TSC2 modulates actin cytoskeleton and focal adhesion through TSC1-binding domain and the Rac1 GTPase

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Tuberous sclerosis complex (TSC) 1 and TSC2 are thought to be involved in protein translational regulation and cell growth, and loss of their function is a cause of TSC and lymphangioleiomyomatosis (LAM). However, TSC1 also activates Rho and regulates cell adhesion. We found that TSC2 modulates actin dynamics and cell adhesion and the TSC1-binding domain (TSC2-HBD) is essential for this function of TSC2. Expression of TSC2 or TSC2-HBD in TSC2−/− cells promoted Rac1 activation, inhibition of Rho, stress fiber disassembly, and focal adhesion remodeling. The down-regulation of TSC1 with TSC1 siRNA in TSC2−/− cells activated Rac1 and induced loss of stress fibers. Our data indicate that TSC1 inhibits Rac1 and TSC2 blocks this activity of TSC1. Because TSC1 and TSC2 regulate Rho and Rac1, whose activities are interconnected in a reciprocal fashion, loss of either TSC1 or TSC2 function may result in the deregulation of cell motility and adhesion, which are associated with the pathobiology of TSC and LAM.

Introduction

Tumor suppressors tuberous sclerosis complex (TSC) 1 and TSC2, also named as hamartin and tuberin, respectively, play a critical role in protein translational regulation and cell growth from *Drosophila melanogaster* to mammals (Krymskaya, 2003; Kwiatkowski, 2003; Manning and Cantley, 2003). TSC1 and TSC2 proteins form a cytosolic heterodimer and exert their function as negative regulators of the mammalian target of rapamycin (mTOR) signaling pathway (Nellist et al., 1999; Goncharova et al., 2002; Kwiatkowski et al., 2002). TSC2 encodes in its COOH terminus a GTPase-activating protein (GAP) for small GTPase Rheb (Ras homologue enriched in brain), whose activity antagonizes mTOR signaling (Gao et al., 2002; Garami et al., 2003; Inoki et al., 2003a; Li et al., 2004). Growth factors, insulin, nutrients, and the cellular energy levels regulate the activity of TSC2 (McManus and Alessi, 2002; Inoki et al., 2003b).

TSC1 (gene encoding protein TSC1, hamartin) and *TSC2* (gene encoding protein TSC2, tuberin) genes are susceptibility factors for TSC (Crino and Henske, 1999; Sparagana and Roach, 2000; Cheadle et al., 2000) and lymphangioleiomyomatosis (LAM; Sullivan, 1998; Carsillo et al., 2000; Johnson and Tattersfield, 2002). The pathobiology of TSC and LAM are generally thought to be linked to abnormal cell growth. However, the neurological manifestations of TSC have been defined as a "neuronal migration disorder" and occur due to aberrant neuronal motility during brain development (Crino and Henske, 1999; Vinters et al., 1999; Gutmann et al., 2000; Sparagana and Roach, 2000); and LAM is a potentially metastatic disease (Yu et al., 2001; Henske, 2003; Karbowniczek et al., 2003), suggesting a role for TSC1 and TSC2 in cell motility. Furthermore, TSC1-deficient murine embryonic fibroblasts have an impaired ability to form serum-induced stress fibers and focal adhesions (Kwiatkowski et al., 2002). Conversely, overexpression of TSC1 or TSC2 in human kidney epithelial cells results in increased E-cadherin expression, increased cell adhesion, and reduced chemotactic migration (Astrinidis et al., 2002; Li et al., 2003). Importantly, TSC1 binds to the ezrin-radixin-moesin (ERM) family of actin-binding proteins (Lamb et al., 2000). In cultured cortical neurons, TSC1 physically anchors intermediate filaments to the actin cytoskeleton by binding to both neurofilament light chains and the ERM proteins (Haddad et al., 2002). Together, these data suggest the potential involvement of TSC2 and TSC1 in cell motility. However, the precise mechanism and

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Abbreviations used in this paper: ERM, ezrin-radixin-moesin; GAP, GTPase-activating protein; LAM, lymphangioleiomyomatosis; LAMD, human LAM-derived; mTOR, mammalian target of rapamycin; PDGFR, PDGF receptor; S6K, p70 S6 kinase; SE, standard error; TSC, tuberous sclerosis complex; TSC2-HBD, TSC1-binding domain of TSC2.

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the relevance of these findings to aberrant neuronal motility in TSC and LAM metastasis remains an enigma.

The Rho family of small GTPases, RhoA, Rac, and Cdc42, are key regulators of actin cytoskeletal remodeling, cell adhesion, and migration. RhoA promotes the formation of stress fibers that are linked to focal adhesions; Rac induces the formation of membrane ruffles and lamellipodia; and Cdc42 induces filopodia formation (Etienne-Manneville and Hall, 2002; Burridge and Wennerberg, 2004). Reciprocal activation of RhoA, Rac, and Cdc42 is critical for the regulation of cell adhesion and motility (Horwitz and Parsons, 1999; Etienne-Manneville and Hall, 2002), and dysregulation of this balance promotes cell transformation and metastasis (Sahai and Marshall, 2002). Recent studies suggest that TSC1 regulates Rho activity through the Rho-activating domain within its NH_2 terminus by an unknown mechanism (Lamb et al., 2000). Interestingly, the Rho-activating domain of TSC1 (amino acids 145–510) overlaps with the domain that binds TSC2: the amino acids 302–430 of TSC1 (Hodges et al., 2001) associate with amino acids 1–418 of TSC2 and are required for TSC1–TSC2 complex formation, which potentially stabilizes each protein (Nellist et al., 1999; Henske, 2003; Krymskaya and Shipley, 2003). These data suggest that the interaction of TSC1 with TSC2 may be important for TSC1-dependent Rho activation and cell adhesion. However, how TSC2 and TSC1 complex formation is involved in regulating actin remodeling and adhesion has not been identified.

Here, we show that TSC2 regulates the actin cytoskeleton and focal adhesion, and the TSC1-binding domain of TSC2 (TSC2-HBD), which corresponds to amino acids 1–460 in the NH_2 terminus of TSC2, is both necessary and sufficient for this function. Importantly, down-regulation of TSC1 with siRNA in TSC2−/− cells also induces disassembly of stress fibers and focal adhesion remodeling, indicating that TSC1 is required for TSC2-dependent actin remodeling. Furthermore, we show that the role of TSC2 in modulating actin dynamics is distinct from its function as a negative regulator of the rapamycin-sensitive mTOR/p70 S6 kinase (S6K) signaling pathway.

Results

TSC2 is necessary for dynamic membrane protrusions during wound closure

To explore whether or not TSC2 regulates cell motility, we performed live imaging of the wound closure of TSC2-deficient

