

Regulation of RhoA-dependent ROCKII activation by Shp2

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Contractile forces mediated by RhoA and Rho kinase (ROCK) are required for a variety of cellular processes, including cell adhesion. In this study, we show that RhoA-dependent ROCKII activation is negatively regulated by phosphorylation at a conserved tyrosine residue (Y722) in the coiled-coil domain of ROCKII. Tyrosine phosphorylation of ROCKII is increased with cell adhesion, and loss of Y722 phosphorylation delays adhesion and spreading on fibronectin, suggesting that

this modification is critical for restricting ROCKII-mediated contractility during these processes. Further, we provide evidence that Shp2 mediates dephosphorylation of ROCKII and, therefore, regulates RhoA-induced cell rounding, indicating that Shp2 couples with RhoA signaling to control ROCKII activation during deadhesion. Thus, reversible tyrosine phosphorylation confers an additional layer of control to fine-tune RhoA-dependent activation of ROCKII.

Introduction

Actin reorganization regulated by RhoA, a small GTPase, is essential for many cellular processes, including adhesion, rounding, migration, contraction, and proliferation (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Aznar and Lacal, 2001; Etienne-Manneville and Hall, 2002). Rho kinase (ROCK) is one of the downstream effectors of RhoA signaling that phosphorylates myosin light chain (MLC), activating myosin to cross-link actin filaments and generating contraction force (Amano et al., 1996; Kimura et al., 1996; Kureishi et al., 1997; Fukata et al., 1998; Feng et al., 1999). Two isoforms of ROCK, ROCKI and ROCKII, which belong to a family of serine/threonine kinases, are activated via interaction with RhoA GTPase (Riento and Ridley, 2003; Mueller et al., 2005). The kinase domain of ROCKs is localized in the N-terminal region. The C-terminal region of ROCKs, which contains the rho binding domain (RBD) and pleckstrin homology domain, is responsible for autoinhibition of the kinase activity through interaction with the N-terminal kinase region (Amano et al., 1999). Proteolytic cleavages of the inhibitory C-terminal region of ROCKI by caspase-3 (Coleman et al., 2001; Sebbagh et al., 2001) and of ROCKII by granzyme B (Sebbagh et al., 2005) result in irreversible activation of ROCK kinases and membrane blebbing in apoptosis. Under the normal physiological conditions, ROCK

is mainly regulated by binding of the GTP-bound form of RhoA to the RBD, which stimulates ROCK kinase activity by disrupting the intramolecular interaction (Fujisawa et al., 1996; Dvorsky et al., 2004).

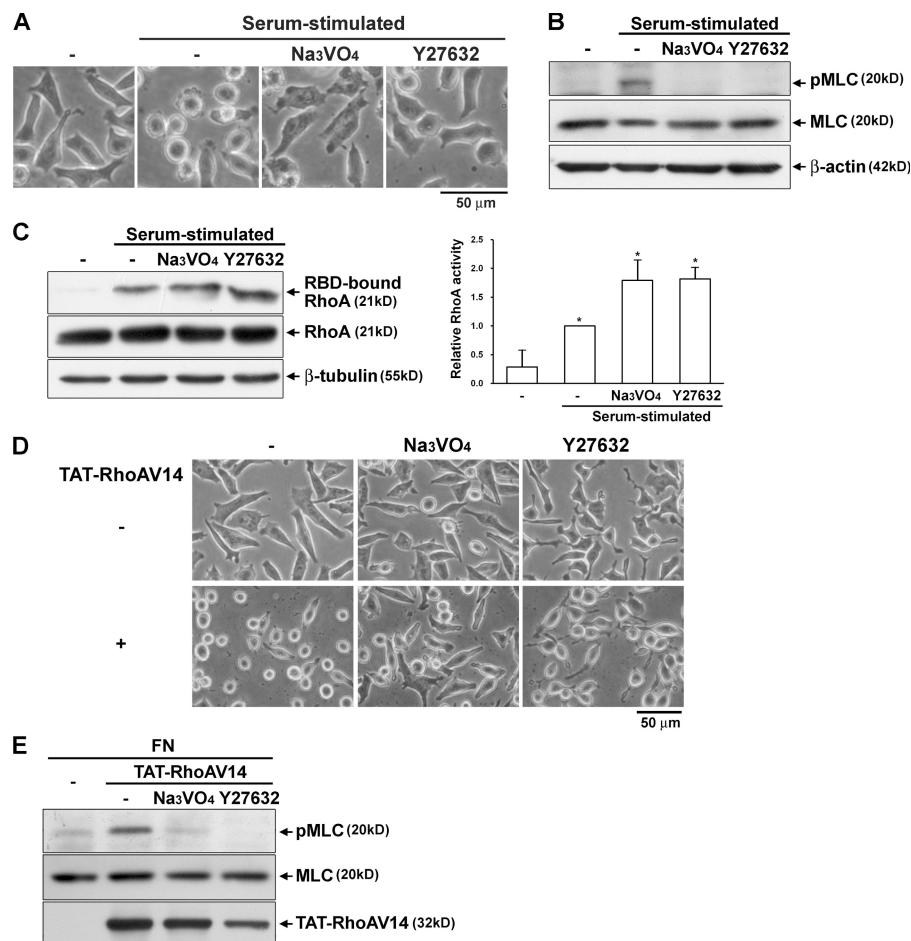
RhoA/ROCK-mediated signaling influences the interactions between the actin cytoskeleton and integrins to regulate integrin activity involved in cell shape and adhesive properties (Cali et al., 1999; Worthylake and Burridge, 2003; Nelson et al., 2004; Basile et al., 2007). It has been shown that RhoA promotes the formation of large integrin-based focal adhesions by increasing actomyosin-dependent integrin clustering and is required for focal adhesion complex formation during adhesion (Hotchin and Hall, 1995; Narumiya et al., 1997). In contrast, an excessive level of ROCK-mediated contractility is able to either disrupt the interaction between extracellular matrix and integrin or to constrain integrin mobility through enrichment of the cortical actin, resulting in cell deadhesion and rounding (Alblas et al., 2001; Liu et al., 2002; Maddox and Burridge, 2003). During cell migration, RhoA/ROCK activation generates protrusive forces at the front and retraction forces at the rear (Worthylake et al., 2001; Kurokawa and Matsuda, 2005; Wong et al., 2006). Presumably, too much ROCK activity would disrupt the adhesion linkage and affect motility. Because it has been shown that RhoA is consistently activated in the protruding regions of randomly migrating fibroblasts (Pertz et al., 2006), in this study we were interested in the negative control for RhoA/ROCK activation contributing to optimization of the localized contractile force in the adhesion and deadhesion processes.

