vivo. To assess this issue, we disrupted p120RasGAP expression by the siRNA technique (Elbashir et al., 2001). We succeeded in the effective and specific inhibition of p120RasGAP protein expression in HeLa cells, whereas the intracellular amount of p190RhoGAP expression was unaffected (Fig. 7 A). Expression of p130Cas was also substantially blocked by treating cells with its specific siRNA, and this was used as a control, together with oligonucleotides with an irrelevant sequence (Fig. 7 A). We found that the activation of RhoA is significantly hampered during cell adhesion, when p120RasGAP expression is inhibited (Fig. 7 B). We also confirmed that overexpression of EGFP-p190RhoGAP in HeLa cells blocked the activation of RhoA in adhesion (Fig. 7 B). In cells in which p120RasGAP expression was inhibited, stress fibers were not formed and cortical actin structures of membrane ruffles were significantly augmented, similar to that seen in the EGFP-p190RhoGAP overexpressing cells (Arthur and Burridge, 2001; Fig. 7 C). Therefore, the presence of p120RasGAP at a normal level seems to be necessary for the efficient activation of RhoA in adhesion of HeLa cells.

Discussion

We show here that paxillin, through its Tyr31/118 phosphorylation, participates in suppression of RhoA activity in cell adhesion and migration in NMuMG cells. We have shown previously that tyrosine phosphorylation of paxillin and p130Cas is the prominent event induced upon integrin engagement in NMuMG cells (Nakamura et al., 2001). We have also demonstrated that overexpression of paxillin and p130Cas exert opposing effects on cell migratory activities,

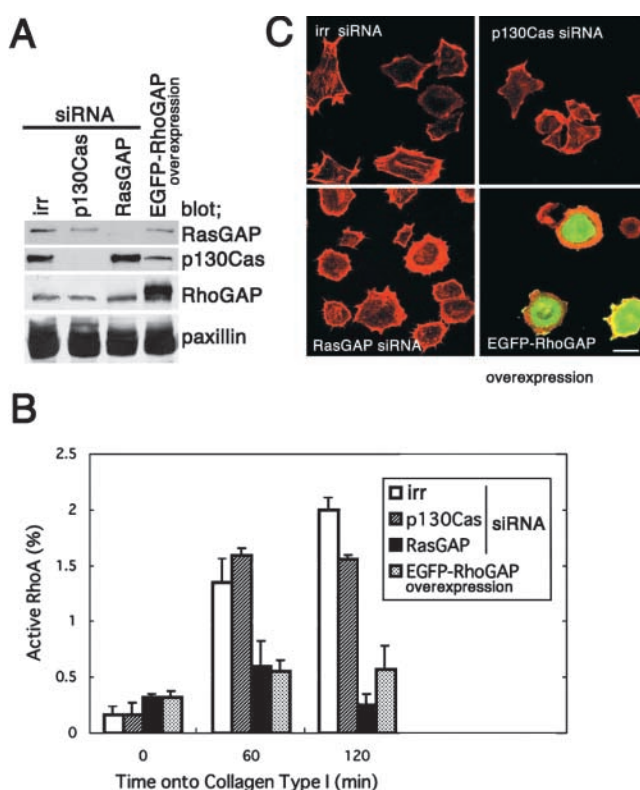


Figure 7. Blockage of p120RasGAP expression hampers efficient activation of RhoA in cell adhesion. HeLa cells were treated with 60 nM oligonucleotides in culture medium, designed for the siRNA-mediated specific disruption of p120RasGAP expression or of p130Cas expression, or transfected with pcDNA/EGFP-p190RhoGAP for its overexpression, as indicated. Oligonucleotides with an irrelevant sequence (irr), were included as a negative control. After 24 h, cells were starved of serum for a further 24 h, detached from culture dishes by incubation with PBS containing 5 mM EDTA, and washed and replated onto collagen type I coated dishes in the presence of 1% serum. (A) Protein expression in each cell preparation is shown, by immunoblotting. (B) RhoA activity was measured at each time point, as in Fig. 1 F. Results are means \pm SEM from three independent experiments. (C) F-actin was visualized in red, in cells pretreated as indicated and fixed 60 min after adhesion. Expression of EGFP-p190RhoGAP, detected by autofluorescence from the tag, was shown in green. Bar, 20 μ m.