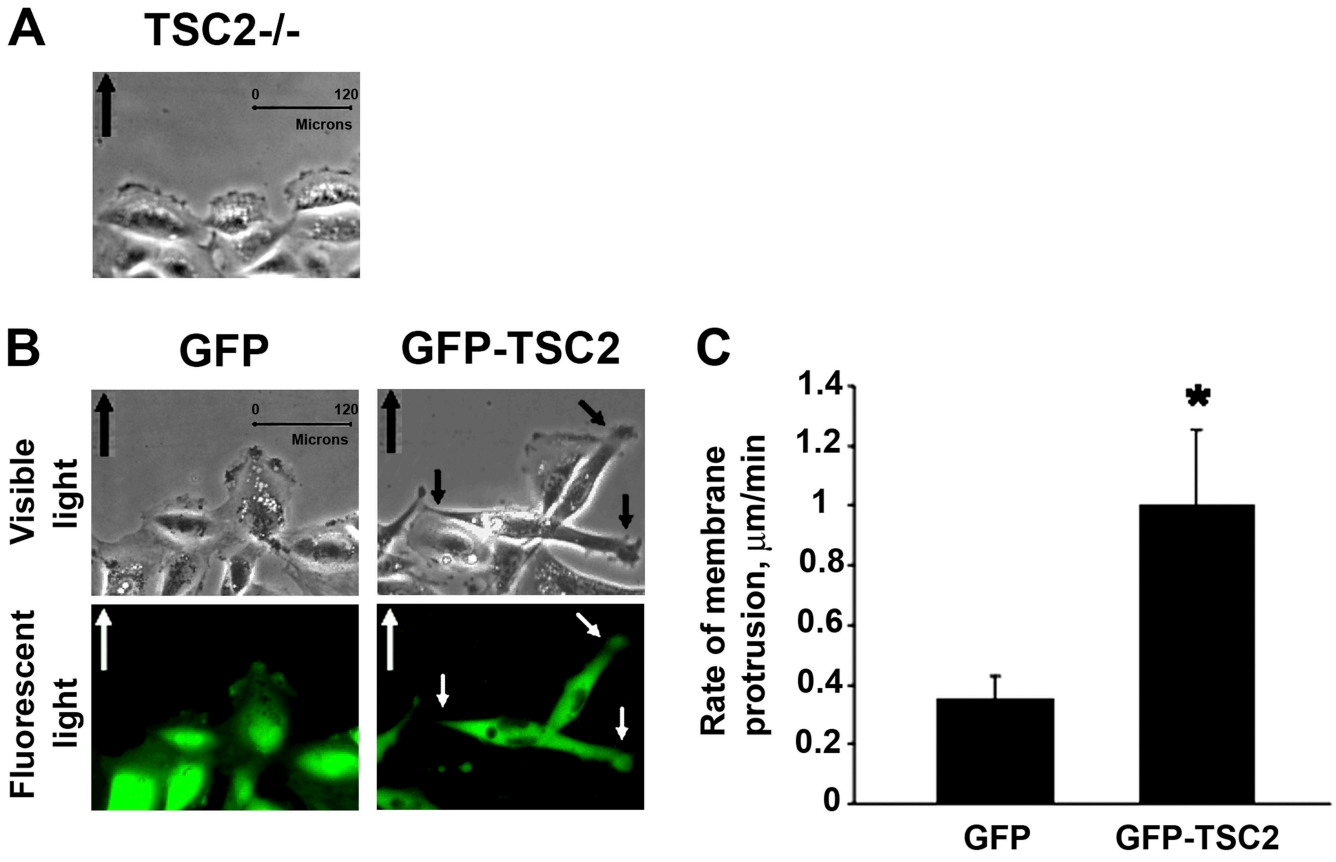


Figure 1. **Re-expression of TSC2 changes TSC2−/− cell morphology and dynamics during wound closure.** (A) Phase-contrast micrograph of time-lapse analysis of TSC2−/− cell motility during wound closure at 4 h after wound scraping; images are representative of three independent experiments. (B) Phase-contrast and fluorescence micrographs demonstrate the representative phenotypes of GFP- or GFP-TSC2–infected cells. TSC2−/− cells, infected with GFP-TSC2 or control GFP replication-deficient adenovirus constructs, were first serum-deprived, and then subjected to live image analysis of wound closure in the presence of 2% FBS. Short arrows indicate differences in membrane protrusion; long arrows indicate direction of cell movement. Bars, 120 μm. Images were taken using a Leitz Inverted Microscope in both the phase-contrast and green fluorescence channels. Images are representative from three independent experiments. (C) Statistical analysis of the rate of membrane protrusion in TSC2−/− cells infected either with GFP or GFP-TSC2. *, $P < 0.0001$ for GFP-TSC2-infected cells versus GFP-infected cells by ANOVA (Bonferroni-Dunn test).

smooth muscle ELT3 cells, which were either untreated or transduced with a replication-deficient adenovirus expressing GFP-tagged wild-type TSC2 or control GFP. As shown in Fig. 1 A and Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1), TSC2−/− cells retained their motile properties (Irani et al., 2002); however, their movement was characterized by the formation of short lamellipodia at the leading edge of migrating cells. Expression of GFP-tagged TSC2 markedly changed the pattern of cell dynamics; during wound closure, moving cells formed dynamic membrane protrusions, and the rate of membrane extension for GFP-TSC2–infected cells was 1.00 ± 0.25 μm min^{-1} compared with 0.35 ± 0.08 μm min^{-1} for GFP-infected cells (Fig. 1, B and C; and Videos 2 and 3, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). Expression of control GFP in TSC2−/− cells had little effect on the pattern of cell movement (Fig. 1 B and Videos 4 and 5, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). These data demonstrate that reexpression of TSC2 markedly changes TSC2−/− cell morphology during cell motility and suggest that TSC2 might be important for the formation of membrane protrusions during directional movement.

TSC2 and TSC2-HBD regulate stress fiber disassembly

Because cell motility is regulated by actin remodeling, we examined actin rearrangements in TSC2−/− cells. F-actin staining revealed abundant stress fiber formation (Fig. 2 A, top). Surprisingly, PDGF, which promotes stress fiber disassembly and lamellipodia formation in most cell types (Zigmond, 1996) and promoted stress fiber disassembly in 3T3 cells (Fig. 2 A, bottom), had little effect on actin rearrangements and lamelli-

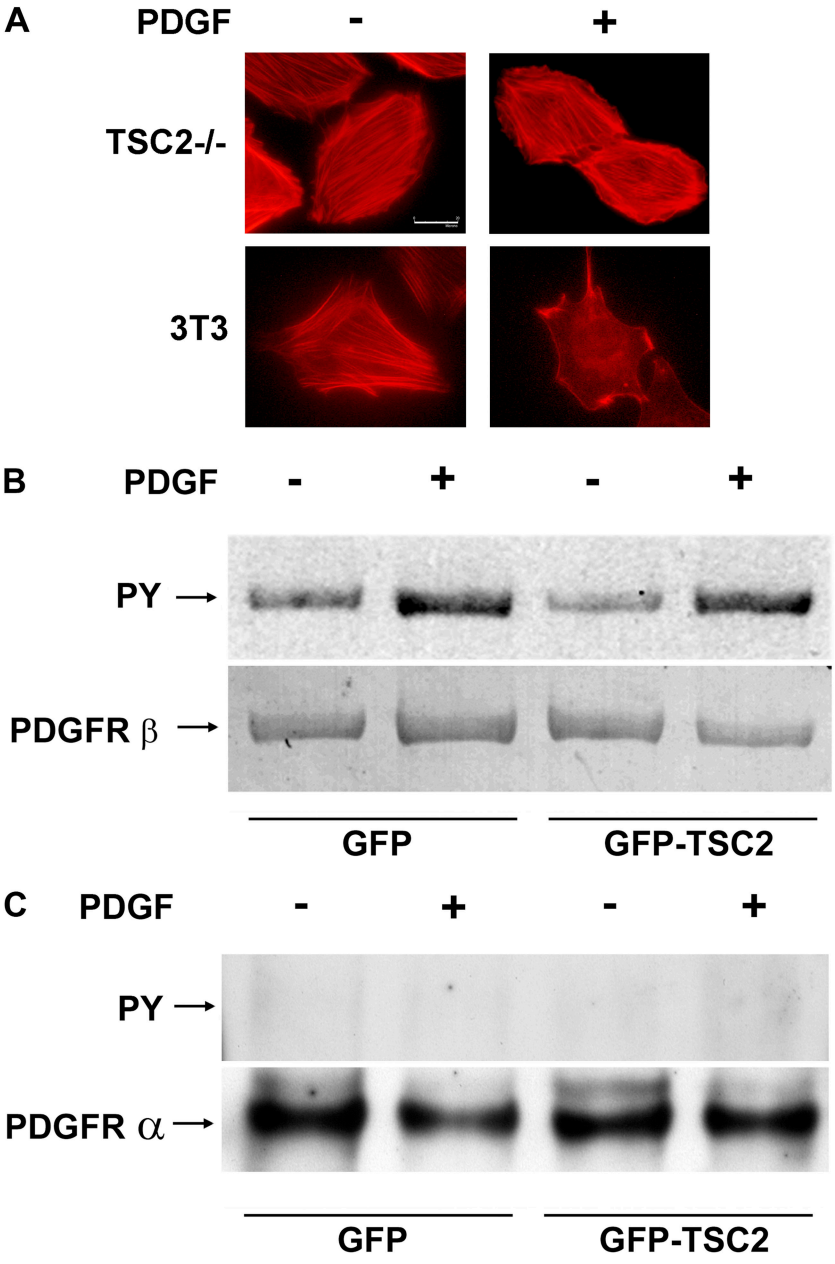


Figure 2. **PDGF has little effect on stress fiber disassembly in TSC2−/− cells.** (A) Rhodamine phalloidin staining of F-actin of serum-deprived TSC2−/− and 3T3 cells, which were either stimulated with 10 ng/ml of PDGF (+) or diluent (−) for 10 min. PDGFR activation in TSC2−/− cells: serum-deprived cells, transfected with either GFP or GFP-TSC2, were stimulated with PDGF. Equalized in protein content whole cell lysates were subjected to immunoprecipitation with either anti-PDGFRβ (B) or PDGFRα (C) antibodies; and then immunoblot analysis was performed with either anti-PDGFRβ, PDGFRα, or anti-phosphotyrosine (PY) antibodies.

