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Semaphorin 4D/Plexin-B1–mediated R-Ras GAP activity inhibits cell migration by regulating β_1 integrin activity

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Plexins are cell surface receptors for semaphorins and regulate cell migration in many cell types. We recently reported that the semaphorin 4D (Sema4D) receptor Plexin-B1 functions as a GTPase-activating protein (GAP) for R-Ras, a member of Ras family GTPases implicated in regulation of integrin activity and cell migration (Oinuma, I., Y. Ishikawa, H. Katoh, and M. Negishi. 2004. *Science*. 305:862–865). We characterized the role of R-Ras downstream of Sema4D/Plexin-B1 in cell migration. Activation of Plexin-B1 by Sema4D suppressed the ECM-dependent R-Ras activation, R-Ras–mediated phosphatydylinositol 3-kinase activation, and β_1 integrin activation through its R-Ras GAP domain, leading to inhibition of cell migration. In addition, inactivation of R-Ras by overexpression of the R-Ras–specific GAP or knockdown of R-Ras by RNA interference was sufficient for suppressing β_1 integrin activation and cell migration in response to the ECM stimulation. Thus, we conclude that R-Ras activity is critical for ECM-mediated β_1 integrin activation and cell migration and that inactivation of R-Ras by Sema4D/Plexin-B1–mediated R-Ras GAP activity controls cell migration by modulating the activity of β_1 integrins.

Introduction

Semaphorins comprise a large family of secreted and transmembrane molecules that play central roles in axon guidance in the developing nervous system (Kolodkin et al., 1993; Tamagnone et al., 1999). The function of semaphorins is mediated by plexins, which are classified into four subfamilies: Plexin-A, -B, -C, and -D (Tamagnone et al., 1999). Semaphorins were originally identified as repulsive axonal guidance molecules, but they have recently been shown to regulate integrin-mediated cell migration in a variety of cells (Tamagnone and Comoglio, 2004). Sema3A exerts an essential permissive role in the execution of vasculature remodeling by inhibiting integrin-mediated adhesion of endothelial cells to the ECM (Serini et al., 2003). Activation of Plexin-B1 negatively regulates integrin-based cell adhesion and migration of NIH-3T3 cells (Barberis et al., 2004). Plexin-C1 inhibits integrin-mediated adhesion and chemokine-induced migration of dendritic cells (Walzer et al., 2005). Thus, semaphorin/plexin signaling plays an important role in the migration of a variety of cells. However, the molecular mechanisms underlying the inhibition of integrin-mediated cell migration by semaphorins through plexins remain unclear.

Rho family small GTPases are signal transduction molecules that remodel the actin cytoskeleton and play fundamental roles in numerous cellular processes (Negishi and Katoh, 2002). The small GTPase Rnd1, a constitutively active GTPase (Nobes et al., 1998), is known to interact directly with the cytoplasmic domain of Plexin-B1 (Oinuma et al., 2003). We recently revealed that Plexin-B1 functions as an R-Ras GTPase-activating protein (GAP) and directly and specifically down-regulates R-Ras activity in response to Sema4D, inducing repulsive response in hippocampal neurons, and that the expression of R-Ras GAP activity of Plexin-B1 requires Rnd1 association with the receptor (Oinuma et al., 2004a). Furthermore, expression of constitutively active R-Ras prevents growth cone collapse induced by Sema4D/Plexin-B1 as well as Sema3A/Plexin-A1, whereas R-Ras siRNA caused a growth cone collapse similar to those induced by semaphorins (Oinuma et al., 2004a).

Integrins are a family of α/β heterodimeric cell surface receptors that bind to the ECM, such as collagens and fibronectins, and play a central part in regulating cell growth, survival, migration, and tumor metastasis (Hood and Cheresh, 2002). Activation of integrins is essential for cell adhesion and cell migration, and several studies show that the Ras family of small

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Abbreviations used in this paper: GAP, GTPase-activating protein; HS, horse serum; PE, phycoerythrin; PI3-K, phosphatydylinositol 3-kinase; WT, wild type.

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GTPases regulates integrin activity (Kinbara et al., 2003). Among the Ras family GTPases, activated R-Ras was shown to induce integrin activation and increase cell adhesion and matrix assembly, suggesting that R-Ras plays an important role in the regulation of integrin activity (Zhang et al., 1996; Sethi et al., 1999). However, how R-Ras activity is regulated and how R-Ras activates integrins remain obscure. Significantly, Sema4D was the first extracellular stimulus shown to influence the activity of R-Ras. These facts collectively prompted us to speculate that plexins regulate integrin-mediated cell migration by their R-Ras GAP activity.

In this study, we characterized the role of R-Ras downstream of Sema4D/Plexin-B1 in regulation of integrin activation and cell migration. The activation of R-Ras by ECM is required for ECM-mediated integrin activation and cell migration, and Sema4D/Plexin-B1 inhibits integrin activation and cell migration through R-Ras GAP activity. We also revealed that down-regulation of phosphatydylinositol 3-kinase (PI3-K) activity is responsible for Sema4D/Plexin-B1–induced suppression of β_1 integrin activity and cell migration.

Results

Sema4D antagonizes integrin-mediated cell migration

We examined the effect of Sema4D on integrin-mediated migration of PC12 cells in a cell migration assay (Fig. 1, A and B). Transwell chambers were coated on the lower side with varying concentrations of collagen I. PC12 cells exhibited a collagen concentration–dependent promotion of cell migration, which was antagonized by Sema4D. The collagen-dependent PC12 cell migration is mediated by α_1 and β_1 integrin subunits, as functional blocking antibodies against α_1 (Fig. 1, C and D) and β_1 (Fig. 1, E and F) integrin subunits strongly impaired the migration. These results indicate that Sema4D antagonizes the collagen receptor, α_1/β_1 integrin–dependent PC12 cell migration.

Sema4D through Plexin-B1 inhibits ECM-mediated activation of R-Ras and β_1 integrins

R-Ras is implicated in integrin-mediated cell migration, and expression of a constitutively active form of R-Ras has been shown to stimulate cell migration (Keely et al., 1999). We previously reported that Sema4D stimulation down-regulates NGF-stimulated R-Ras activity via the R-Ras GAP activity of Plexin-B1 to induce neurite retraction (Oinuma et al., 2004a). We next tested whether stimulation of PC12 cells with collagen and Sema4D affects R-Ras activity. PC12 cells were plated onto collagen-coated dishes and lysed, and the lysates were incubated with the GST-fused Ras binding domain of c-Raf-1 (GST-RBD) to pull down activated R-Ras (de Rooij and Bos, 1997). As shown in Fig. 2 A, cells plated on collagen-coated dishes showed a collagen concentration–dependent increase in endogenous R-Ras activity, whereas those kept in suspension or plated onto the non–integrin-dependent substrate poly-D-lysine did not. Furthermore, the collagen-dependent activation of R-Ras was inhibited by a functional blocking antibody against β_1 integrins, P5D2, and was enhanced by affinity-related activation of β_1 integrins by the monoclonal antibody 8A2, which mechanically induces a

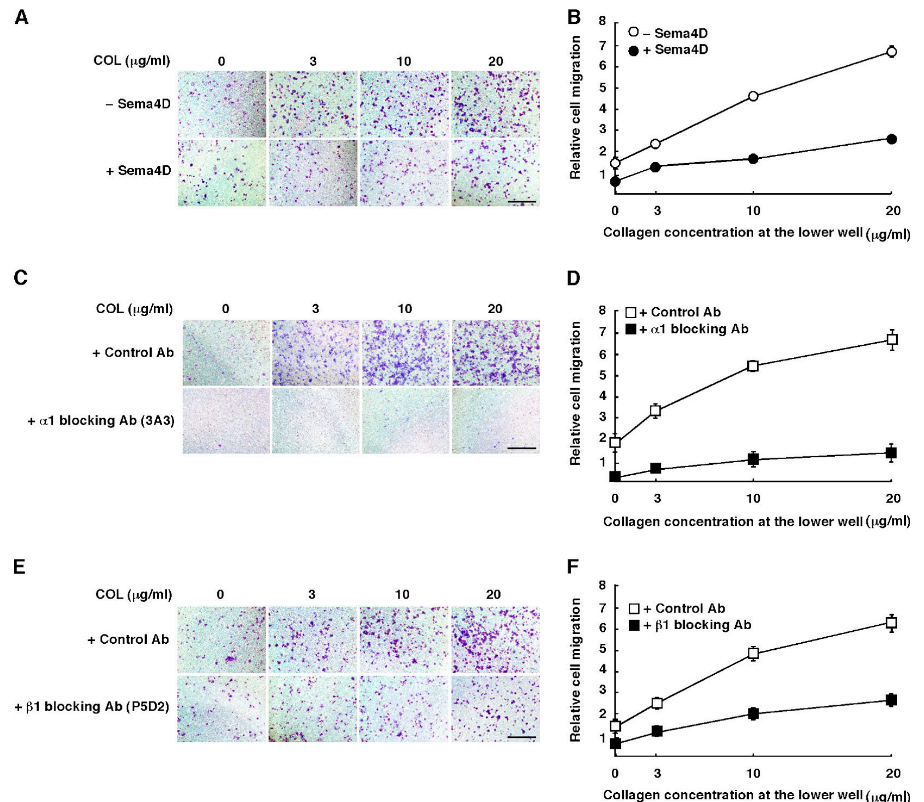


Figure 1. **Sema4D antagonizes integrin-mediated cell migration.** (A) PC12 cells were tested in the transwell assay either in the presence or absence of Sema4D. Transwell chambers were coated on the lower side with varying concentrations of collagen I. Migrated cells were visualized by the staining of crystal violet. (C and E) PC12 cells were pretreated with 5 μg/ml of functional blocking monoclonal antibodies against α_1 and β_1 integrin subunits (3A3 and P5D2, respectively) or control mouse IgG1 and subjected to the transwell assay. Migrated cells were visualized by the staining of crystal violet. (B, D, and F) Relative cell migration was determined by the number of the migrated cells normalized to the total number of cells. Results are the means ± SEM of three independent experiments. Bars, 1 mm.