through their tyrosine phosphorylation (Yano et al., 2000). Tyrosine phosphorylation of p130Cas has been shown to act to enhance membrane ruffle formation, possibly by binding to Crk proteins (Dolfi et al., 1998; Hashimoto et al., 1998; Girardin and Yaniv, 2001). p130Cas does not bind to p120RasGAP. Therefore, both p130Cas and paxillin appear to contribute to the efficient formation of membrane ruffles in cell adhesion and migration, but by distinct signaling pathways; the former is likely to act by enhancing Rac1 activity (Dolfi et al., 1998; Hashimoto et al., 1998; Girardin and Yaniv, 2001), whereas the latter is likely to act by suppressing RhoA activity (as shown in this paper). The fact that a dysfunction in Tyr31/118 phosphorylation causes substantial loss of membrane spreading and ruffling suggests that appropriate paxillin phosphorylation is essential for these events in NMuMG cells, even in the presence of intact p130Cas molecules.

We showed that paxillin, through its Tyr31/181 phosphorylation, binds to p120RasGAP. It has been shown that the major cellular complex formed by p120RasGAP is that with p190RhoGAP (Moran et al., 1991; Settleman et al., 1992a, 1992b), although about half a dozen of tyrosine phosphorylated proteins have been identified as binding to the p120RasGAP SH2 domains. Among the several different GAP proteins for RhoA, p190RhoGAP has been shown to be the major GAP regulating integrin-mediated RhoA activity, and suppresses RhoA-mediated stress fiber formation (Ridley et al., 1993; Arthur and Burridge, 2001). p190RhoGAP has also been shown to be necessary for efficient membrane ruffle formation and cell migration: suppression of RhoA activity by p190RhoGAP in turn promotes membrane protrusion, ruffling, and spreading (Arthur and Burridge, 2001). We showed that paxillin binds to p120RasGAP in a similar manner to that proposed for p190RhoGAP binding to p120RasGAP, where the two SH2 domains and two sites of tyrosine phosphorylation appear to be required. However, it should be noted that p190RhoGAP can also bind to p120RasGAP independently of the tyrosine phosphorylations (Hu and Settleman, 1997; Roof et al., 1998). However, this tyrosine phosphorylation-independent association appears to comprise only 10–20% of the complexes formed *in vivo* (Roof et al., 1998). Therefore, the binding of paxillin to p120RasGAP and the binding of p190RhoGAP to p120RasGAP may be substantially mutually exclusive, unless these interactions take place at distinct places within a cell. All three proteins were found colocalized at the leading cell edges in migrating NMuMG cells. We also provide several lines of evidence that Tyr31/118 phosphorylation of paxillin is competitive with p190RhoGAP in binding to p120RasGAP, and the phosphorylation status of paxillin at Tyr31/118 affects the intracellular amount of p190RhoGAP complexed with p120RasGAP.

The regulatory mechanism of the GAP activity of p190RhoGAP has not been established. It has been hypothesized from experiments using Rat-2 fibroblasts expressing the NH₂-terminal half of p120RasGAP bearing the SH2-SH3-SH2 domain (GAP-N) that p190RhoGAP complexed with p120RasGAP may have higher and more efficient GAPing activity for Rho *in vivo* than that of the monomeric form of p190RhoGAP (McGlade et al., 1993). In contrast, there is also an opposite belief that the p120RasGAP/p190RhoGAP complex may be a default status for both proteins with regard to their GAP activities *in vivo*: these proteins in complex are soluble, having either the SH2 domains or the tyrosine phosphorylation sites occupied by complex formation, and may therefore be unable to efficiently access each of their membrane bound targets, Ras and Rho (Bryant et al., 1995). A biochemical assay has revealed that p120RasGAP molecules in complex with p190RhoGAP exhibits an approximately fourfold decrease in GAP activity, as compared to that of the monomeric form (Moran et al., 1991). We observed that inhibition of p120RasGAP protein expression in HeLa cells hampers the efficient activation of RhoA upon cell adhesion. This result, as well as the above described results of the expression of the 2X mutant in NMuMG cells, are consistent with the latter hypothesis.

Unfortunately, the siRNA method has so far been applicable only for several cell lines in our laboratory, and we were not able to perform the same experiment using NMuMG cells.