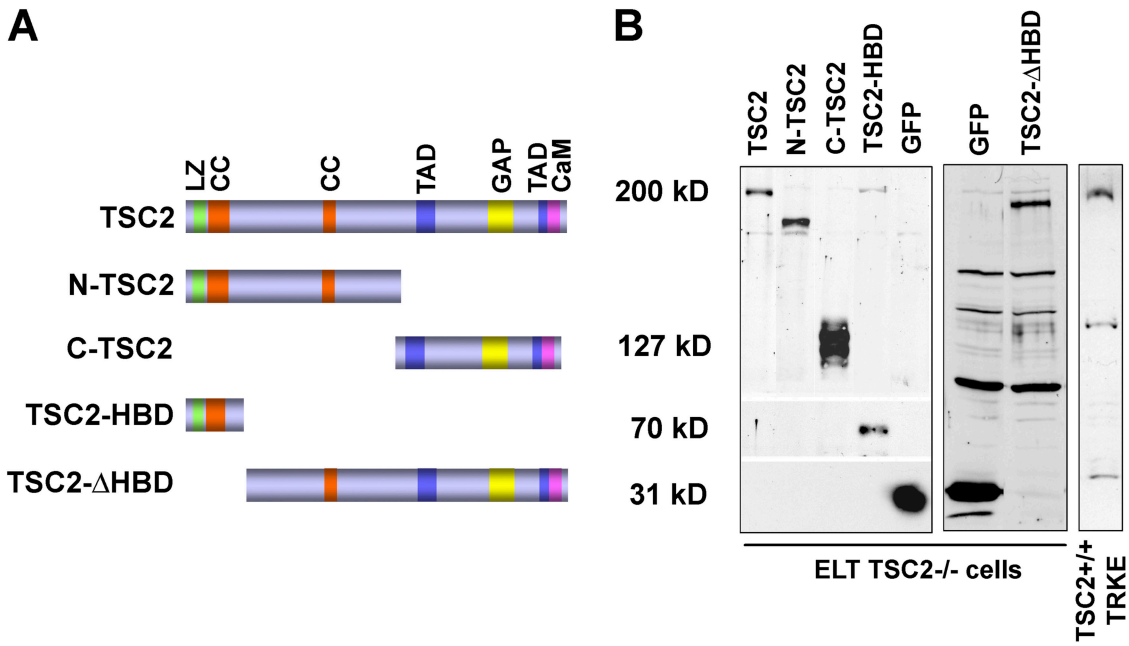


Figure 3. **Schematic representation and expression of GFP-tagged TSC2 constructs in the TSC2−/− cells.** (A) TSC2 includes leucine zipper (LZ), two coiled-coiled (CC), two transcription-activating domains (TAD), GAP homology (GAP), and calmodulin (CaM)-binding domain. (B) To identify expression of TSC2 mutants, cells were transfected with pEGFP vectors expressing GFP-tagged TSC2, N-TSC2, C-TSC2, TSC2-HBD, TSC2-ΔHBD, or control GFP. After 24 h of transient transfection, cells were lysed, and whole cell lysates were subjected to 8% SDS-PAGE followed by immunoblot analysis with anti-GFP antiserum. TSC2-positive rat TRKE cells, used as a positive control, were lysed and subjected to 8% SDS-PAGE followed by immunoblot analysis with anti-TSC2 antibody.

podia formation in TSC2−/− cells (Fig. 2 A, top). This finding was more surprising because we previously demonstrated that PDGF stimulates migration of TSC2−/− ELT3 cells (smooth muscle cells derived from Eker rat uterine leiomyomas; Irani et al., 2002). Because Zhang et al. (2003) demonstrated that PDGF receptor (PDGFR) α and PDGFRβ levels were reduced in TSC2−/−TP53−/− murine embryonic fibroblasts, we examined their expression in TSC2−/− rat ELT3 cells. Immunoblot analysis revealed that PDGFRα and PDGFRβ are expressed in these cells, and TSC2 expression had little effect on both receptor levels (Fig. 2, B and C). Stimulation of cells with PDGF-BB induced activation of PDGFRβ, but has little effect on PDGFRα (Fig. 2, B and C, respectively). These data suggest that PDGF-induced signaling is not defective in rat TSC2−/− cells at the receptor levels.

To examine whether TSC2 is required for actin remodeling, and to identify which domain of TSC2 is important for stress fiber disassembly, we tested a panel of GFP-tagged deletion constructs of TSC2 (Fig. 3). The reexpression of full-length TSC2 in TSC2−/− cells markedly promoted stress fiber disassembly and the formation of cortical actin compared with cells transfected with control GFP (Fig. 4 A). Interestingly, the expression of TSC2-ΔHBD in TSC2−/− cells containing the Rheb GAP domain (Fig. 4 A) or the expression of C-TSC2 (not depicted) had little effect on stress fiber disassembly. In contrast, expression of N-TSC2 (not depicted) or TSC2-HBD was sufficient to induce stress fiber disassembly (Fig. 4 A).

Stress fiber assembly is regulated by activation of Rho GTPase, which could be activated by TSC1 through its Rho-activating domain (Lamb et al., 2000). Because TSC2 binds TSC1 (Nellist et al., 1999, 2001; Hodges et al., 2001) by the domain that overlaps the Rho-activating domain of TSC1 and potentially inhibits TSC1-dependent Rho activation, we investigated whether or not stress fiber disassembly involves TSC1. As seen in Fig. 4 A, microinjection of siRNA in TSC2−/− cells directed against TSC1 promoted stress fiber disassembly similar to the effects of the expression of TSC2 or TSC2-HBD. Because TSC2-ΔHBD had no effect on stress fiber disassembly, but TSC2-HBD and siRNA TSC1 were sufficient to produce this effect in TSC2−/− cells, these data suggest that TSC2-dependent actin remodeling involves TSC1.

Quantitative analysis of F-actin staining revealed that 94.6 ± 1.7% of GFP-transfected cells showed stress fibers; in contrast, TSC2, TSC2-HBD, or siRNA TSC1 promoted marked stress fiber disassembly by 42.3 ± 1.9%, 52.7 ± 1.5%, and 31.7 ± 2.6%, respectively (Fig. 4 B). Because TSC2 is a susceptibility factor for LAM disease, we tested whether TSC2 or TSC2-HBD will affect actin remodeling in primary cultures of cells derived from the LAM tumors (Goncharova et al., 2002). As seen in Fig. 4 C, TSC2 and TSC2-HBD expression also promoted stress fiber disassembly in human LAM-derived (LAMD) cells, suggesting that TSC2 might be important for abnormal metastatic cell growth associated with TSC2 deficiency in LAM (Henske, 2003).

TSC2 or TSC2-HBD promotes focal adhesion remodeling

Because actin rearrangements are accompanied by focal adhesion remodeling, we examined whether or not TSC2-induced stress fiber disassembly correlates with changes in focal adhe-