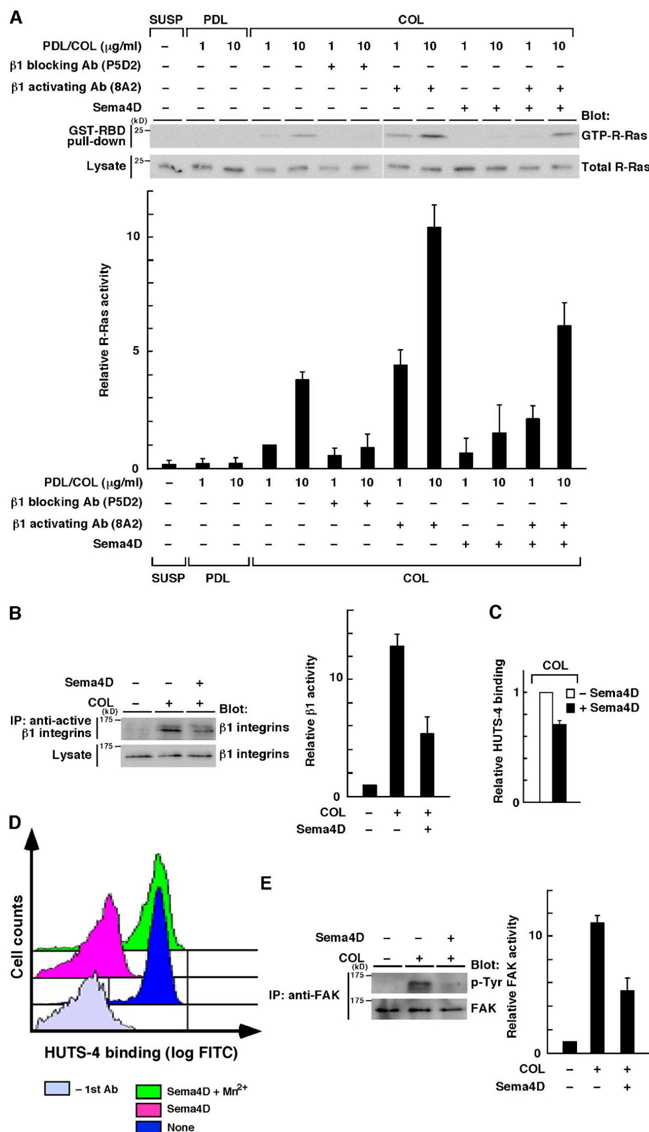


Figure 2. **Sema4D inhibits ECM-mediated activation of R-Ras and functional activation of β_1 integrins.** (A) PC12 cells were collected and kept in suspension (SUSP) or replated onto poly-D-lysine (PDL)– or collagen (COL)–coated (1 or 10 μg/ml) dishes with or without Sema4D in the plating media. 15 min after plating, the cells were lysed and the lysates were incubated with GST-RBD, and bound R-Ras protein and total lysates were analyzed by immunoblotting. For the indicated samples, cells were treated with 5 μg/ml of monoclonal β_1 integrin blocking (P5D2) or activating (8A2) antibody before replating. Relative R-Ras activity was determined by the amount of R-Ras bound to GST-RBD normalized to the amount of R-Ras in cell lysates analyzed by NIH Image software (bottom). (B and E) PC12 cells were seeded onto noncoated or collagen-coated (10 μg/ml) dishes, with or without Sema4D in the plating media. Cells were lysed, and the lysates were immunoprecipitated with an antibody against the active β_1 integrins, HUTS-4 (B), or an antibody against FAK (E) to measure the activity of β_1 integrins or tyrosine phosphorylated FAK, respectively. (C) The ELISA using HUTS-4 antibody was performed to confirm the effect of Sema4D on activity of β_1 integrins under a detergent-free condition. Results are the means ± SEM of three independent experiments. (D) PC12 cells were treated for 3 h at 37°C with control medium or with medium containing Sema4D or Sema4D plus 1 mM Mn^{2+}. Cells were incubated with HUTS-4 antibody or buffer alone (−1st Ab), followed by labeling with the FITC-conjugated secondary antibody. Fluorescence intensity was determined by flow cytometry analysis. Error bars indicate SEM.

high-affinity state of β_1 integrins. These data suggest that β_1 integrins are required for R-Ras activation upon ECM-mediated adhesion. Sema4D stimulation strongly inhibited the collagen-induced activation of R-Ras, and affinity-related activation of β_1 integrins by the 8A2 antibody attenuated the inhibitory effect of Sema4D on ECM-mediated R-Ras activation. R-Ras is known to regulate β_1 integrin activation (Zhang et al., 1996). To examine the effect of Sema4D on β_1 integrin activity, we measured the activity of β_1 integrins in cells with or without Sema4D stimulation by the immunoprecipitation assay with the monoclonal antibody against active conformations of β_1 integrins, HUTS-4, which detects hybrid domain swing-out in β_1 integrins, a process most commonly associated with ligand binding (Mould et al., 2003). Sema4D antagonized the collagen-dependent activation of β_1 integrins (Fig. 2 B). Inhibition of β_1 integrin activity by Sema4D was also observed in the ELISA using the HUTS-4 antibody, which was performed under a detergent-free condition (Fig. 2 C). To further ascertain that Sema4D indeed affects the activity of β_1 integrins, we performed flow cytometry analysis using the HUTS-4 antibody. As shown in Fig. 2 D, cells treated with Sema4D showed a decrease in the level of HUTS-4 binding (FITC staining). Mn^{2+} treatment, which induces the activation of β_1 integrins, resulting in the effective interaction with the ECM ligands and increased HUTS-4 binding (Luque et al., 1996), completely overcame the Sema4D-induced decrease in HUTS-4 binding. These results suggest that decreased HUTS-4 binding induced by Sema4D is due to affinity modulation of β_1 integrins. FAK is known to be autophosphorylated at tyrosine upon integrin activation (Hildebrand et al., 1993), and FAK phosphorylation downstream of β_1 integrins is the important step for integrin-mediated cell migration (Parsons et al., 2000; Sieg et al., 2000). As shown in Fig. 2 E, Sema4D inhibited the collagen-mediated FAK tyrosine phosphorylation. These results suggest that Sema4D inhibits ECM-mediated activation of R-Ras and β_1 integrins.

We also confirmed the involvement of the endogenous Plexin-B1 receptor in Sema4D-dependent inhibition of R-Ras activity and integrin functions. As shown in Fig. 3, both Sema4D-dependent inhibition of collagen-mediated activation of R-Ras and cell migration were blocked by the monoclonal antibody against Plexin-B1, which recognizes the extracellular ligand binding region of the receptor. These results suggest that Sema4D through Plexin-B1 inhibits ECM-mediated activation of R-Ras, functional activation of β_1 integrins, and inhibition of cell migration.

Inhibition of cell migration by Sema4D is mediated by suppression of β_1 integrin activity

We tested whether Sema4D-mediated inhibition of cell migration is mediated by suppression of β_1 integrin activity. PC12 cells preincubated with 5 μg/ml β_1 integrin activating monoclonal antibody (8A2) were subjected to the transwell assay. We tested the migration at relatively low concentrations of collagen (~3.0 μg/ml) because this antibody inhibits cell migration at

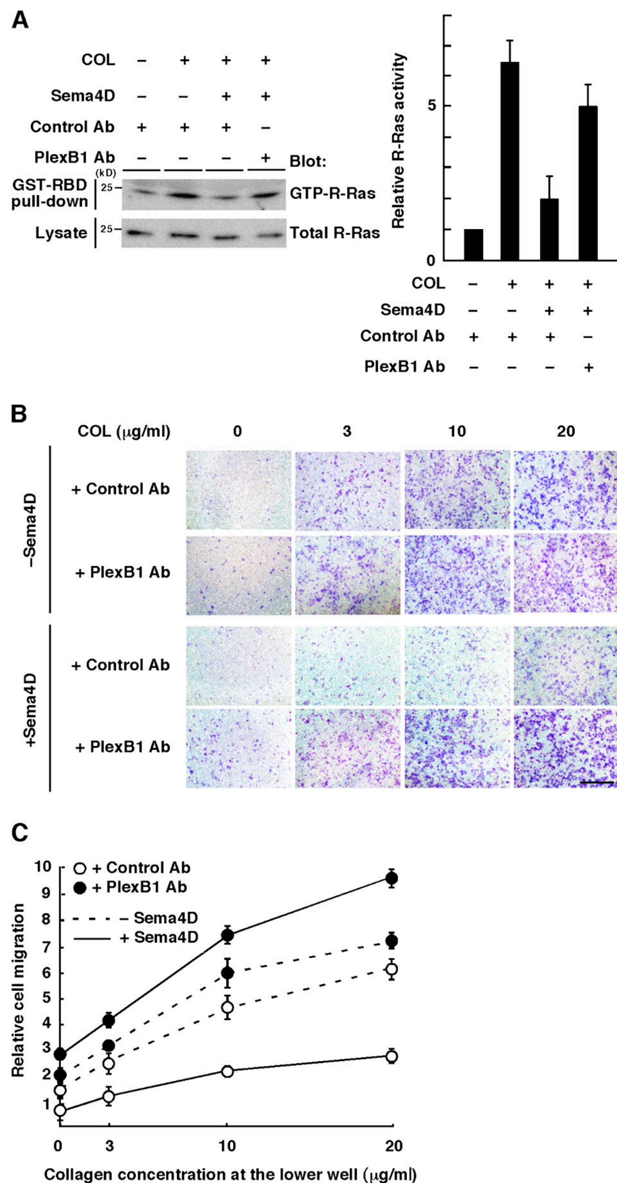


Figure 3. **Sema4D-dependent inhibition of R-Ras activity and ECM-mediated cell migration involves the endogenous Plexin-B1 receptor.** (A) PC12 cells were pretreated with 5 μg/ml of a mouse monoclonal antibody against the extracellular ligand binding region of Plexin-B1 (PlexB1 Ab) or the control mouse IgG2b (Control Ab). PC12 cells were seeded onto noncoated or collagen-coated (10 μg/ml) dishes with or without Sema4D in the plating media, and relative R-Ras activity was determined as described in the legend to Fig. 2 A. (B) PC12 cells were pretreated with 5 μg/ml of the monoclonal antibody against Plexin-B1 and were subsequently subjected to the transwell assay in either the presence or absence of Sema4D. Migrated cells were visualized by the staining of crystal violet. (C) Relative cell migration was determined by the number of the migrated cells normalized to the total number of cells. Results are the means ± SEM of three independent experiments. Bar, 1 mm.

high concentration of the ECM ligands by freezing β_1 at a high-affinity state (Kuijpers et al., 1993). As shown in Fig. 4, affinity-related activation of β_1 integrins by 8A2 stimulation overcame the inhibitory effect of Sema4D on collagen-mediated cell migration, whereas a control IgG2a antibody did not. These results suggest that the inhibition of β_1 integrin activity is required for the inhibition of cell migration by Sema4D.