Based on our results and results from the literature as already mentioned, we propose a model as to how Tyr31/118-phosphorylated paxillin participates in the suppression of RhoA activity in cell adhesion and migration. Paxillin is phosphorylated both at Tyr31 and Tyr118 upon integrin activation at the leading cell edges and binds to p120RasGAP. This binding is in competition with p190RhoGAP binding to p120RasGAP, and thus increases the amounts of p190RhoGAP not complexed with p120RasGAP. p190RhoGAP, freed from p120RasGAP, may then efficiently access RhoA molecules at or near the cell periphery and suppress their activity, as a monomeric protein or even by binding to other SH2-containing proteins via the freed phosphorylation sites.

Unlike in HeLa cells, we found that RhoA activity in the p120RasGAP-deficient cells derived from p120RasGAP gene knockout mice (Kulkarni et al., 2000) is not significantly higher than that in these cells back-transfected with full-length p120RasGAP, during adhesion to fibronectin (unpublished data). The p120RasGAP-deficient cells appear to have originated from fibroblasts, as they were established by culturing embryonic tissue after early passage cells underwent crisis, and they respond to PDGF (Kulkarni et al., 2000). It is documented that RhoA activity is relatively high in cultured fibroblasts such as NIH3T3, and activation of Rac by Tiam1 in NIH3T3 cells induces an epithelial-like morphology with functional cadherin-based adhesions (Sander et al., 1999). In cells such as NIH3T3 cells and p120RasGAP-deficient cells, robust stress fibers are formed directly at the cell periphery, as seen in the 2X-expressing NMuMG cells. The p120RasGAP-deficient cells back transfected with p120RasGAP also form robust stress fibers directly at the cell periphery (unpublished data). We have shown that 2X NMuMG cells fail to immediately disassemble their stress fibers and focal adhesions upon cell–cell collision, which occurs quickly upon cell collision in the parental cells to form the cadherin-mediated cell–cell adhesions (Nakamura et al., 2000). We have also found that expression of the 2X mutant in fibroblasts such as 3Y1 cells and NIH3T3 cells, does not further enhance stress fiber formation (Nakamura et al., 2000). Thus, the suppression of RhoA activity, via the possible interaction of p120RasGAP with Tyr31/118-phosphorylated paxillin, appears to be cell-type specific, and does not always function efficiently in cultured fibroblasts, which may have the intrinsic ability to activate RhoA at a high level to form robust stress fibers at the cell periphery (see below).

In addition to the leading cell edges, Tyr31/118-phosphorylated paxillin also accumulates at focal adhesions underneath the cell body in migrating NMuMG cells. Nevertheless, p120RasGAP does not accumulate at focal adhesions in NMuMG cells. Several different proteins bind to the phosphorylated Tyr31/118 of paxillin. Therefore, Tyr31/118-phosphorylated paxillin may bind to proteins other than p120RasGAP at focal adhesions and be engaged in different signaling pathways, which may or may not contribute to the formation of stress fibers. The ability of Tyr31/118-phos-

phorylated paxillin to engage in different downstream signaling pathways may result from the different roles played by the paxillin Tyr31/118 phosphorylation in different cellular contexts, as seen in previous reports (Petit et al., 2000; Yano et al., 2000) and in different cell types such as NMuMG epithelial cells and several fibroblasts as discussed above. Like paxillin, many other integrin-assembly proteins and their phosphorylation events occur both at membrane ruffles and focal adhesions. The mechanisms of how such proteins possibly select different downstream signaling pathways still remain largely elusive.

It has been reported from analysis of p120RasGAP-deficient cells that complex formation of p120RasGAP and p190RhoGAP appears to be important for cell polarization, by regulating stress fiber and focal adhesion reorientation (Kulkarni et al., 2000). It has also been reported that overexpression of the GAP-N protein of p120RasGAP causes irregular and branched formation of stress fibers (McGlade et al., 1993). Significant alterations in stress fiber organization was also observed in NMuMG cells when GAP-N was overexpressed (unpublished data). However, we have yet to analyze whether the p120RasGAP and p190RhoGAP complex is involved in cell polarity or branching activity in NMuMG cells. Moreover, we have not yet fully analyzed the biological significance of the p120RasGAP molecules associated with paxillin. It is possible that p120RasGAP bind to paxillin only to release p190RhoGAP. Alternatively, binding of Tyr31/118-phosphorylated paxillin to p120RasGAP may act to recruit p120RasGAP molecules to sites of integrin activation, similar to the case of p120RasGAP binding to activated growth factor receptors (Kazlauskas et al., 1990; Moran et al., 1991; Molloy et al., 1992).