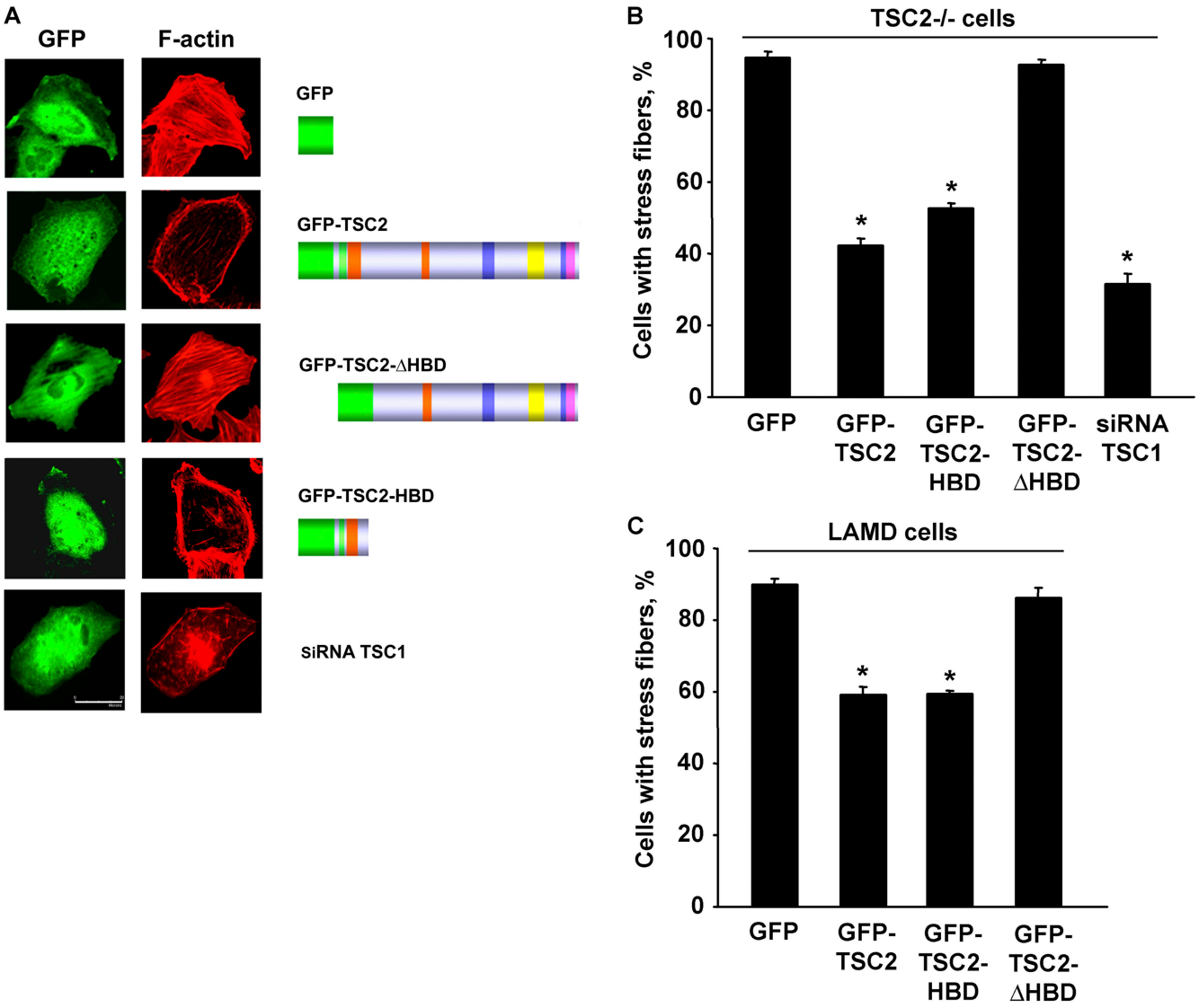


Figure 4. **TSC2, TSC2-HBD, and siRNA TSC1 promote stress fiber disassembly.** (A, left) F-actin staining (red) of TSC2−/− cells transfected with GFP-TSC2 or the indicated GFP-tagged TSC2 constructs identified by immunostaining with anti-GFP antibody (green) or microinjected with siRNA TSC1 and GST to identify injected cells (green). (right) Schematic representation of TSC2 constructs. Bar, 20 μm. (B) Quantitative analysis of F-actin staining. Data represent the percentage of cells with stress fibers per total number of cells transfected with pEGFP, pEGFP-TSC2, pEGFP-TSC2-HBD, or pEGFP-TSC2-ΔHBD taken as 100%. A total of 205 of GFP-TSC2–transfected, 360 of GFP-TSC2-HBD–transfected, 302 of GFP-TSC2-ΔHBD–transfected, 331 of GFP-transfected, and 644 siRNA TSC1-microinjected cells were analyzed from three independent experiments. Data represent the mean ± SE. *, $P < 0.0001$ for GFP-TSC2–, GFP-TSC2-HBD–transfected cells, or siRNA TSC1 + GST-microinjected cells versus GFP-transfected cells by ANOVA (Bonferroni-Dunn test). (C) Statistical analysis of F-actin staining of LAMD cells transfected with GFP, GFP-TSC2, GFP-TSC2-HBD, and GFP-TSC2-ΔHBD. Data represent the mean ± SE from two independent experiments. *, $P < 0.001$ for GFP-TSC2– and GFP-TSC2-HBD–transfected cells versus GFP-transfected cells by ANOVA (Bonferroni-Dunn test).

sion formation. Immunohistochemical analysis with anti-vinculin antibody showed that in TSC2−/− cells focal adhesions were localized throughout the cell as well as at the cell periphery (Fig. 5 A, top; and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). Expression of TSC2 promoted marked changes in the shape and size of the focal adhesions: most focal adhesions in the center of the cells were disassembled, and the quantity of focal adhesions per cell was attenuated by 30.3 ± 5.9% compared with control. At the same time, the size of focal adhesions at the cell periphery was also markedly decreased (Fig. 5 A, middle; and Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). Importantly, TSC2-HBD, which is involved in TSC1–TSC2 complex formation, also promoted focal adhesion disassembly in the center of the cells (Fig. 5 A, bottom; and Video 8, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). TSC1 regulates cell adhesion, and its inactivation results in the loss of focal adhesion (Lamb et al., 2000). To determine if focal adhesion remodeling in TSC2−/− cells requires TSC1, we microinjected siRNA TSC1 and found that down-regulation of TSC1 promoted focal adhesion disassembly in TSC2−/− cells (Fig. 5 B). These results demonstrate that expression of TSC2-HBD and the down-regulation of TSC1 with siRNA TSC1 are sufficient for focal adhesion disassembly, which suggest that TSC2 involves TSC1 in regulating focal adhesion remodeling.

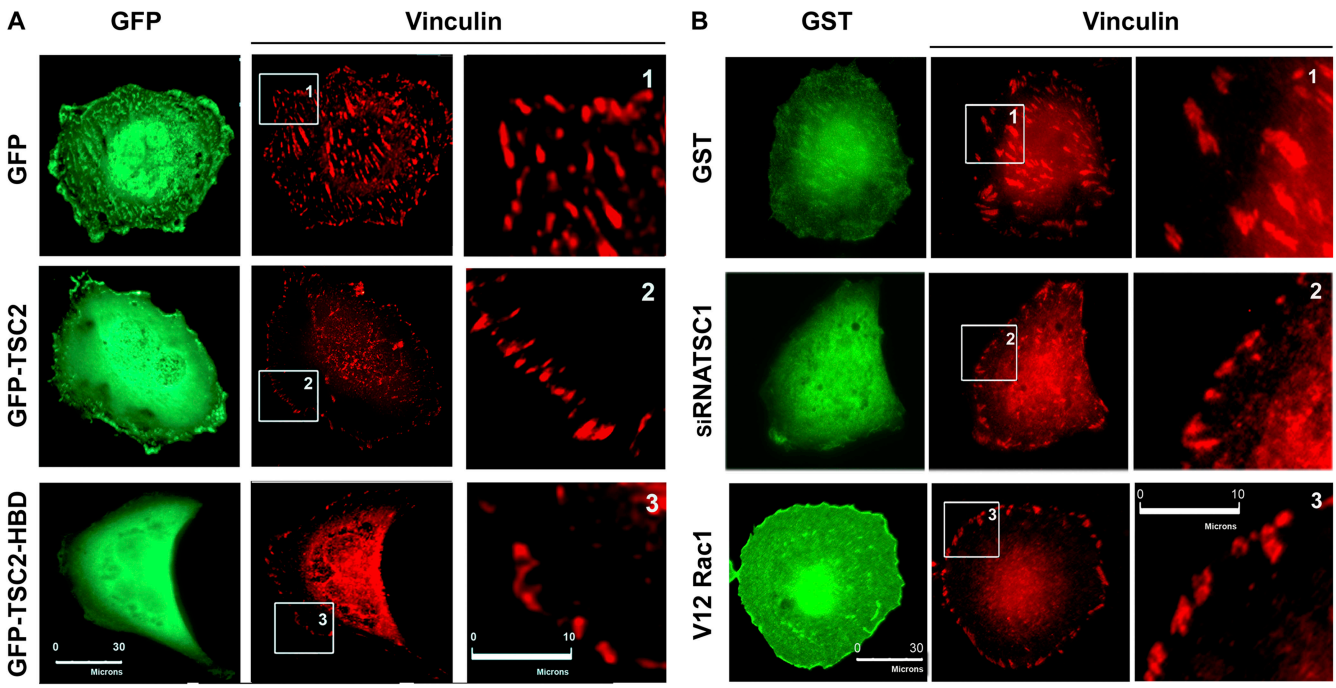
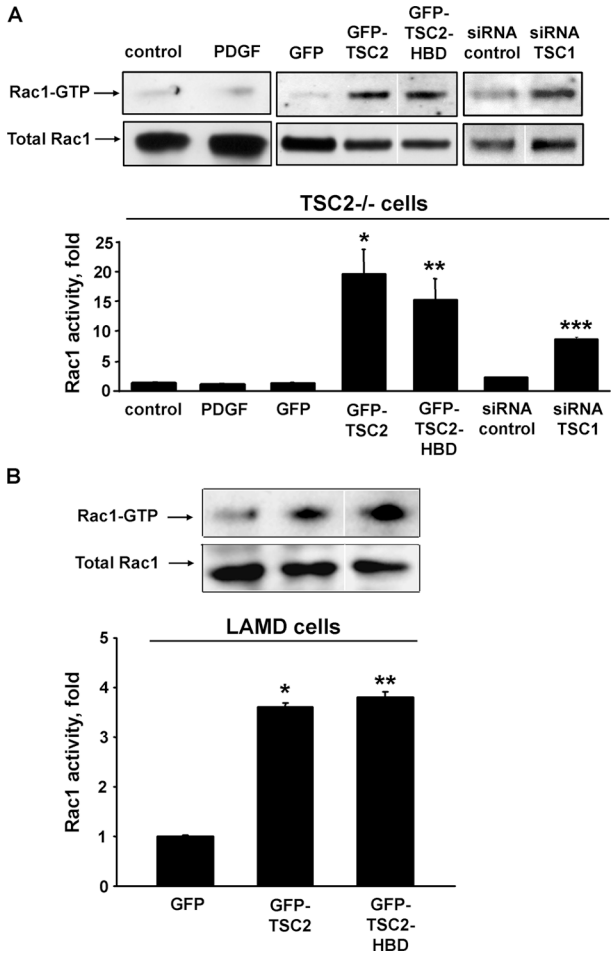


Figure 5. **TSC2, TSC2-HBD, and siRNA TSC1 modulate focal adhesion formation.** (A) Cells were transfected with control pEGFP (top), pEGFP-TSC2 (middle), or pEGFP-TSC2-HBD (bottom) plasmid, and then immunostained with anti-GFP (green) and anti-vinculin (red) antibodies to detect focal adhesions. Images are representative of three separate experiments. (B) Cells were transfected with control GST, GST-tagged V12Rac1, or siRNA TSC1 comicroinjected with GST to identify microinjected cells, followed by immunostaining with anti-GST (green) and anti-vinculin (red) antibodies. Representative images from 138 microinjected cells. Changes in the shape and size of the focal adhesion are represented in enlarged insets (1–3). Bars: (A and B) 30 μm; (insets) 10 μm.