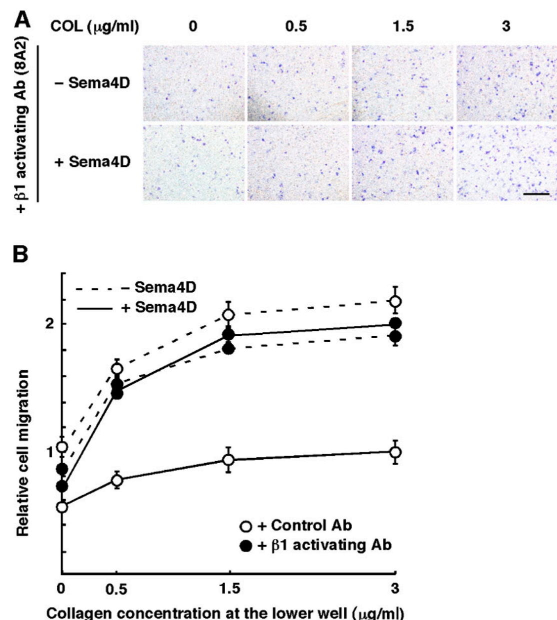


Figure 4. **Affinity-related activation of β_1 integrins overcomes the inhibitory effect of Sema4D on collagen-mediated PC12 cell migration.** (A and B) PC12 cells were pretreated with 5 μg/ml of β_1 integrin activating monoclonal antibody (8A2) or control IgG2a (Control Ab) and were subsequently subjected to the transwell assay in either the presence or absence of Sema4D. Migrated cells were visualized by the staining of crystal violet. (B) Relative cell migration was determined by the number of migrated cells normalized to the total number of cells. Results are the means ± SEM of three independent experiments. Bar, 1 mm.

Plexin-B1 inhibits ECM-dependent activation of R-Ras and β_1 integrins through its R-Ras GAP activity

We recently reported that Plexin-B1 encodes R-Ras GAP within its cytoplasmic tail and that Plexin-B1 associated with the Rho family GTPase Rnd1 functions as a specific GAP toward R-Ras (Oinuma et al., 2004a). We examined whether Sema4D/Plexin-B1–Rnd1–mediated R-Ras GAP activity suppresses adhesion-dependent R-Ras activation. COS-7 cells expressing R-Ras–wild type (WT) were plated onto fibronectin-coated dishes or nonadherent control dishes and lysed, and the lysates were incubated with GST-RBD to pull down activated R-Ras. The same cell lysates were also used for the immunoprecipitation assay using HUTS-4 for measurement of the activity of β_1 integrins. In COS-7 cells, fibronectin stimulation activated both R-Ras and β_1 integrins (Fig. 5 A). As shown in Fig. 5 B, expression of Plexin-B1–WT and Rnd1 inhibited the fibronectin-mediated R-Ras activation in the presence of Sema4D. However, this inhibitory effect was not observed in cells expressing Plexin-B1–GGA, a mutant lacking the ability to associate with Rnd1, or Plexin-B1–RA, a mutant lacking primary and secondary arginine residues required for the catalytic activity of GAP. These results suggest that Sema4D/Plexin-B1–Rnd1–mediated R-Ras GAP activity inhibits adhesion-dependent R-Ras activation. To examine the effect of Sema4D/Plexin-B1–Rnd1–mediated R-Ras GAP activity on β_1 integrin activity, we measured the activity of β_1 integrins in cells expressing Plexin-B1 and Rnd1 with or without Sema4D stimulation by the immunoprecipitation assay. As shown in Fig. 5 C, expression of Plexin-B1

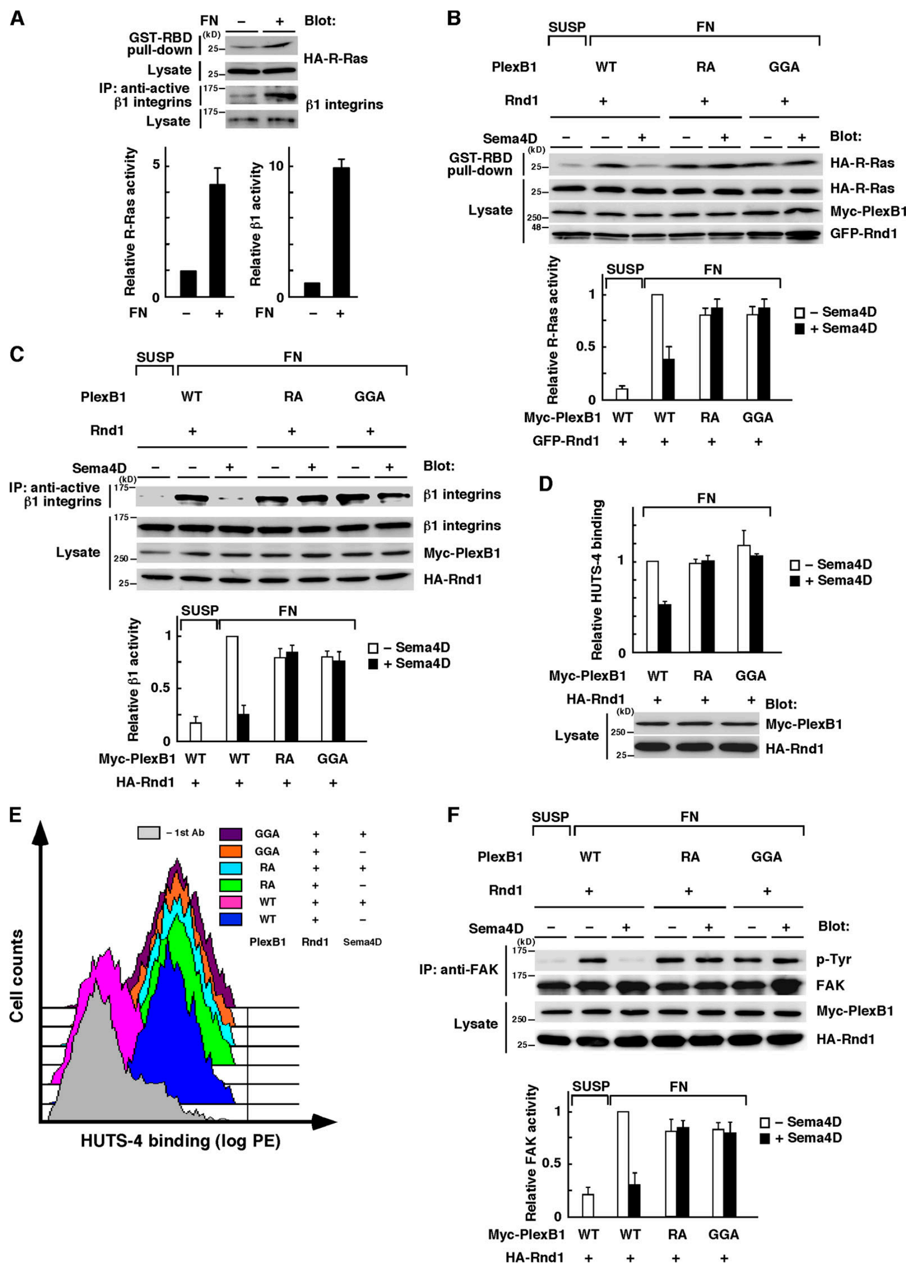


Figure 5. **Plexin-B1–mediated R-Ras GAP activity inhibits ECM-dependent activation of R-Ras and β_1 integrins.** (A) COS-7 cells transfected with HA-tagged wild-type R-Ras were detached with 1.5 mM EDTA, replated onto the dishes coated with or without 10 µg/ml fibronectin (FN), and incubated at 37°C for 15 min. (top) The cell lysates were incubated with either the antibody against the active conformations of β_1 integrins HUTS-4 or GST-RBD to see the activity of β_1 integrins or R-Ras, respectively. (bottom) Relative activity of R-Ras or β_1 integrins was normalized to the amount of R-Ras or β_1 integrins in cell lysates analyzed by NIH Image software. (B, C, and F) COS-7 cells transfected with mutants of Myc-tagged Plexin-B1 and HA-tagged Rnd1 were detached with 1.5 mM EDTA. The cells were either kept in suspension (SUSP) or replated onto the dishes coated with 10 µg/ml fibronectin and incubated at 37°C for 15 min in the presence or absence of Sema4D. Relative activity of R-Ras (B) and β_1 integrins (C) and tyrosine phosphorylation of FAK (F) were measured. (D, top) The ELISA using HUTS-4 antibody was performed to confirm the effect of Sema4D/Plexin-B1–mediated R-Ras GAP activity on activity of β_1 integrins under the detergent-free condition. (bottom) Expression levels of each construct were verified by immunoblot analysis. Results are the means ± SEM of three independent experiments. (E) COS-7 cells transiently cotransfected with GFP-Rnd1 and Plexin-B1 were stimulated for 5 min with Sema4D and analyzed by two-color flow cytometry. HUTS-4 binding (PE staining) was analyzed on a gated subset of cells, positive for GFP expression to discriminate β_1 integrin activity of transfected cells from that of untransfected cells. Level of HUTS-4 binding in cells expressing GFP-Rnd1 and various Plexin-B1 expression constructs, with or without Sema4D stimulation, was analyzed. Error bars indicate SEM.

and Rnd1 strongly inhibited the fibronectin-mediated β_1 integrin activation in the presence of Sema4D, whereas inhibition of β_1 integrin activation was not observed in cells expressing Plexin-B1–GGA or Plexin-B1–RA. The same results were also obtained by the ELISA using the HUTS-4 antibody performed under a detergent-free condition (Fig. 5 D). We also confirmed the results by flow cytometry analysis. COS-7 cells transiently cotransfected with GFP-Rnd1 and Plexin-B1 were treated with Sema4D, and GFP expression and HUTS-4 binding (phycoerythrin [PE] staining) were simultaneously analyzed by two-color flow cytometry. HUTS-4 binding (PE staining) was analyzed on a gated subset of cells positive for GFP expression to discriminate β_1 integrin activity of transfected cells from that of untransfected cells. As shown in Fig. 5 E, a Sema4D-dependent decrease in HUTS-4 binding was observed in Plexin-B1–WT and Rnd1-expressing cells. However, cells coexpressing Rnd1 with Plexin-B1–RA or Plexin-B1–GGA, which lacks R-Ras GAP activity, did not show a Sema4D-dependent reduction in HUTS-4 binding. In addition, Sema4D/Plexin-B1–Rnd1 also inhibited the fibronectin-mediated FAK tyrosine phosphorylation, whereas inhibition of FAK phosphorylation was not observed in cells expressing Plexin-B1–GGA or Plexin-B1–RA (Fig. 5 F). These results suggest that Sema4D/Plexin-B1–Rnd1–mediated R-Ras GAP activity inhibits adhesion-dependent activation of R-Ras and thereby inhibits functional activation of β_1 integrins.