In conclusion, we have shown that paxillin, through its phosphorylation at Tyr31/118, contributes to efficient membrane spreading and ruffling, by suppressing RhoA activity. The flexible structure of membrane ruffles at the leading cell edges might be advantageous for the efficient perception of cell-cell collision and the subsequent formation of cell-cell adhesion. For the integrity of cytoarchitecture and its efficient remodeling, spatially and temporally coordinated regulation of the different Rho-family GTPases, such as RhoA, Rac1, and Cdc42, are necessary within a single cell. The complementary, but potentially opposing effects between paxillin tyrosine phosphorylation and p130Cas tyrosine phosphorylation may more generally be involved in the hierarchical and/or mutually exclusive regulation of activities of these GTPases in cell adhesion and migration (for review see Hall, 1998; also see Sander et al., 1999; Cox et al., 2001): such are issues that deserve further experimental scrutiny.

Materials and methods

Cells

NMuMG cells (Miettinen et al., 1994) stably expressing EGFP-tagged paxillin cDNAs, and their treatment with TGF- β 1 (R&D Systems) have been described previously (Nakamura et al., 2000). COS7, HeLa, and 3Y1 cells were cultured as described previously (Yano et al., 2000; Mazaki et al., 2001). Cell migration was induced by scratching the confluent cultures as described (Nakamura et al., 2000). Cell adhesion to collagen was performed as described (Mazaki et al., 2001) by plating cells onto 10 μ g/ml collagen type I-coated plates in the presence of 1% serum.

Antibodies

Phosphorylation site-specific antibodies against each of the four major tyrosine phosphorylation sites of paxillin were generated by Biosource International (Nakamura et al., 2000). Src (Ab327) and Csk antibodies were as described previously (Sabe et al., 1994). Other antibodies against the following proteins were purchased from commercial sources: paxillin, Crk-1/II, Fak, Pyk2, p130Cas, p120RasGAP, and p190RhoGAP were from TDL; Rac1, Cdc42, p85 of PI3K, Shc, and Shp-2 were from UBI; RhoA, Nck, Crk-L, and p120RasGAP (clone B4F8) were from Santa Cruz Biotechnology, Inc.; and HA and GST were from Berkeley Antibody. Affinity-purified donkey antibodies to rabbit or mouse IgG conjugated either with horseradish peroxidase or Cy5 were from Jackson ImmunoResearch Laboratories.

cDNAs and their expression

Human p120RasGAP cDNA was amplified by PCR from the first strand cDNAs prepared from mRNAs of human fetal brain (CLONTECH Laboratories, Inc.), and ligated into pBabePuro/EGFP (Nakamura et al., 2000) to be fused in-frame to the COOH terminus of EGFP, or into pBabeHygro/HA₃ to be tagged with three repeats of the HA sequence at the NH₂ terminus. GST fusion forms of the SH2-SH3-SH2 domain or its derivatives were constructed in pGEX4T-1 (Amersham Biosciences), according to a previous report (Hu and Settleman, 1997). Mutations were introduced into the SH2 domains, in which Arg207 and Arg377 were changed to leucines (Songyang et al., 1995). Human p190RhoGAP cDNA (KIAA 0174) was obtained from Kazusa DNA Research Institute, and used after its missense mutations were corrected. The RhoGAP cDNA was ligated into pEGFP, to be expressed as NH₂-terminal tagged proteins. Nucleotide sequences were confirmed for all the plasmids after construction. cDNAs in pBabeHygro each encoding HA₃-tagged RhoA(G14V), RhoA (T19V), Rac1(G12V), Rac1(T17N), Cdc42(G12V), and Cdc42(T17N) were from K. Kaibuchi (Nagoya University, Nagoya, Japan). pGEX-2T/p120RasGAP SH2(N) and pGEX-Rhotekin RBD were gifts from B. Mayer (University of Connecticut, Farmington, CT) and M.A. Schwartz (University of Virginia, Charlottesville, VA), respectively.

cDNAs in the pBabe vectors were expressed by the use of the BOS23-derived retrovirus-mediated infection technique (Pear et al., 1993), and cells were selected for three days in the presence of 1 μ g/ml Puromycin (Sigma-Aldrich) or 0.6 mg/ml of Hygromycin B (Life Technologies) before being subjected to analysis, as described previously (Nakamura et al., 2000). For transient expression, COS7 and HeLa cells were transfected with plasmid DNAs using Polyfect (QIAGEN). Expression and purification of GST fusion proteins in an *Escherichia coli* system were as described (Sabe et al., 1994).