Because the cortical actin staining observed in TSC2-transfected cells was reminiscent of the effects of Rac1 (Etienne-Manneville and Hall, 2002) and because small GTPase Rac1 induces the formation of membrane ruffles and lamellipodia, which ultimately results in focal adhesion remodeling, we examined the effect of the constitutively active form of Rac1 (V12Rac1) on focal adhesion formation in TSC2−/− cells. Vinculin immunostaining showed that V12Rac1 expression induced marked changes in focal adhesion formation similar to effects of TSC2 (Fig. 5 B), suggesting a potential link between TSC2 and Rac1 signaling.

Figure 6. **TSC2, TSC2-HBD, and siRNA TSC1 activate Rac1.** (A) TSC2−/− cells were stimulated with 10 ng/ml of PDGF or diluent for 10 min or transfected with plasmids expressing GFP-tagged TSC2 or TSC2-HBD, or GFP as a control, or microinjected with siRNA TSC1 or control siRNA, and then subjected to the Rac1 activity assay. (top) Immunoblot analysis with anti-Rac1 antibody to detect Rac1 in pull-down assay with PAK-1 PBD agarose (top images) and in whole cell lysates (bottom images). Images are representative of three separate experiments. (bottom) Quantitative analysis of Rac1 activity assays using Gel-Pro Analyzer Software. Rac1 activity in cells transfected with control pEGFP plasmid was taken as a onefold. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-HBD versus GFP; ***, $P < 0.001$ for siRNA TSC1 versus control siRNA. Data represent the mean ± SE from three independent experiments. (B) LAMD cells were transfected with GFP-TSC2, GFP-TSC2-HBD, or GFP as a control, and then Rac1 activity assay was performed. (top) Immunoblot analysis with anti-Rac1 antibody to detect Rac1 in pull-down assay with PAK-1 PBD agarose (top images) and in whole cell lysates (bottom images). Images are representative of two separate experiments. (bottom) Quantitative analysis of Rac1 activity. Data represent the mean ± SE from two independent experiments. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-HBD versus GFP by ANOVA (Bonferroni-Dunn test). White lines indicate that intervening lanes have been spliced out.

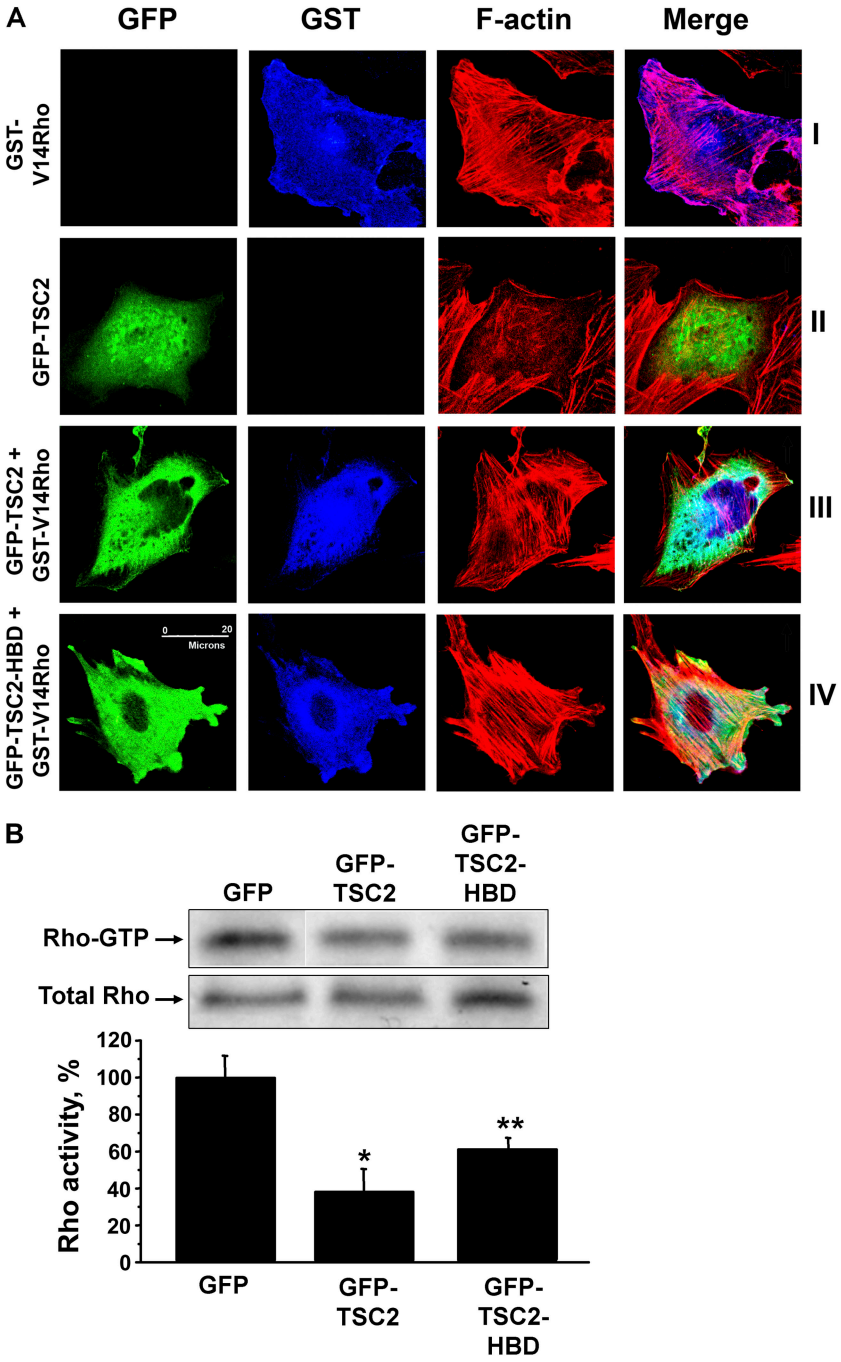


Figure 7. **TSC2 and TSC2-HBD inhibit Rho activity in TSC2−/− cells.** (A) TSC2−/− cells were transfected with pEBG-V12Rho or GFP-TSC2; GFP-TSC2 was cotransfected with pEBG-V12Rho plasmids; or pEGFP-TSC2-HBD was cotransfected with pEBG-V12Rho plasmid; and then cells were stained with anti-GFP to detect GFP, GFP-TSC2, or GFP-TSC2-HBD (green), anti-GST to detect GST, or GST-V12Rho (blue) and rhodamine phalloidin to detect F-actin (red). Images are representative of three independent experiments. Bar, 20 μm. (B) Cells were transfected with pEGFP-TSC2, pEGFP-TSC2-HBD, and control pEGFP plasmids expressing GFP-tagged TSC2, GFP-TSC2-HBD, and control GFP, respectively, and then Rho activity assay was performed. Immunoblot analysis of Rho-GTP pull-down with Rhotekin-RBD agarose (top) and whole cell lysates (bottom) was performed with anti-Rho antibodies. White lines indicate that intervening lanes have been spliced out. Quantitative analysis of three independent experiments was performed using Gel-Pro Analyzer Software. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-HBD versus GFP by ANOVA (Bonferroni-Dunn test).

Activation of Rac1 by TSC2

To clarify the function of Rac1 in TSC2-induced stress fiber disassembly and focal adhesion remodeling, we next examined Rac1 activation in TSC2−/− cells. Stimulation of TSC2−/− cells with PDGF had little effect on the basal Rac1 activity (Fig. 6 A), whereas PDGF-stimulated Rac1 activity in 3T3 cells (not depicted), which were used as a model cell line. By expressing TSC2 in TSC2−/− cells, we found that TSC2 alone was sufficient to markedly increase Rac1 activity compared with cells transfected with control GFP (Fig. 6 A). Similarly, Rac1 activity was increased in LAMD cells transfected with TSC2, indicating that TSC2 may elicit activation of Rac1 (Fig. 6 B). Importantly, down-regulation of TSC1 with TSC1 siRNA or expression of TSC2-HBD was also sufficient for stimulating Rac1 activity (Fig. 6 A), suggesting that TCS2 interaction with TSC1 may be involved in the regulation of Rac1 activity. We conclude that TSC2 acts upstream of Rac1 in pathways regulating actin and focal adhesion remodeling.