R-Ras activity is required for the ECM-mediated activation of β_1 integrins

We next examined whether regulation of R-Ras activity plays key roles in the ECM-mediated activation of β_1 integrins. As shown in Fig. 6 A, in untransfected cells, activity of β_1 integrins was increased upon adhesion to fibronectin. This activation was

completely blocked by the down-regulation of endogenous R-Ras activity by the expression of the myristoylated GAP domain of p98–R-RasGAP (Myr–R-RasGAP), which exhibits a specific GAP activity toward R-Ras (Yamamoto et al., 1995). R-Ras is implicated in integrin regulation, and the constitutively active form of R-Ras has been shown to increase the affinity of β_1 integrins for fibronectin (Zhang et al., 1996) and to stimulate cell migration (Keely et al., 1999). Expression of R-Ras–QL actually induced remarkable activation of β_1 integrins, and this was not further enhanced by fibronectin. We also tested whether R-Ras activity affects FAK tyrosine phosphorylation. As shown in Fig. 6 B, expression of Myr–R-RasGAP completely blocked the fibronectin-induced FAK phosphorylation, whereas R-Ras–QL markedly stimulated FAK phosphorylation independent of fibronectin, indicating that endogenous R-Ras activity is also required for ECM-mediated FAK phosphorylation. We further confirmed requirement of R-Ras in the ECM-mediated functional activation of β_1 integrins. We reduced expression of R-Ras in COS-7 cells by R-Ras–specific siRNA expression vector and examined the effect on the activation of β_1 integrins and phosphorylation of FAK. As shown in Fig. 6 (C and D), expression of R-Ras siRNA effectively reduced endogenous R-Ras protein, and reduction in R-Ras protein blocked both the fibronectin-dependent activation of β_1 integrins and phosphorylation of FAK. The ELISA using HUTS-4, under detergent-free conditions, also confirmed suppression of β_1 integrin activation by inactivation of R-Ras by expression of Myr–R-RasGAP or knockdown of R-Ras by R-Ras RNA interference (Fig. 6, E and F). We also confirmed these results by two-color flow cytometry. COS-7 cells transiently transfected with Myr–R-RasGAP or an R-Ras siRNA together with GFP were stained with HUTS-4, and HUTS-4 binding (PE staining) was analyzed

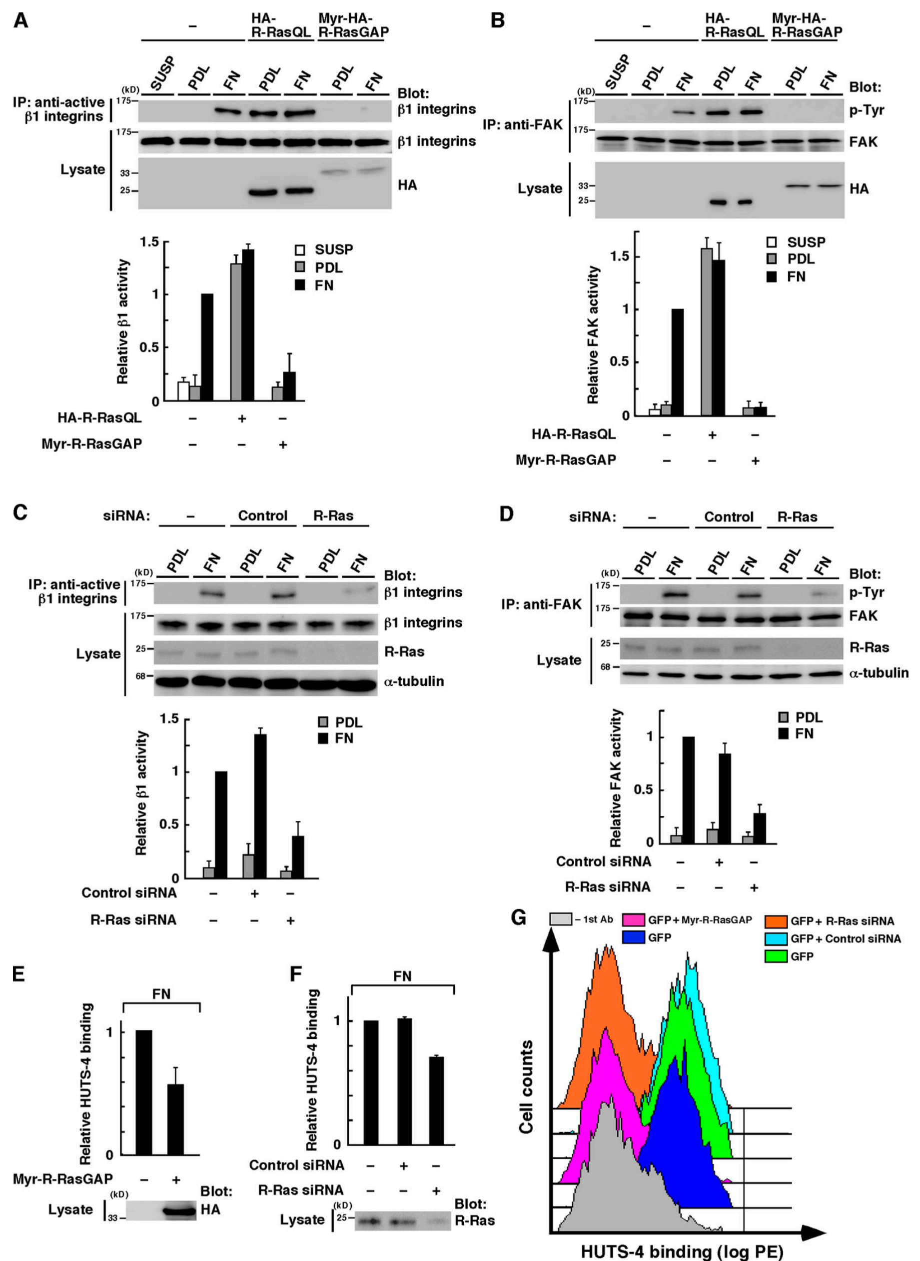


Figure 6. **R-Ras activity is required for ECM-mediated activation of β_1 integrins.** (A–D) COS-7 cells expressing the indicated expression plasmids were detached with 1.5 mM EDTA in PBS and kept in suspension (SUSP) or replated onto the dishes coated with 20 μg/ml poly-D-lysine (PDL) or 10 μg/ml fibronectin (FN). After 15 min, activity of β_1 integrins (A and C) and tyrosine phosphorylated FAK (B and D) were measured. (E and F, top) The ELISA using HUTS-4 was performed under a detergent-free condition. (bottom) Expression levels of each construct or the levels of endogenous R-Ras protein in cells transfected with siRNAs were verified by immunoblot analysis. Results are the means ± SEM of three independent experiments. (G) COS-7 cells transiently transfected with Myr–R-RasGAP or an R-Ras siRNA together with GFP were stained with HUTS-4, and HUTS-4 binding (PE staining) was analyzed on GFP-positive cells.

on GFP-positive cells. As shown in Fig. 6 G, the level of HUTS-4 binding was reduced in cells expressing Myr–R-RasGAP or R-Ras siRNA. These results demonstrate that activation of the endogenous R-Ras protein is essential for the ECM-mediated functional activation of β_1 integrins.

Plexin-B1 inhibits cell migration through R-Ras GAP activity

We next examined the effect of Sema4D/Plexin-B1 signaling on integrin-mediated cell migration. COS-7 cells expressing a control GFP alone exhibited a fibronectin concentration–dependent promotion of cell migration, and ectopic expression of GFP–R-Ras–WT enhanced this fibronectin-dependent cell migration (Fig. 7, A and B). Coexpression of Plexin-B1–WT and Rnd1 with R-Ras–WT blocked the R-Ras–induced promotion of cell migration toward fibronectin, in the presence of Sema4D at the lower well (Fig. 7 C). On the other hand, expression of Plexin-B1–RA, a mutant of Plexin-B1 that lacks R-Ras GAP activity, did not exhibit the Sema4D-dependent inhibition of cell migration toward fibronectin (Fig. 7 D). Association of Rnd1 with Plexin-B1 is essential for the expression of R-Ras GAP activity of Plexin-B1 (Fig. 5 B), and inhibition of cell migration was not observed in the cells without Rnd1 or in the cells expressing Plexin-B1–GGA, a mutant of Plexin-B1 unable to interact with Rnd1 (Fig. 7 E). The Plexin-B subfamily has been shown to activate RhoA via its COOH-terminal PDZ domain binding motif (Perrot et al., 2002; Swiercz et al., 2002; Oinuma et al., 2003). However, Plexin-B1–ΔC, a mutant of Plexin-B1 that lacks the PDZ domain binding motif but still has R-Ras GAP activity (Oinuma et al., 2004a), inhibited fibronectin-dependent cell migration in the presence of Sema4D (Fig. 7 E). Cell migration mediated by constitutively active R-Ras, R-Ras–QL, was not suppressed by the Sema4D/Plexin-B1–Rnd1 complex (Fig. 7 E). Expression levels of these constructs used in the assay were similar, as verified by immunoblot analysis (not depicted). Furthermore, R-Ras activity is essential for ECM-mediated cell migration, as both inactivation of R-Ras by expression of Myr–R-RasGAP or knockdown of R-Ras by R-Ras RNA interference almost completely suppressed the fibronectin-dependent cell migration (Fig. 7 F).