Protein binding and immunoblotting

In vitro protein binding assays using GST fusion proteins were performed as described (Settleman et al., 1992a; Sabe et al., 1994) with slight modifications. Briefly, cell lysates were prepared in 1% Triton X-100 buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 5 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM Na₃VO₄, 10 μ M Na₃MgO₄, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 3 μ g/ml pepstatin A, 10 μ g/ml aprotinin; Settleman et al., 1992a). Washing of precipitates was done with 0.1% Triton X-100 buffer, which contains Triton X-100 at 0.1% instead of at 1% and 1 mM EDTA instead of Mg²⁺ in the above buffer. In each experiment, 250 μ g of cell lysate and 5 μ g of GST-fusion protein bound to glutathione-Sepharose beads (Amersham Biosciences) were used. Immunoprecipitation was also done using 500 μ g of cell lysate prepared in 1% Triton X-100 buffer and the appropriate antibodies bound to Protein A-Sepharose beads (Amersham Biosciences), or to anti-mouse IgG-Sepharose beads (Sigma-Aldrich), as described (Tachibana et al., 1995). Determination of protein concentrations, SDS-PAGE and immunoblotting were done as described (Sabe et al., 1994). In each figure, 10 μ g of total cell lysates was included as a control, if necessary. Antibodies retained on the membranes were visualized using an enzyme-linked chemiluminescence method (Amersham Biosciences).

For peptide binding analysis, the NH₂-terminal succinimidyl-6-(biotinamide) hexanoate linked peptides, either nonphosphorylated or phosphorylated on tyrosines, were synthesized in milligram quantities by Peptide Institute (Osaka, Japan). Sequences of each peptide, as well as the phosphorylation sites, were EETpPYSYPTGNH for Tyr31, GNHTpYQEIA-VPP for Tyr40, EEHVpYSFPNKQK for Tyr118, and LSLpYGVpPETNS for Tyr181. Peptide competition analyses were done as described (Hu and Settleman, 1997).

siRNA-mediated disruption of p120RasGAP expression

Inhibition of p120RasGAP expression in HeLa cells was done by use of the siRNA technique (Elbashir et al., 2001). Oligonucleotides for human

p120RasGAP siRNA were designed (5'-AACUGCCCACUUCGUUGCUU-GUU-3' and 5'-CAAGCAACGAAGUGGGCAGUUUU-3'), and transfected into cells using Polyfect (QIAGEN), according to the manufacturer's instructions. As controls, oligonucleotides for human p130Cas siRNA (5'-ACCACCACGCAGUCUACGACGUU-3' and 5'-CGUCGUAGACUGCG-UGGUGUUU-3'), and oligonucleotides with a similar length but irrelevant sequence (irr) (5'-AUCCGAAGAGAAAGCAGUCCUU-3' and 5'-GGGACUGCUUUCUUCGGAUUU-3') were used.

RhoA activity

RhoA activities were measured using Rhotekin GST-RBD in cell adhesion to extracellular matrices in the presence of 1% serum, as described (Ren et al., 1999). Precipitated Rho proteins were quantified by immunoblotting using the monoclonal antibody against RhoA, coupled with their densitometric analysis using NIH Image 1.61 software (National Institutes of Health [Research Service Branch]).

Laser confocal microscopy

Cells were fixed in 4% paraformaldehyde at 37°C, 4 h after the induction of migration or 1 h after their adhesion to collagen, unless otherwise indicated, and analyzed using laser confocal microscopy with the attached computer software (LSM 510 version 2.5; Carl Zeiss), as previously described (Nakamura et al., 2000). Optical measurement of protein expression in a single cell was performed as described previously (Uchida et al., 2001). Antibodies were used at 1 µg/ml for the anti-HA antibody, and 2.5 µg/ml for the anti-p190RhoGAP antibody. Fluorescence dye-conjugated secondary antibodies were used at 1:1,000 dilution. F-actin was visualized by incubation for 1 h with Texas red-X phalloidin (Molecular Probes) at 1:600 dilution. EGFP-tagged proteins were visualized by autofluorescence from the tag. Each figure of microscopic analysis shows representative results that were observed in a majority of the cDNA-transfected cells in at least three independent experiments (>60–70% of the population in 100–200 cells examined) unless otherwise indicated.

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