TSC2 and TSC2-HBD inhibit Rho activity

The small GTPase RhoA is necessary for stress fiber and focal adhesion formation (Etienne-Manneville and Hall, 2002) and TSC1 activates Rho (Lamb et al., 2000). To determine whether or not TSC2-induced stress fiber disassembly was by impaired signaling downstream of Rho, we cotransfected TSC2 or TSC2-HBD with constitutively active Rho, V14Rho. As seen

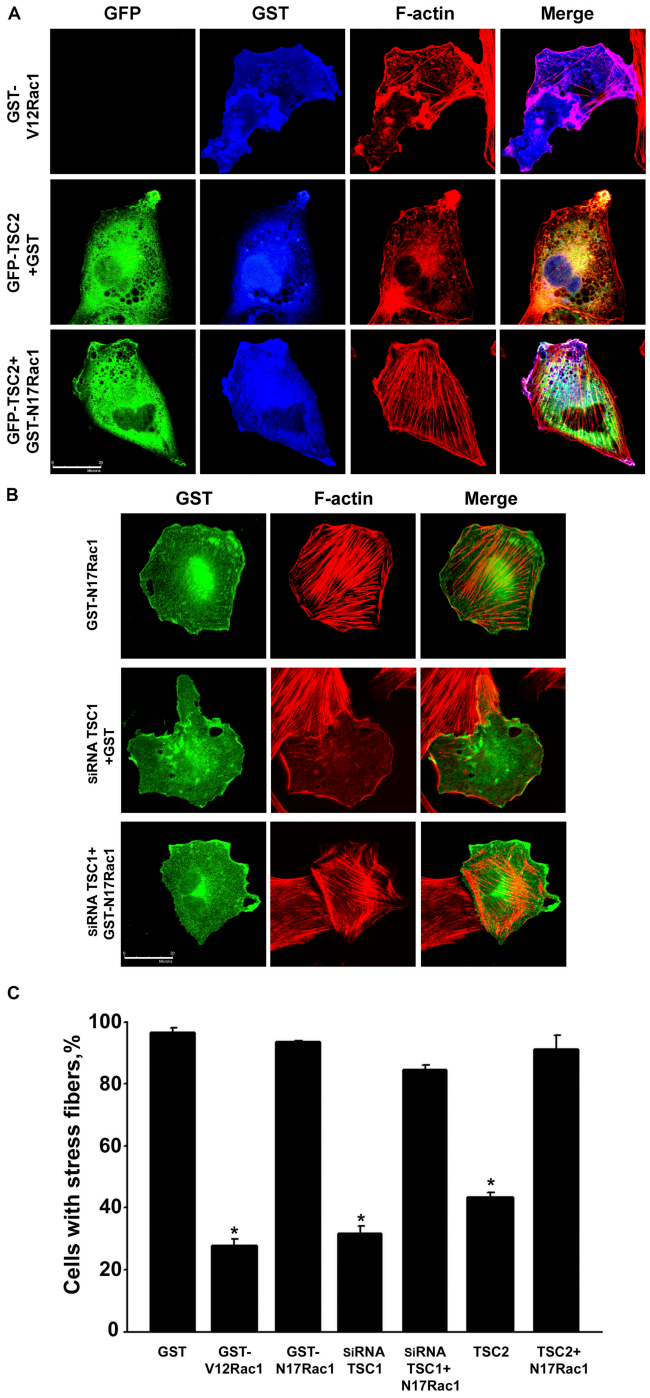


Figure 8. **Dominant-negative Rac1 abrogates TSC2- and siRNA TSC1-induced stress fiber disassembly.** (A) Serum-deprived cells were transfected with pEBG-V12Rac1 expressing activated Rac1 or the cells were cotransfected with pEGFP-TSC2 and pEBG-N17Rac1 plasmids, expressing GFP-TSC2 and GST-N17Rac1, respectively, and were stained with anti-GFP to detect GFP-TSC2 (green), anti-GST to detect GST, GST-V12Rac1, GST-N17Rac1 (blue), and phalloidin rhodamine to detect F-actin (red). Images are representative of three independent experiments. Bar, 20 μm. (B) Cells, microinjected with GST-N17Rac1, comicroinjected with siRNA TSC1 and GST to identify microinjected cells, or comicroinjected with siRNA TSC1 and N17Rac1, were stained with anti-GST antibody to detect GST and GST-N1Rac1 (green) and phalloidin rhodamine to detect F-actin (red). Images are representative of three independent experiments. (C) Quantitative analysis of F-actin staining. Data represent the percentage of cells with stress fibers per total number of cells transfected with plasmids expressing GST, GST-V12Rac1, GST-N17Rac1, GFP-TSC2, or coexpressed GFP-TSC2 and GST-N17Rac1, siRNA TSC1 and GST-N17Rac1, or siRNA TSC1 and control GST taken as 100%. Data represent the mean ± SE from three independent experiments. *, $P < 0.001$ for GST-V12Rac1 versus GST, siRNA TSC1 + GST versus siRNA TSC1 + GST-N17Rac1, and GFP-TSC2 versus GFP-TSC2 + GST-N17Rac1 by ANOVA (Bonferroni-Dunn test).

in Fig. 7 A (panels III and IV, respectively), in cells cotransfected with V14Rho, stress fibers were maintained compared with cells expressing TSC2 alone (Fig. 7 A, panel II). These data suggest that stress fiber disassembly induced by TSC2 and TSC2-HBD is not due to failure in pathways downstream of Rho that regulate assembly and maintenance of stress fibers.

To investigate whether or not TSC2 may modulate Rho activity, we examined if TSC2 expression affected endogenous Rho activity in TSC2−/− cells. As seen in Fig. 7 B, TSC2 modestly but reproducibly attenuated Rho activity by 64 ± 13% compared with GFP-transfected cells. TSC2-HBD alone was also sufficient for the attenuation of Rho activity by 40 ± 9% compared with control GFP (Fig. 7 B). Together, these results indicate that TSC2-induced actin rearrangements involve activation of Rac1 and inhibition of Rho.

Rac1 acts upstream of Rho in TSC2-dependent actin remodeling

Rho and Rac1 both regulate stress fiber formation and focal adhesion remodeling in a reciprocal manner, as such activation of Rac1 results in the inhibition of Rho and vice versa (Horwitz and Parsons, 1999). To clarify the hierarchy of Rho inhibition and Rac1 activation in TSC2-dependent stress fiber disassembly, we analyzed whether or not the activated form of Rac1, V12Rac1, could promote actin rearrangements in TSC2−/− cells; and then we performed cotransfection experiments of dominant-negative GST-tagged Rac1 (N17Rac1) with TSC2. Expression of V12Rac1 promoted stress fiber disassembly in the cell center and formation of cortical actin at the cell periphery similar to the effect of TSC2 (Fig. 8 A, top and middle, respectively). In contrast, in cells coexpressing N17Rac1 and TSC2, stress fibers were maintained (Fig. 8 A, bottom), suggesting that TSC2-induced stress fiber disassembly requires Rac1 activation. Furthermore, when N17Rac1 was comicroinjected with siRNA TSC1, stress fibers also remained (Fig. 8 B, bottom), indicating that TSC1-dependent stress fiber formation requires the negative regulation of Rac1 activity. Quantitative analysis of these experiments is presented in Fig. 8 C. Because stress fibers are maintained by active Rho, and inactive Rac1 coexpressed with TSC2 or siRNA TSC1 does not promote stress fiber disassembly, this data indicates that TSC2 requires activation of Rac1, followed by inhibition of Rho, in regulating actin remodeling.

TSC2-HBD is not required for the negative regulation of ribosomal protein S6 activation

To address the relationship between the role that TSC2 plays in regulating actin dynamics and its function as a negative regulator of protein translation through mTOR, we examined the effect of

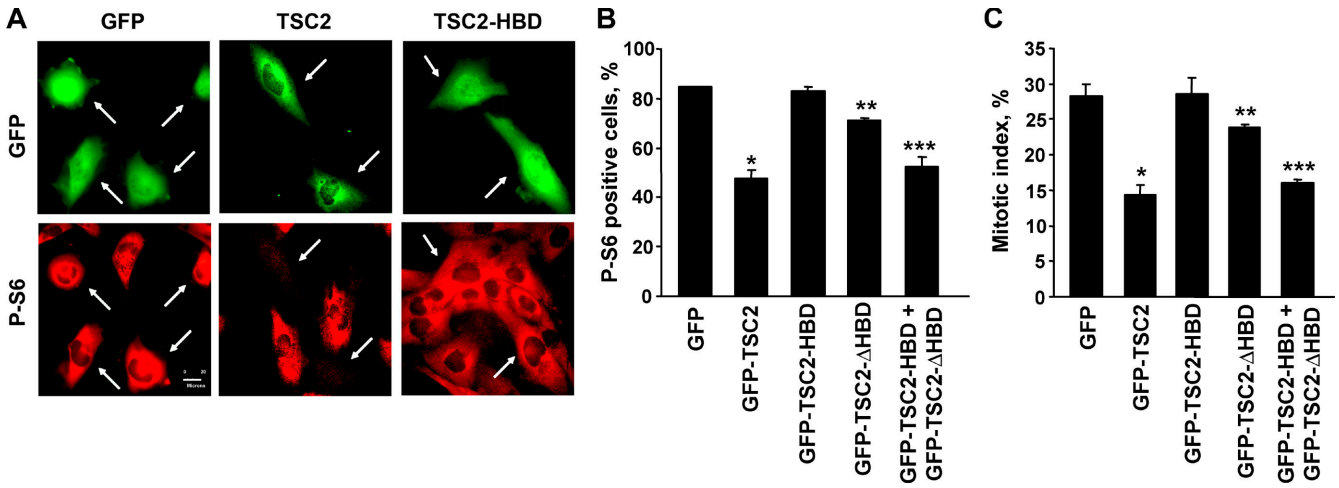


Figure 9. **Expression of TSC2, but not TSC2-HBD, inhibits ribosomal protein S6 phosphorylation and DNA synthesis in TSC2−/− cells.** TSC2−/− cells were transfected with plasmids expressing GFP-tagged TSC2, TSC2-ΔHBD, or TSC2-HBD, or comicroinjected with pEGFP-TSC2-HBD and pEGFP-TSC2-ΔHBD plasmids, serum-deprived for 24 h, and immunostained with anti–phospho-S6 (red) antibody and anti-GFP antiserum (green); or BrdU incorporation analysis was performed. (A) Representative images of three separate experiments were taken. Arrows indicate transfected cells. (B) Quantitative analysis of P-S6 immunostaining. Data represent the percentage of P-S6–positive transfected cells per total number of transfected cells. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-ΔHBD versus GFP; ***, $P < 0.001$ for GFP-TSC2-HBD + GFP-TSC2-ΔHBD versus GFP. (C) BrdU incorporation analysis of transfected cells. Mitotic index represents the percentage of BrdU-positive transfected cells compared with the total number of transfected cells. Data are mean ± SE of three separate experiments. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-ΔHBD versus GFP; ***, $P < 0.001$ for GFP-TSC2-HBD + GFP-TSC2-ΔHBD versus GFP.

rapamycin, a specific mTOR inhibitor, on the actin cytoskeleton in the TSC2−/− cells at a dose that completely abrogates S6K activity. We found that rapamycin had little effect on actin dynamics (unpublished data), which serves as evidence that the regulation of cell dynamics is a novel function of TSC2, which is independent from its function in protein translational regulation.