We further confirmed that the R-Ras GAP activity exhibited by endogenous Plexin-B1 is required for Sema4D-mediated

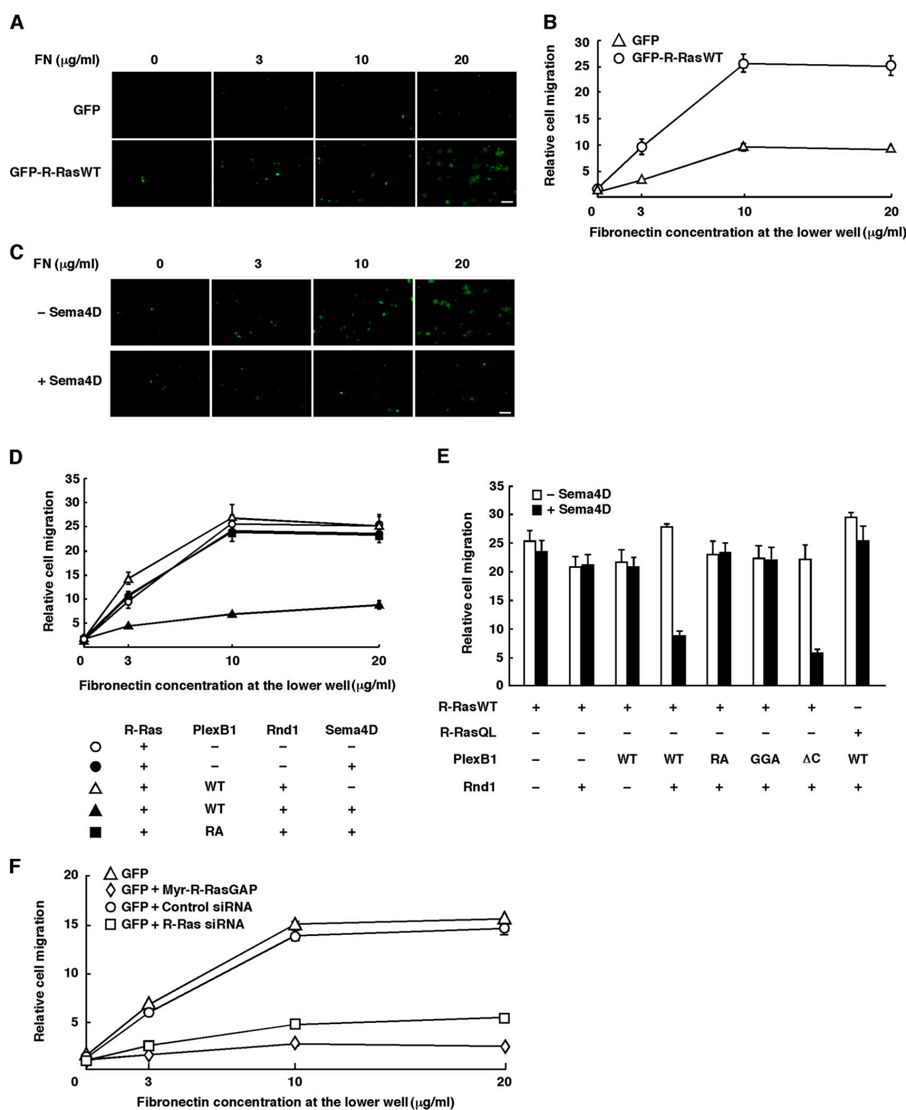


Figure 7. **Plexin-B1 inhibits ECM-mediated cell migration through R-Ras GAP activity.** (A and B) COS-7 cells expressing GFP or GFP–R-Ras–WT were used in a cell migration assay, using transwell chambers coated on the lower side with varying concentrations of fibronectin (FN). Migrated cells were visualized by the fluorescence of GFP (A), and relative cell migration was determined (B). (C) COS-7 cells expressing GFP–R-Ras–WT together with Plexin-B1–WT and Rnd1 were tested in the transwell assay in either the presence or absence of Sema4D. (D) COS-7 cells expressing the listed plasmids were tested in the transwell assay using the chambers coated with varying concentrations of fibronectin in either the presence (closed symbols) or absence (open symbols) of Sema4D. Migrated cells were visualized by the fluorescence of GFP. (E) Relative cell migration at the point of 20 μg/ml fibronectin with or without Sema4D was determined. Expression levels of the constructs were verified by immunoblot analysis (not depicted). (F) COS-7 cells transfected with the indicated plasmids were tested in a cell migration assay using the transwells coated on the lower sides with varying concentrations of fibronectin. Relative cell migration was determined by the number of the migrated cells normalized to the total number of the transfected cells. Results are the means ± SEM of three independent experiments. Bars, 1 mm.

inhibition of ECM-mediated PC12 cell migration. We recently reported that the cytoplasmic region of Plexin-B1 by nature takes the intramolecularly tethered form and that disruption of the interaction between the NH_2-terminal region (N-Cyt) and the COOH-terminal region (C-Cyt) within the cytoplasmic domain (Fig. 8 A) by Rnd1 binding to N-Cyt is essential for exhibiting the R-Ras GAP activity. C-Cyt associates with N-Cyt–GGA, which has no ability to interact with Rnd1, and Rnd1 cannot disrupt this interaction (Oinuma et al., 2004b). As shown in Fig. 8 B, overexpression of Plexin-B1–N-Cyt–GGA could effectively block the Sema4D/Plexin-B1–Rnd1 complex–mediated R-Ras GAP activity, suggesting that Plexin-B1–N-Cyt–GGA could be an effective tool to inhibit the R-Ras GAP activity of Plexin-B1 in a dominant-negative manner. Overexpression of Plexin-B1–N-Cyt–GGA in PC12 cells almost completely blocked the Sema4D-mediated inhibition of ECM-mediated cell migration (Fig. 8, C and D).

We also examined the role of endogenous R-Ras protein in PC12 cell migration. Transfection of the R-Ras siRNA effectively reduced the expression of endogenous R-Ras protein in PC12 cells, whereas the control siRNA did not work (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200508204/DC1), and expression of R-Ras siRNA almost completely suppressed the collagen-dependent cell migration (Fig. S1, B and C), suggesting that R-Ras is a prime regulator for integrin-mediated cell migration in PC12 cells. These results demonstrate that activation of endogenous R-Ras protein is essential for the ECM-mediated cell migration and that regulation of R-Ras activity through Sema4D/Plexin-B1–mediated R-Ras GAP activity plays a key role in ECM-mediated cell migration.

Sema4D/Plexin-B1–Rnd1 inhibits PI3-K activity through its R-Ras GAP activity

PI3-K is the predominant effector of R-Ras (Marte et al., 1997; Suire et al., 2002), and R-Ras–mediated cell migration is sensitive to pharmacological PI3-K inhibitors (Keely et al., 1999; Rincón-Arano et al., 2003). Expression of R-Ras–QL induces the ECM-independent functional activation of β_1 integrins and tyrosine phosphorylation of FAK (Fig. 6, A and B) and causes COS-7 cell migration in the absence of ECM ligands (Fig. 9 A). The D64A mutation of R-Ras or the pharmacological PI3-K inhibitor LY294002 abrogated the cell migration induced by R-Ras–QL (Fig. 9, A and B). R-Ras–QL–64A, the effector loop mutant of R-Ras, impairs the ability of R-Ras to activate PI3-K (Oertli et al., 2000), and R-Ras–QL–mediated phosphorylation of the PI3-K effector Akt (PKB) was abolished by the D64A mutation (Fig. 9 C). We further examined the involvement of PI3-K in R-Ras–QL–induced activation of β_1 integrins and subsequent FAK phosphorylation. As shown in Fig. 9 (D and E), D64A mutation or LY294002 treatment markedly blocked both R-Ras–QL–induced activation of β_1 integrins and phosphorylation

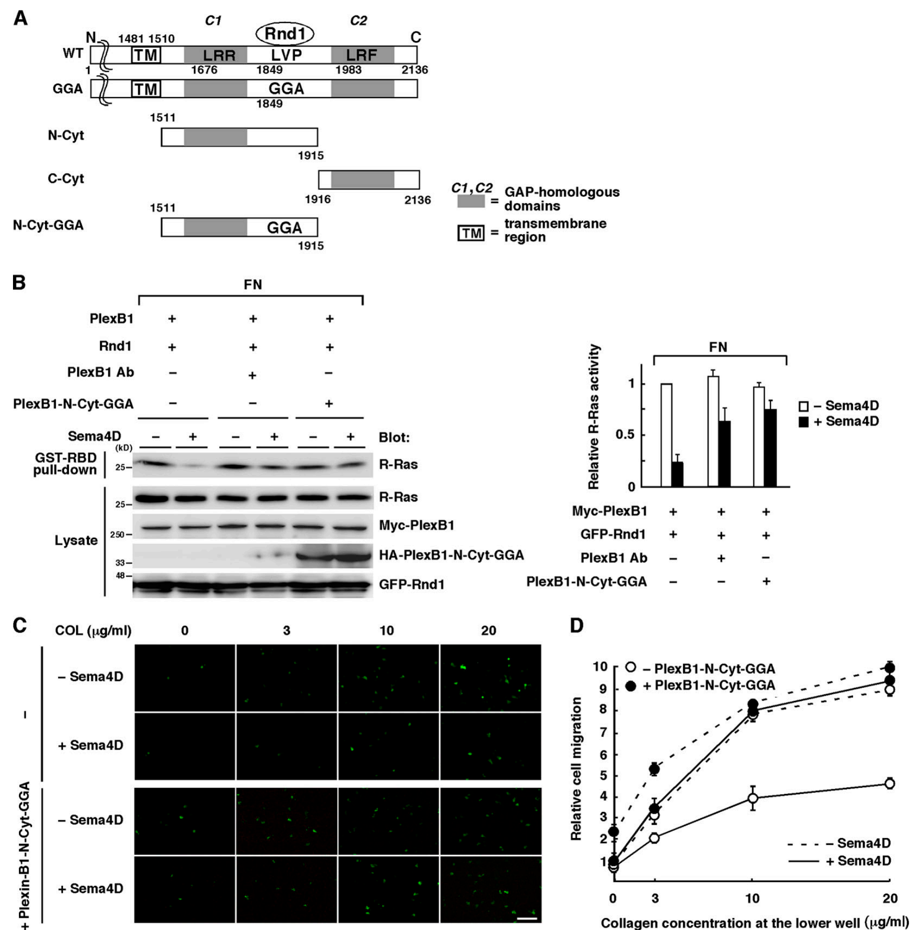


Figure 8. **R-Ras GAP activity of endogenous Plexin-B1 is required for Sema4D-mediated inhibition of integrin-mediated PC12 cell migration.** (A) Schematic representation of the Plexin-B1–N-Cyt–GGA construct used in the experiment. The Rnd1 binding region and the R-Ras GAP domains (*C1* and *C2*) are indicated. Letters indicate the specific amino acid residues within domains (A, Ala; F, Phe; G, Gly; L, Leu; P, Pro; R, Arg; V, Val), and numbers indicate amino acid positions within the sequence. (B) Relative activity of R-Ras was determined as described in the legend to Fig. 5 B. (C) PC12 cells transfected with GFP alone or GFP plus HA–Plexin-B1–N-Cyt–GGA were tested in the transwell assay in either the presence or absence of Sema4D. (D) Relative cell migration was determined by the number of the migrated cells normalized to the total number of cells. Migrated cells were visualized by the fluorescence of GFP. Results are the means ± SEM of three independent experiments. Bar, 1 mm.