Because TSC2 negatively regulates the activity of ribosomal protein S6 by inhibition of its phosphorylation (Goncharova et al., 2002), we investigated whether TSC2-HBD or TSC2-ΔHBD affects ribosomal protein S6 hyperphosphorylation in TSC2−/− cells. Consistent with previously published results (Goncharova et al., 2002), full-length TSC2 inhibited S6 phosphorylation by 56.2 ± 3.9% compared with GFP-transfected cells (Fig. 9, A and B). TSC2-ΔHBD also significantly, however to a lesser extent than full-length TSC2, inhibited S6 phosphorylation by 22.7 ± 2.8%. Importantly, comicroinjection of TSC2-HBD and TSC2-ΔHBD inhibited phospho-S6 by 47.4 ± 3.3%, which was comparable to the inhibitory effect of full-length TSC2 (Fig. 9 B). In contrast, expression of TSC2-HBD had little effect on S6 phosphorylation, suggesting that this domain of TSC2 is not required for the regulation of ribosomal protein S6 activation (Fig. 9, A and B).

In parallel, we examined the effect of TSC2 mutants on DNA synthesis. As we previously demonstrated (Goncharova et al., 2002), TSC2, but not TSC2-HBD, significantly inhibited TSC2−/− cell proliferation by 49.4 ± 4.7%. Importantly, TSC2-ΔHBD alone was sufficient for inhibition of DNA synthesis, however, to a much lesser extent than full-length TSC2. By coexpressing TSC2-HBD and TSC2-ΔHBD we found that DNA synthesis was inhibited by 43.2 ± 1.4%, which was comparable to inhibition by full-length TSC2 (Fig. 9 C). Collectively, these data demonstrate that TSC2-HBD is not required for S6 phosphorylation and regulation of DNA synthesis.

Discussion

TSC1 has been identified as an activator of Rho and a regulator of cell adhesion (Lamb et al., 2000). The Rho-activating domain of TSC1 overlaps with the region that binds TSC2 (Nellist et al., 1999, 2001; Hodges et al., 2001), indicating that TSC2 may modulate TSC1-dependent activation of Rho. Our work identifies a novel function of TSC2 as a modulator of the actin cytoskeleton and focal adhesion remodeling. In our model, TSC1 inhibits Rac1, and TSC2 blocks this activity of TSC1 that is a prerequisite to the activation of Rac1 and the subsequent inhibition of Rho; this, in turn, promotes stress fiber disassembly and focal adhesion remodeling (Fig. 10). Loss of function of either TSC1 or TSC2 due to inactivating mutations potentially promotes deregulation of the TSC1–TSC2 complex formation followed by deregulation of Rac1 and Rho activities, which, consequently, induces abnormal cell motility associated with the pathobiology of LAM and TSC.

Actin dynamics is a major cellular process regulating cell morphogenesis, movement, and behavior. In the normal cellular context, directional cell movement requires the formation of membrane protrusions and new focal adhesions, and stabilization of existing adhesions, which are regulated by Rho GTPases. Activity of these GTPases is modulated by a plethora of signaling molecules among which TSC2 appears as an important physiological modulator. Activation of Rac1 by reexpression of TSC2 in TSC2−/− cells resulted in the reorganization of the actin cytoskeleton and focal adhesion remodeling. Through attenuation of Rho activity, TSC2 facilitates stress fiber disassembly and focal adhesion remodeling, thus consequently promoting dynamic membrane protrusions. Our work indicates that TSC2 functions as a modulator of Rac1 and Rho activation and actin remodeling, which involves TSC1. TSC1, a

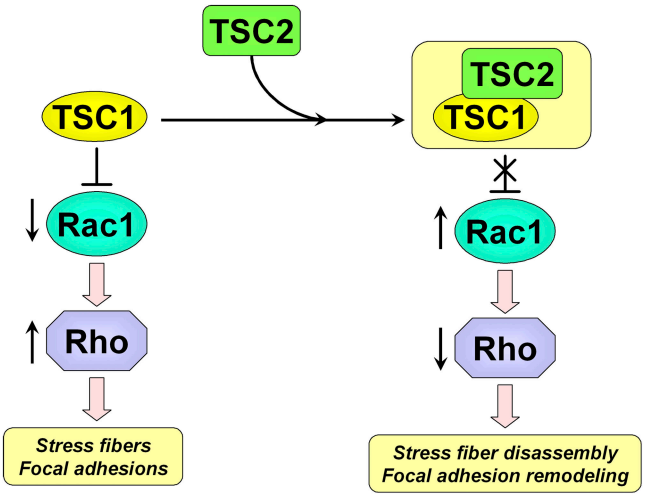


Figure 10. **Schematic representation of the TSC2-dependent modulation of actin cytoskeleton and focal adhesion.** TSC1 inhibits Rac1, and TSC2 blocks this activity of TSC1. TSC2 through its specific TSC1-binding domain forms a complex with TSC1, which is a prerequisite for Rac1 activation and Rho inhibition. This, in turn, promotes stress fiber disassembly and focal adhesion remodeling. Dysregulation of TSC2 function due to inactivating mutations promotes deregulation of the TSC1–TSC2 complex formation, followed by deregulating Rac1 and Rho activation, which, in turn, results in abnormal cell motility and adhesion associated with LAM and TSC pathobiology.

binding partner of TSC2 (Nellist et al., 1999; Henske, 2003; Krymskaya and Shipley, 2003), regulates the activity of Rho and associates with the cortical proteins ERM, which serve as molecular bridges between the plasma membrane and the cortical actin (Lamb et al., 2000). The fact that TSC2 binds TSC1 through TSC2-HBD (van Slegtenhorst et al., 1998; Benvenuto et al., 2000; Hodges et al., 2001), which overlaps with the Rho-activating domain of TSC1 (Lamb et al., 2000), suggests the critical importance of the TSC2–TSC1 interaction for TSC1-dependent Rho activation and cell adhesion. Our data show that Rho can be inhibited by the reexpression of TSC2, which contradicts a previous paper by Astrinidis et al. (2002) in which the stable expression of TSC2 in the same TSC2−/− cells results in Rho activation. The possible reason for the discrepancy between our data and the previous paper lies within the potential differences in the mode of cell motility that differ in their requirement for Rho activation (Martin, 2003; Sahai and Marshall, 2003) due to differences in experimental approaches. Using transient TSC2 expression by mammalian expression vector or adenoviral infection in TSC2−/− cells, we show that TSC2 promotes "classical" lamellipodial or mesenchymal motility driven by activated Rac1 and the down-regulation of Rho activation. In contrast to this classical type of motility, some tumor cell lines require Rho activation to promote a rounded bleb-associated mode of motility (Sahai and Marshall, 2003). Astrinidis et al. (2002) use ELT3 and MDCK cell clones with stable overexpression of TSC2. We can't rule out that cell lines stably overexpressing TSC2 had changed their phenotype with concomitant alterations in the mode of motility that requires Rho activation as it is demonstrated by Astrinidis et al. (2002). Our data also show that Rac1 acts upstream of Rho in TSC1–TSC2–dependent actin remodeling. How TSC1–TSC2 activates Rac1 and inhibits Rho remains to be elucidated. One possibility is that TSC1–TSC2 may modulate the activity of kinases regulating Rac1/RhoGDI association (DerMardirossian et al., 2004). Additionally, differential cellular localization of TSC2 and its mutants (unpublished data) suggests that TSC2 functions in regulating Rac1 activity or in the complex formation with TSC1 may depend on the subcellular localization of TSC2, TSC1, and Rac1. Another avenue to explore in elucidating the relationship between TSC1–TSC2 and Rac1 is to determine the modulation of growth factor–stimulated Rac1 activity by TSC1–TSC2.