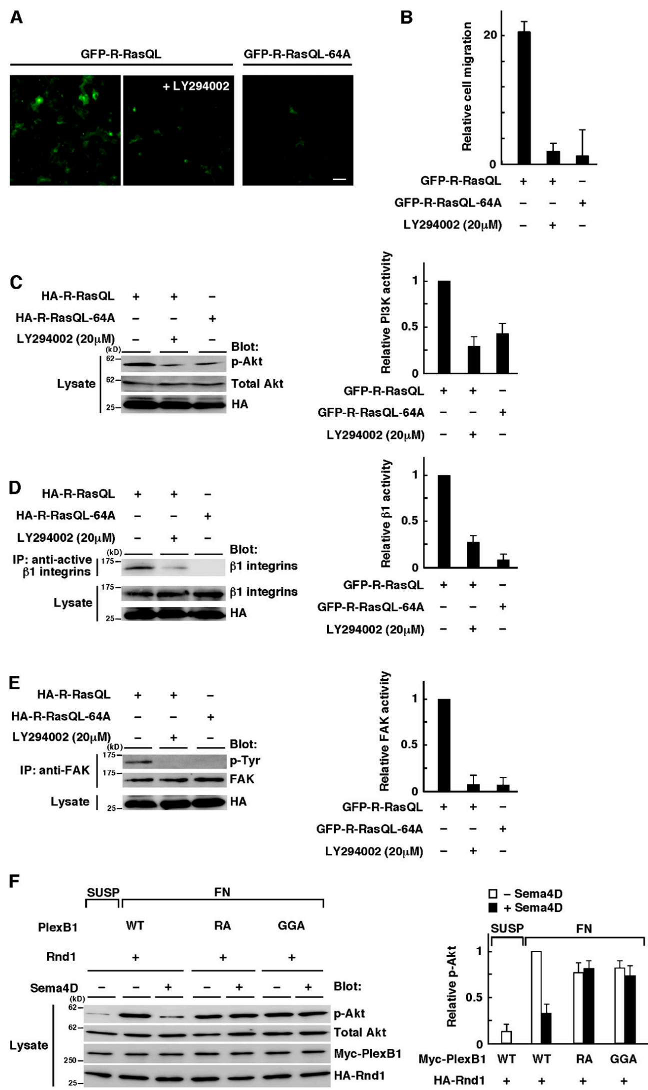


Figure 9. **Sema4D/Plexin-B1–Rnd1 inhibits PI3-K activity through inactivation of R-Ras.** (A) COS-7 cells transfected with GFP–R-Ras–QL or its effector loop mutant, R-Ras–QL–64A, were tested in a cell migration assay using the noncoated bare transwells in either the presence or absence of 20 μM LY294002. Bar, 1 mm. (B) Relative cell migration was determined. Results are the means ± SEM of three independent experiments. (C) Phosphorylated and total Akt were analyzed by immunoblotting with phospho-Akt (Ser473) and total Akt antibodies. (D and E) The state of β_1 integrin activity (D) and FAK tyrosine phosphorylation (E) were analyzed. (F) COS-7 cells prepared as described in the legend to Fig. 5 were directly lysed on dishes, and the lysates were analyzed by the immunoblot analysis with phospho-Akt (Ser473) and total Akt antibodies. Results are the means ± SEM of three independent experiments.

of the downstream effector FAK. It has been reported that prominent PI3-K–dependent phosphorylation of Akt occurs in response to β_1 integrin–mediated adhesion (Velling et al., 2004). We examined the effect of Sema4D/Plexin-B1–mediated R-Ras GAP activity on PI3-K activity by measuring the phosphorylation of Akt. As shown in Fig. 9 F, expression of Plexin-B1–WT and Rnd1 inhibited the fibronectin-mediated Akt phosphorylation in the presence of Sema4D. However, this inhibition was not observed in cells expressing Plexin-B1–GGA or Plexin-B1–RA that had no ability to exhibit R-Ras GAP activity (Fig. 5 B). These results suggest that PI3-K activity is necessary for R-Ras–mediated activation of β_1 integrins and that Sema4D/Plexin-B1–Rnd1 inactivates PI3-K through down-regulation of R-Ras activity.

Down-regulation of PI3-K activity is responsible for Sema4D/Plexin-B1–induced suppression of β_1 integrin activity

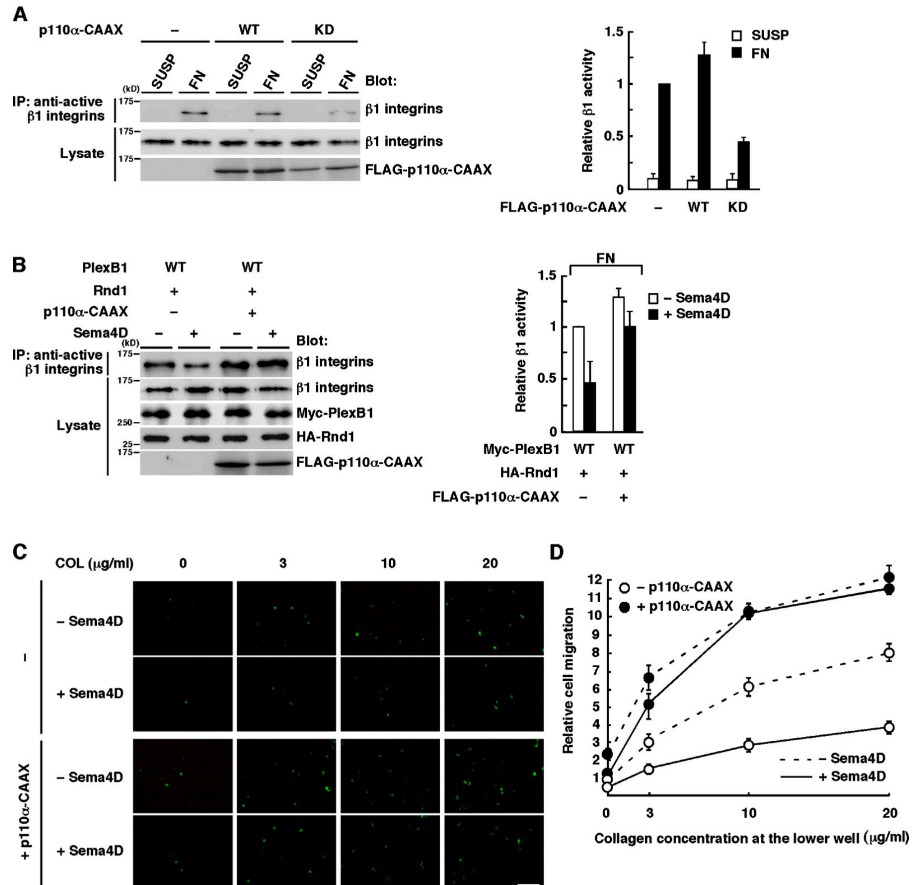
To clear out the role of PI3-K downstream of Sema4D/Plexin-B1, leading to suppression of β_1 integrin activity, we transfected p110α-CAAX, a constitutively active form of PI3-K (Berrier et al., 2000), and tested the ability of Sema4D/Plexin-B1 to inhibit β_1 integrin activation. In COS-7 cells, overexpression of p110α-CAAX by itself did not induce β_1 integrin activation in the absence of fibronectin (Fig. 10 A; Oertli et al., 2000). On the other hand, overexpression of the kinase-dead form of p110α blocked the fibronectin-dependent β_1 integrin activation. These results suggest that PI3-K activity is necessary but that PI3-K activity by itself is not sufficient for inducing β_1 integrin activation. We next examined whether the down-regulation of PI3-K activity downstream of Sema4D/Plexin-B1 is necessary for inhibition of β_1 integrin activity. As shown in Fig. 10 B, overexpression of p110α-CAAX blocked Sema4D/Plexin-B1–dependent inactivation of β_1 integrins. We also examined the ability of Sema4D to inhibit cell migration in cells expressing p110α-CAAX. As shown in Fig. 10 (C and D), overexpression of p110α-CAAX in PC12 cells almost completely blocked the Sema4D-mediated inhibition of cell migration. These results suggest that down-regulation of PI3-K activity, downstream of Sema4D/Plexin-B1, is responsible for suppression of β_1 integrin activity and inhibition of the ECM-mediated cell migration.

Discussion

Cell migration is a fundamental cellular process in many cell types, and semaphorins are known to act as a negative regulator for integrin-mediated cell migration. We show that the Sema4D receptor, Plexin-B1, down-regulates R-Ras activity and inhibits ECM-mediated integrin activation and cell migration through its R-Ras GAP activity.