TSC2 regulates cell growth and proliferation through the mTOR/S6K signaling pathway, which is sensitive to rapamycin, thus inhibiting mTOR. In TSC2−/− cells, we determined that actin remodeling was insensitive to rapamycin, suggesting that the rapamycin-sensitive mTOR pathway does not contribute to biochemical events that occur as a part of the regulatory mechanisms of cell dynamics. Further support for this observation was provided by the phosphorylation level of ribosomal protein S6, which was unaffected by expression of TSC2-HBD, a domain of TSC2, which was necessary and sufficient to regulate Rac1/Rho activities and actin remodeling. Importantly, TSC2-ΔHBD, which contains the GAP homology domain, is not involved in TSC2-dependent actin rearrangement but is sufficient for the modulation of mTOR/S6K activity, which suggests that different domains of TSC2 are involved in the regulation of cell dynamics and protein translation/cell growth through activation of two independent signaling pathways. Because TSC2 is an upstream regulator of mTOR, and in yeast TOR controls the actin cytoskeleton (Schmelzle and Hall, 2000), further studies are needed to address the question of whether or not the rapamycin-insensitive component of mTOR is involved in TSC2-dependent actin dynamics.

Our current findings provide evidence that TSC2 plays an important role in regulating cell dynamics. Notably, the function of TSC2-HBD, which is essential for the TSC2-dependent regulation of the actin cytoskeleton and focal adhesion, is not required for the regulation of S6 phosphorylation and cell growth, indicating that TSC2 may influence both cell dynamics and protein translation. It is important to note that activation of Rac1 is required for neurite outgrowth (Kozma et al., 1997; Yamaguchi et al., 2001). In contrast, Rho activation promotes neurite retraction and an inhibition of neurite outgrowth (Katoh et al., 1998; Yamaguchi et al., 2001). Because the loss of TSC1 or TSC2 functions promotes aberrant neuronal motility during brain development (Crino and Henske, 1999; Vinters et al., 1999; Gutmann et al., 2000; Sparagana and Roach, 2000), TSC1 and TSC2 loss of functions may deregulate Rac and Rho activities, which may then contribute to TSC pathology. Because TSC and LAM disease severity is predominantly associated with the loss of TSC2 function, the perturbed balance between TSC2 and TSC1 interaction could be a key event in the pathobiology of TSC and LAM.

Materials and methods

Cell culture

LAMD and TSC2−/− ELT3 cells were derived from the Eker rat uterine leiomyoma (Howe et al., 1995) and maintained as previously described (Goncharova et al., 2002). TSC2−/− ELT3 cells were a gift from C.L.

Walker (National Institute of Environmental Health Sciences Center for Research on Environmental Disease, Smithville, TX). 3T3 fibroblasts were purchased from American Type Culture Collection (CCL-92) and maintained in DME supplemented with 10% FBS. All experiments were performed on serum-deprived cells before experiments for 24 h.

Plasmid and adenovirus construction

The pEGFP-TSC2, encoding GFP-tagged TSC2; pEGFP-N-TSC2, encoding 1–1113 amino acids of TSC2; and pEGFP-C-TSC2, encoding 1114–1784 amino acids of TSC2 mammalian expression constructs were created as previously described (Finlay et al., 2004). The pEGFP-TSC2-HBD or pEGFP-TSC2-ΔHBD plasmids, expressing 1–460 amino acids or 461–1784 amino acids of TSC2, respectively, were produced by digesting the pEGFP-TSC2 plasmid, expressing wild-type human TSC2, with SalI and FspI or FspI and XbaI endonucleases, respectively. These fragments were ligated with pEGFP-C3 vector digested with SmaI and SalI or SmaI and XbaI, respectively. Successful insertion of TSC2-HBD or TSC2-ΔHBD into pEGFP plasmid was confirmed by analytical digest and sequence analysis with pEGFP-C Sequencing Primer (BD Biosciences). Recombinant adenovirus expressing GFP and TSC2 cDNA constructs were created using the AdEasy vector system as described previously (Finlay et al., 2004). The expression of GFP-tagged TSC2 mammalian expression constructs and adenoviruses was confirmed by transient transfection or adenovirus infection of TSC2−/− cells with these vectors and immunoblot analysis of whole cell lysates with anti-GFP antibody (Fig. 3).

Transient transfection and replication-deficient adenovirus infection

Transient transfection was performed using the Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. Infection with replication-deficient adenovirus was performed as described previously (Lanuti et al., 1999). Transfection and virus infection were used in parallel and yielded similar results regarding the regulation of cell cytoskeleton, motility, and the modulation of Rac1 and Rho GTPases activities. pEBG-V14Rho, pEBG-N17Rac1, and pEBG-V12Rac1 expression vectors were a gift from M.M. Chou (University of Pennsylvania, Philadelphia, PA).

Wound assay and live imaging of wound closure

Cells, plated on chamberslides, were wounded by scraping a 10-μl pipette tip through the cell monolayer, and then gently washed with PBS, incubated with fresh media supplemented with 2% FBS for 2 h, followed by supravital analysis. Supravital analysis was performed in the micro-incubator (model CSMI; Harvard Apparatus) with constant 37°C temperature on an inverted microscope (model TE300; Nikon) equipped with a digital video camera (model Evolution QEi; Media Cybernetics) under 100 magnification for 8.3 h. Images were taken every 10 min in both the phase-contrast and fluorescence channels and were analyzed using Image-Pro Plus 5.0.0.39 software (Media Cybernetics).

Immunocytochemistry

Cells were washed three times with PBS, fixed with 3.7% PFA for 15 min, treated with 0.1% Triton X-100 for 30 min at RT, and blocked with 0.5% TSA Fluorescein System blocking reagent (NEN Life Science Products) in TBS. After incubation with rhodamine phalloidin (Molecular Probes) or primary and then secondary antibodies conjugated with either Alexa Fluor488, Alexa Fluor594, or Alexa Fluor633 (see online supplemental materials) cells were mounted in Vectashield mounting medium (Vector Laboratories). Immunostaining was analyzed using the scanning laser confocal microscopic system (model TCS SP2; Leica), a microscope (model Eclipse TE2000-E; Nikon) equipped with a digital video camera (model Evolution QEi; Media Cybernetics), or a microscope (model Eclipse E400; Nikon) equipped with a digital camera (model Coolpix 995; Nikon) under 1,000 magnification. Three-dimensional analysis was performed using Z-series images taken with z interval 0.1 μm, which were then three-dimensionally deconvoluted using AutoDebur + AutoVisualize Software 9.3 (AutoQuant Imaging, Inc.).

Immunoblot analysis

Serum-deprived TSC2−/− cells were stimulated with 10 ng/ml of PDGF-BB (Calbiochem) for 10 min followed by immunoblot analysis with anti-PDGFRβ (Santa Cruz Biotechnology, Inc.), anti-PDGFRα, and antiphosphotyrosine antibodies (Upstate Cell Signaling Solutions) as previously described (Goncharova et al., 2002).

Rac and Rho activity assays

Cells were transfected with pEGFP-TSC2, pEGFP-TSC2-HBD, pEGFP-TSC2-ΔHBD, control pEGFP plasmid, siRNA TSC1, or siGLO RISC-Free siRNA (Dharmacon Research, Inc.) as a control and growth arrested, and then Rho or Rac activities were measured using Rac or Rho Activation Assay Kits (Upstate Cell Signaling Solutions) according to the manufacturer's protocol. Rho and Rac1 were detected using anti-Rho (A, B, or C) or anti-Rac1 antibodies, respectively (Upstate Cell Signaling Solutions). siRNA TSC1 sequences were a gift from R. Lamb (Institute for Cancer Research, London, UK); anti-TSC1 antibody was a gift from M. Nellist (Erasmus University, Rotterdam, Netherlands).

Microinjection

Microinjection was performed using Eppendorf Microinjection System as described previously (Goncharova et al., 2002). 18 h after injection, cells were subjected to immunocytochemical or BrdU incorporation assays.

BrdUrd incorporation

Cells, transfected with plasmids expressed GFP-conjugated TSC2, TSC2-HBD, TSC2-ΔHBD, or GFP as a control, or coinjected with plasmids expressed TSC2-HBD and TSC2-ΔHBD, were maintained for 24 h in serum-free medium, and BrdU incorporation was assessed (Goncharova et al., 2002). The mitotic index was defined as the percentage of BrdU-positive transfected/injected cells per field/total number of transfected/injected cells per field.

Statistical analysis

Statistical analysis of F-actin staining or immunostaining was performed by using microscope (model Eclipse E400; Nikon) images taken at 200 magnification followed by quantitative analysis using Gel-Pro Analyzer Software. Data points from individual assays represent the mean values ± standard error (SE). Statistically significant differences among groups were assessed with the analysis of variance (ANOVA; Bonferroni-Dunn test), with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. All experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples.

Online supplemental material

Down-regulation of TSC1 level by siRNA TSC1 and primary and secondary antibodies used for immunocytochemical analysis are listed in supplemental materials. The corresponding movie files of live cells are organized as follows: Video 1 for Fig. 1 A and Videos 2–5 for Fig. 1 B. Movie files demonstrating three-dimensional projection of cells immunostained with anti-vinculin antibody are organized as follows: Videos 6–8 for Fig. 5. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1.

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