R-Ras is implicated in integrin regulation, and a constitutively active form of R-Ras has been shown to increase the affinity of integrins for fibronectin (Zhang et al., 1996) and to stimulate cell migration (Keely et al., 1999). We have examined a role of R-Ras in ECM-mediated integrin activation and cell migration and showed that R-Ras is markedly activated by the ECM and that this activation is required for activation of β_1 integrins and subsequent cell migration, as inactivation of R-Ras activity by expression of the GAP domain of p98–R-RasGAP or knockdown of R-Ras by R-Ras–specific siRNA markedly reduces ECM-mediated integrin activation and cell migration. Our results also revealed that β_1 integrins are required for R-Ras activation upon ECM-mediated adhesion. This suggests a positive feedback during cell-substrate adhesion, implicating R-Ras activation and the consequent further strengthening of integrin-mediated functions. Therefore, R-Ras is a central regulator for ECM-mediated integrin activation and cell migration, and the regulation of R-Ras activity is critical for integrin-mediated cell migration.

Figure 10. **Down-regulation of PI3-K activity is responsible for Sema4D/Plexin-B1–induced suppression of β_1 integrin activity.** (A, left) Lysates of COS-7 cells expressing p110α-CAAX, a constitutively active form of PI3-K, or its kinase-dead (KD) form were immunoprecipitated with HUTS-4. (right) Relative β_1 integrin activity was analyzed. (B) Activity of β_1 integrins in COS-7 cells expressing the indicated expression plasmids with or without Sema4D stimulation were examined. (C and D) PC12 cells transfected with GFP alone (open symbols) or GFP plus p110α-CAAX (closed symbols) were tested in the transwell assay in either the presence or absence of Sema4D. Results are the means ± SEM of three independent experiments. Bar, 1 mm.



Semaphorins are implicated in migration of a variety of cells. Stimulation of Plexin-B1 by Sema4D is reported to hamper integrin-based adhesion and cell migration in NIH-3T3 cells (Barberis et al., 2004). We have reported that Plexin-B1 encodes an R-Ras GAP in the cytoplasmic tail and that stimulation of the Plexin-B1–Rnd1 complex by Sema4D induces the R-Ras GAP activity and resultant repulsive response of neuronal growth cone (Oinuma et al., 2004a). We demonstrate here that Plexin-B1/Rnd1–mediated R-Ras GAP activity is also involved in Sema4D-induced inhibition of integrin activation and cell migration. Furthermore, the COOH-terminal PDZ domain binding motif of Plexin-B1 is dispensable for suppression of integrin activity and cell migration by Sema4D. In addition to Sema4D, class 3 semaphorins have been shown to control adhesion and migration of endothelial cells by inhibiting integrin function (Serini et al., 2003), and Sema3A signaling–deficient mice have shown defective migration of neural crest cells (Kawasaki et al., 2002). Furthermore, Plexin-C1, a receptor of semaphorin A39R, was recently reported to inhibit integrin-mediated adhesion and chemokine-induced migration (Walzer et al., 2005). The R-Ras GAP–homologous domains are well conserved among plexin families, including Plexin-A and -C1. In addition, we recently reported that the down-regulation of R-Ras activity is also required for the Sema3A/Plexin-A–induced repulsive response in hippocampal neurons (Oinuma et al., 2004a). We speculate that the direct regulation of R-Ras activity by plexins is likely to be a mutual signaling pathway among plexin families and that this R-Ras GAP activity of plexin families may be a critical signaling system for semaphorin-regulated cell migration.

Semaphorins were initially identified as repulsive factors for axon guidance, and many neurons use members of the integrin family of cell surface receptors for responses to neurite growth promoting factors, and integrin activation regulates neurite outgrowth (Hynes, 2002). Recently, expression of constitutively active R-Ras was shown to promote integrin-dependent neurite outgrowth of retinal neurons, suggesting that R-Ras activity plays an important role in integrin-dependent neurite outgrowth (Ivins et al., 2000). Therefore, it is proposed that the down-regulation of R-Ras activity by Plexin-B1 via R-Ras GAP activity suppresses R-Ras–mediated integrin activation and thereby induces growth cone collapse and inhibition of neurite outgrowth. With respect to signaling of other repulsive factors, the ephrin-B1 receptor EphB2, another family of the repulsive factor receptor, was also reported to suppress integrin-mediated functions by inactivating R-Ras (Zou et al., 1999), suggesting that repulsive guidance cues inhibit integrin-mediated functions by inactivating R-Ras in general and that R-Ras acts as a common regulator of integrin activation and cell migration (Serini and Bussolino, 2004).

We also examined the downstream signaling of Sema4D/Plexin-B1–mediated R-Ras GAP activity leading to inactivation of β_1 integrins and found that down-regulation of PI3-K activity is responsible for Sema4D/Plexin-B1–induced suppression of

β_1 integrin activity and cell migration. PI3-K activity is known to be required for R-Ras–mediated enhancement of cell migration (Keely et al., 1999; Rincón-Arano et al., 2003). PI3-K has emerged as the predominant effector for R-Ras, and R-Ras is a more potent activator of PI3-K than other Ras family members (Marte et al., 1997; Suire et al., 2002). On the other hand, PI3-K activity has been shown to promote interaction between talin with the β_1 integrin cytoplasmic tail, leading to the clustering and activation of integrins (Calderwood et al., 1999; Martel et al., 2001; Calderwood et al., 2002). Integrin activation by mechanical stretch is also mediated by PI3-K and is followed by an increase in integrin binding to the extracellular matrix proteins (Katsumi et al., 2005). Therefore, elevated PI3-K activity by activated R-Ras may trigger a sequence of events leading to clustering and activation of integrins, although overexpression of p110α-CAAX by itself is not sufficient for inducing β_1 integrin activation (Fig. 10 A; Oertli et al., 2000). We used the monoclonal antibody HUTS-4, which detects hybrid domain swing-out in β_1 integrins, a process most commonly associated with ligand binding affinity (Mould et al., 2003), to measure activity of β_1 integrins and revealed that Sema4D/Plexin-B1–mediated R-Ras GAP activity suppresses affinity of β_1 integrins through inactivation of PI3-K activity. Consistent with our results, a previous report demonstrated that an R-Ras–mediated increase in affinity of the β_1 integrins is dependent on PI3-K activity by performing the ligand binding assay in mast cells (Kinashi et al., 2000). On the other hand, Oertli et al. (2000) have shown that PI3-K activity is not required for R-Ras–mediated integrin activation in CHO cells by using a ligand-mimetic antibody, PAC-1. Therefore, we speculate that this discrepancy may be due to the differences in ways to measure integrin activity or that R-Ras may regulate integrin activity via both PI3-K–dependent and –independent pathways, depending on the cell type.

In conclusion, our results demonstrate that R-Ras activity is required for ECM-mediated integrin activation and cell migration and that the Sema4D/Plexin-B1–Rnd1 complex regulates integrin activation and cell migration through the R-Ras GAP activity. However, a variety of molecules such as ErbB-2 and Met have been known to be involved in plexin signaling, inducing diverse physiological functions (Giordano et al., 2002, Swiercz et al., 2004). It was recently shown that Plexin-B1 enhances chemotaxis of endothelial cells through the activation of multiple intracellular tyrosine kinase cascades independent of the R-Ras GAP activity (Basile et al., 2005). Regulation of R-Ras activity, tyrosine kinases, and other signaling mechanisms may participate in diverse actions of plexins. Further work will be required to delineate the precise mechanism of R-Ras–mediated integrin activation and its regulation by plexins for cell migration during physiological and pathological processes, including neural cell migration, angiogenesis, and tumor metastasis.

Materials and methods

DNA constructs and site-directed mutagenesis

Plexin-B1 cDNA was provided by L. Tamagnone (Torino University, Torino, Italy). HA-tagged Rnd1; HA- and GFP-tagged human R-Ras and R-Ras–QL (Q87L); the GST-fused Ras binding domain of c-Raf-1 (amino acids 53–130); the NH_2-terminal HA-tagged myristoylated form of R-RasGAP; and Myc-tagged Plexin-B1, Plexin-B1–GGA (L1849G, V1850G, and P1851A), Plexin-B1–RA (R1677A, R1678A, and R1984A), Plexin-B1–ΔC (lacking the last seven COOH-terminal amino acids), and Plexin-B1–N-Cyt–GGA (amino acids 1511–1915) were described previously (Oinuma et al., 2004a,b). The effector loop mutant of R-Ras, R-Ras–DA (D64A), was generated by a PCR-mediated mutagenesis. NH_2-terminal FLAG-tagged p110α was a gift from T. Katada (Tokyo University, Tokyo, Japan), and CAAX sequence was fused to the COOH terminus to create a constitutively active form as described previously (Katoh et al., 2002). The specific siRNA for R-Ras was designed to target 19 nucleotides at nucleotides 359 and 377 (5′-gcaagctcttcactcagat-3′), whereas the control siRNA was designed at nucleotides 426 and 444 (5′-caaggcagatctggagaca-3′), and both were expressed by using a siRNA expression vector (Ambion) as described previously (Oinuma et al., 2004a).

Antibodies and reagents

The pharmacological PI3-K inhibitor LY294002 was purchased from Calbiochem. A soluble form of Sema4D fused to human IgG_1-Fc was a gift from H. Kikutani (Osaka University, Osaka, Japan). We used the following antibodies: mouse monoclonal antibodies against Myc and phosphotyrosine; a rabbit polyclonal antibody against p125-FAK (Upstate Biotechnology); mouse monoclonal antibodies against α-tubulin (Sigma-Aldrich), β_1 integrins (BD Biosciences), and active β_1 integrins, HUTS-4 (Chemicon); a rabbit polyclonal antibody against R-Ras (Santa Cruz Biotechnology, Inc.); a rat monoclonal antibody against HA (Roche); and HRP-conjugated secondary antibodies (DakoCytomation). For functional studies in the transwell assay, we used the following antibodies: the affinity-related β_1 integrin–activating monoclonal antibody 8A2 (IgG2a); the functional blocking monoclonal antibody against the integrin α_1 subunit, 3A3 (IgG1; Serotec); the functional blocking monoclonal antibody against the integrin β_1 subunit, P5D2 (IgG1; Chemicon); and a mouse monoclonal antibody against the extracellular ligand binding region (raised against amino acids 771–1070 of human origin) of Plexin-B1 (IgG2b; Santa Cruz Biotechnology, Inc.). FITC- and PE-conjugated F(ab′)$_2$–specific secondary antibodies for flow cytometry were purchased from Jackson ImmunoResearch Laboratories. The PhosphoPlus Akt Antibody kit (Cell Signaling) was used for the analysis of the phosphorylation state of Akt.

Immunoblotting

Proteins were separated by 12.5% SDS-PAGE and were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 3% low-fat milk in TBS and incubated with primary antibodies. The primary antibodies were detected with HRP-conjugated secondary antibodies and a chemiluminescence detection kit (Chemi-Lumi One; Nacalai Tesque). Images were captured using a LAS 1000 analyzer (Fuji) equipped with Image Gauge 4.0 software (Fuji).

Immunofluorescence microscopy

Cells on coverslips were fixed with 4% PFA in PBS for 15 min and washed with PBS five times. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and incubated with 10% FBS in PBS for 30 min to block nonspecific antibody binding. Cells were incubated with an anti–R-Ras antibody (1:200 dilution) for 1 h and then incubated with an Alexa Fluor 594–conjugated secondary antibody for 1 h. Cells were washed in PBS for 1 h and mounted in 90% glycerol containing 0.1% p-phenylenediamine dihydrochloride in PBS. Images were captured at RT using a microscope (Eclipse E800; Nikon) and a 40 $\times$ 0.75 objective (Nikon) equipped with a digital camera (DC350F; Leica). The images were arranged and labeled using Photoshop software (Adobe).

Cell culture and transfection

COS-7 cells were cultured in DME containing 10% FBS, 4 mM glutamine, 100 U/ml penicillin, and 0.2 mg/ml streptomycin under humidified conditions in 95% air and 5% CO_2 at 37°C. PC12 cells were maintained in RPMI 1640 with 10% horse serum (HS) and 5% FBS. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A soluble form of Sema4D was expressed as a fusion protein with the Fc fragment of human IgG_1. Stimulation with Sema4D was performed by incubation of the cells with Sema4D-Fc–containing medium at 37°C.

Cell migration assay

10^4 cells were detached with 1.5 mM EDTA in PBS, washed three times with serum-free medium, resuspended in DME containing 1% BSA, seeded on the upper side of 8-μm pore filters of Transwell chambers (Costar), which were coated on the lower side with varying concentrations of either fibronectin or collagen I (Sigma-Aldrich), and incubated for 7 h. Cells on the upper side of the filters were mechanically removed, and cells on the

lower side were fixed with 4% PFA. The numbers of migrated cells through the filter were counted by the fluorescence of GFP or the staining with crystal violet (*A*). At the same time, the cells were seeded onto 24-well plastic culture plates to count the total number of transfected cells (*B*). Relative cell migration was then determined by the number of migrated cells normalized to the total number of transfected cells (*A/B*). Unless described, the value from the GFP-transfected cells in the absence of coating was defined as 1. For functional studies using activating or inhibitory monoclonal antibodies, cells were pretreated with 5 μg/ml of antibodies or corresponding negative IgG controls for 5 min before seeding onto the transwells. Images were captured at RT in PBS using a microscope (Eclipse TE300-FN; Nikon) and a Plan Fluor 10 × 0.30 objective (Nikon) equipped with digital camera (DS-L1 and DS-5M; Nikon). The images were arranged and labeled using Photoshop 7.0 software.

Measurement of the activity of β_1 integrins by immunoprecipitation

Measurement of β_1 integrin activity by immunoprecipitation was performed as described previously (Serini et al., 2003). 3×10^6 COS-7 cells were maintained in DME containing 1% FBS after transfection. 16 h after transfection, cells were detached with 1.5 mM EDTA in PBS, washed three times with serum-free medium, and resuspended in 10 ml of 1% BSA in DME with or without Sema4D-Fc. The cell suspension was plated onto 10-cm plates coated with or without 10 μg/ml fibronectin and incubated at 37°C for 15 min. The cells were lysed directly on dishes with ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM sodium vanadate, 25 mM NaF, 10 μg/ml pepstatin, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 5 μg/ml HUTS-4, immunoprecipitated for 2 h, and subsequently incubated with protein G–Sepharose beads (GE Healthcare) for 1 h at 4°C. After the beads were washed twice with the ice-cold cell lysis buffer, the bound proteins were eluted in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting with the monoclonal antibody against β_1 integrins. To measure the activity of β_1 integrins in PC12 cells, 10^6 cells were maintained in RPMI 1640 containing 1% HS for 12 h, detached with 1.5 mM EDTA in PBS, washed three times with serum-free medium, and resuspended in 10 ml of 1% BSA in RPMI 1640 with or without Sema4D-Fc. The cell suspension was plated onto 10-cm plates coated with or without 10 μg/ml collagen I and incubated at 37°C for 3 h. The cells were lysed directly on dishes with ice-cold cell lysis buffer.

Measurement of the activity of β_1 integrins by ELISAs

Measurement of the activity of β_1 integrins by ELISAs under detergent-free condition was performed as described previously (Shih et al., 1999). 10^5 cells transfected in 24-well plastic culture plates were detached with 1.5 mM EDTA in PBS, washed three times with serum-free medium, and resuspended in 1 ml DME containing 1% BSA, with or without Sema4D-Fc. One tenth of the resuspended cells (100 μl) were seeded onto the 96-well assay plates, which were coated with 10 μg/ml of either fibronectin or collagen I. Cell adhesion was allowed for 15 min at 37°C. Then, the cells were delicately washed once with PBS and the adherent cells were fixed with 4% PFA. After the fixative, the cells were thoroughly rinsed with PBS containing 0.1% BSA. To avoid nonspecific binding, the cells were incubated with PBS containing 5% BSA for 3 h at RT. Cells were then incubated overnight at 4°C with 2 μg/ml HUTS-4. After the incubation with primary antibody, the wells were rinsed and blocked with PBS containing 5% BSA for 3 h at RT before they were exposed to an HRP- conjugated secondary antibody. After the incubation, cells were rinsed again with PBS followed by distilled H_2O. The peroxidase color reaction was developed in the dark using *O*-phenylenediamine according to the manufacturer's instructions (ELISA OPD kit; Nacalai Tesque), and the plate was read on a kinetic microtiter plate reader (GENios; Tecan) using the XFluor4 program (Tecan). The antibody concentration and incubation times were optimized to ensure testing in the linear range. Expression levels of the constructs used in the assay were also verified by immunoblot analysis.

Flow cytometry analysis

Analysis of cell surface expression of active β_1 integrins by flow cytometry was performed as described previously (Wang et al., 2002). 10^6 PC12 cells were seeded onto 6-cm noncoated plates in RPMI 1640 containing 10% HS and 5% FBS. 18 h after seeding, cells were treated with medium containing Sema4D-Fc or Sema4D-Fc plus 1 mM Mn^{2+} for 3 h at 37°C. Cells were washed once with PBS and resuspended in blocking solution containing 5% dissociation buffer (Invitrogen) and 2% sheep serum in PBS. Cells were then incubated with 2.5 μg HUTS-4 or buffer alone for 1 h at 4°C, washed with the blocking solution, and labeled with FITC-conjugated secondary antibody for 30 min at 4°C. Cells were then washed and analyzed with an EPICS ELITE flow cytometer using the EXPO32 analysis program (Beckman Coulter). For the analysis of active β_1 integrins in transiently transfected COS-7 cells, 10^6 cells were transfected with a GFP expression vector together with various other expression vectors. Cells were kept for 18 h in DME containing 10% FBS after transfection, stimulated for 5 min at 37°C with or without Sema4D-Fc, and were collected and incubated with HUTS-4 antibody or buffer alone as described previously in this section. Cells were labeled with a PE-conjugated secondary antibody, and expression of GFP and activity of β_1 integrins (PE staining) were simultaneously analyzed by two-color flow cytometry. Analysis of the intensity of PE staining in a GFP-positive population was performed as described previously (Ohgushi et al., 2005). Approximately 10,000 cells were analyzed in each experiment, and the results shown are representative of two independent experiments.

Measurement of R-Ras activity

Measurement of R-Ras activity in cells was performed as described previously (Oinuma et al., 2004a). 7×10^5 COS-7 cells were maintained in DME containing 1% FBS after transfection. The cell suspension was prepared as described (see Measurement of the activity of β_1 integrins by immunoprecipitation) and plated onto plastic dishes coated with or without 10 μg/ml fibronectin and incubated at 37°C for 15 min. The cells were lysed directly on dishes with ice-cold cell lysis buffer (25 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 75 μg of GST-fused Ras binding domain of c-Raf-1 (GST-RBD). To examine the effect of collagen I and Sema4D stimulation on R-Ras activity in PC12 cells, 10^6 cells were maintained in RPMI 1640 containing 1% HS for 12 h, detached with 1.5 mM EDTA in PBS, washed three times with serum-free medium, and resuspended in 10 ml of 1% BSA in RPMI 1640 with or without Sema4D-Fc. For samples indicated, cells were treated with 5 μg/ml of monoclonal β_1 integrin blocking (P5D2) or activating (8A2) antibody before replating. Cells were either kept in suspension or plated onto 6-cm plates coated with (1 or 10 μg/ml) or without collagen I and incubated at 37°C for 15 min. The cells were lysed directly on dishes with ice-cold cell lysis buffer, and the lysates were used in a pull-down assay using GST-RBD.

Detection of FAK tyrosine phosphorylation

Detection of tyrosine phosphorylation of FAK was performed as described elsewhere (Sieg et al., 2000). The cells were lysed directly on dishes with ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 10% glycerol, 1 mM sodium vanadate, 25 mM NaF, 10 μg/ml pepstatin, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 4 μg/ml of the polyclonal antibody against FAK, immunoprecipitated for 2 h, and subsequently incubated with protein A–Sepharose beads (GE Healthcare) for 1 h at 4°C.

Detection of Akt serine phosphorylation

COS-7 cells were maintained in DME with 0.5% FBS after transfection for 36 h. We added 20 μM LY294002 directly to the culture medium after transfection and changed it at every 12 h to reduce the basal levels of PI3-K activity. Cells were directly lysed on dishes with 1× Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting.

Online supplemental material

Fig. S1 shows reduction in endogenous R-Ras protein by RNA interference in PC12 cells and requirement of endogenous R-Ras protein in collagen-mediated PC12 cell migration. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508204/DC1.

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