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Abbreviations used in this paper: FN, fibronectin; MLC, myosin light chain; PTP, protein tyrosine phosphatase; RBD, rho binding domain; ROCK, rho kinase; SRE, serum-responsive element; SRF, serum response factor.

The online version of this paper contains supplemental material.

Figure 1. Involvement of tyrosine dephosphorylation in ROCK-dependent cell rounding. (A–C) Adherent D2 cells plated in culture dishes in serum-free medium for 2 h were serum stimulated and treated with 300 μ M orthovanadate (Na_3VO_4) or 20 μ M Y27632, followed by stimulation with 20% heat-inactivated serum for 10 min. (A) Phase-contrast images of cells with indicated treatment. (B) Western blots of cells probed for phosphorylated[$\text{T}^{18}/\text{S}^{19}$] and total MLC. (C) Endogenous RhoA activity measured by GST-RBD pulldown assay as described in Materials and methods. Values are mean \pm SD of three independent experiments. (D and E) Cells were plated on FN-coated dishes for 2 h in serum-containing medium treated with 20 μ M Y27632 or 300 μ M orthovanadate, followed by addition of 20 μ g/ml of TAT-RhoAV14 for 30 min. (D) Phase-contrast images of cells. (E) Western blot of cells probed by antibodies against phospho-MLC, total MLC, and HA for TAT-RhoAV14. Bars, 50 μ m.



At present, it is known that RhoA/ROCK signaling is negatively controlled by a variety of mechanisms, including cytosolic sequestration of GDP-bound RhoA by guanosine nucleotide dissociation inhibitors, stimulation of the GTPase activity by GAPs (Ridley, 2001), and local degradation of RhoA protein by Smurf1 in the leading edge of migrating cells (Sahai et al., 2007). In addition to these negative controls via RhoA, three proteins that directly inhibit ROCK function through protein interaction have been described. Gem and Rad are two small G proteins that can directly bind and inhibit ROCK I and II, respectively (Ward et al., 2002). RhoE, a Rho protein which is defective in GTPase function, has also been shown to interact with ROCKI and to inhibit ROCKI-induced stress fiber formation (Riento et al., 2003). Recent reports have further shown that RhoE is a target gene of p53 (Ongusaha et al., 2006; Gadea et al., 2007). In response to DNA damage, RhoE expression is up-regulated so that ROCKI activity is repressed to prevent apoptosis (Boswell et al., 2007). The delicate inhibition of RhoA activation and the existence of ROCK inhibitors highlight how important it is to ensure that RhoA/ROCK is not activated at the time and places where too much contractility and inappropriate actin reorganization would interfere with the cellular processes.

Our laboratory has previously used a myeloid leukemia cell line, D2, to show that RhoA/ROCK activation signaling prevents adhesion (Lai et al., 2001), rendering these cells suscep-

tible to phorbol ester-induced apoptosis (Lai et al., 2002, 2003; Chang and Lee, 2006; Chang et al., 2006). In this paper, we report that RhoA/ROCK-mediated deadhesion of D2 cells requires molecular events involving tyrosine dephosphorylation. We also found that ROCKII is phosphorylated at the Y722 site during adhesion and that an Shp2-mediated dephosphorylation event is required for RhoA-induced deadhesion of D2 cells. Y722 phosphorylation decreases ROCKII binding to RhoA-GTP, thereby attenuating RhoA-mediated ROCKII activation. Importantly, expression of Y722F ROCKII markedly delayed D2 cells adhering to the fibronectin (FN) matrix. These results imply another layer of complexity in regulating ROCKII through Y722 phosphorylation and add Shp2 function in ROCKII activation.

Shp2, encoded by the *Ptpn11* gene, is a ubiquitously expressed nonreceptor protein tyrosine phosphatase (PTP) characterized by having two Src homology-2 domains in the N-terminal region that autoinhibit its PTP catalytic function (Chan and Feng, 2007; Mohi and Neel, 2007). To become activated, Shp2 is recruited to the membrane of tyrosine-phosphorylated docking proteins such as SHPS1/SIRP-1 (Fujioka et al., 1996). In receptor tyrosine kinase signaling, Shp2 is activated by the recruitment of the tyrosine phosphorylated scaffolding adapters, including insulin receptor substrate, fibroblast growth factor receptor substrate, and Gab proteins, to its N-Src homology-2 domain, causing catalytic activation of its PTP domain (Neel et al., 2003). Fibroblasts derived from Shp2^(-/-) mice have been shown to have

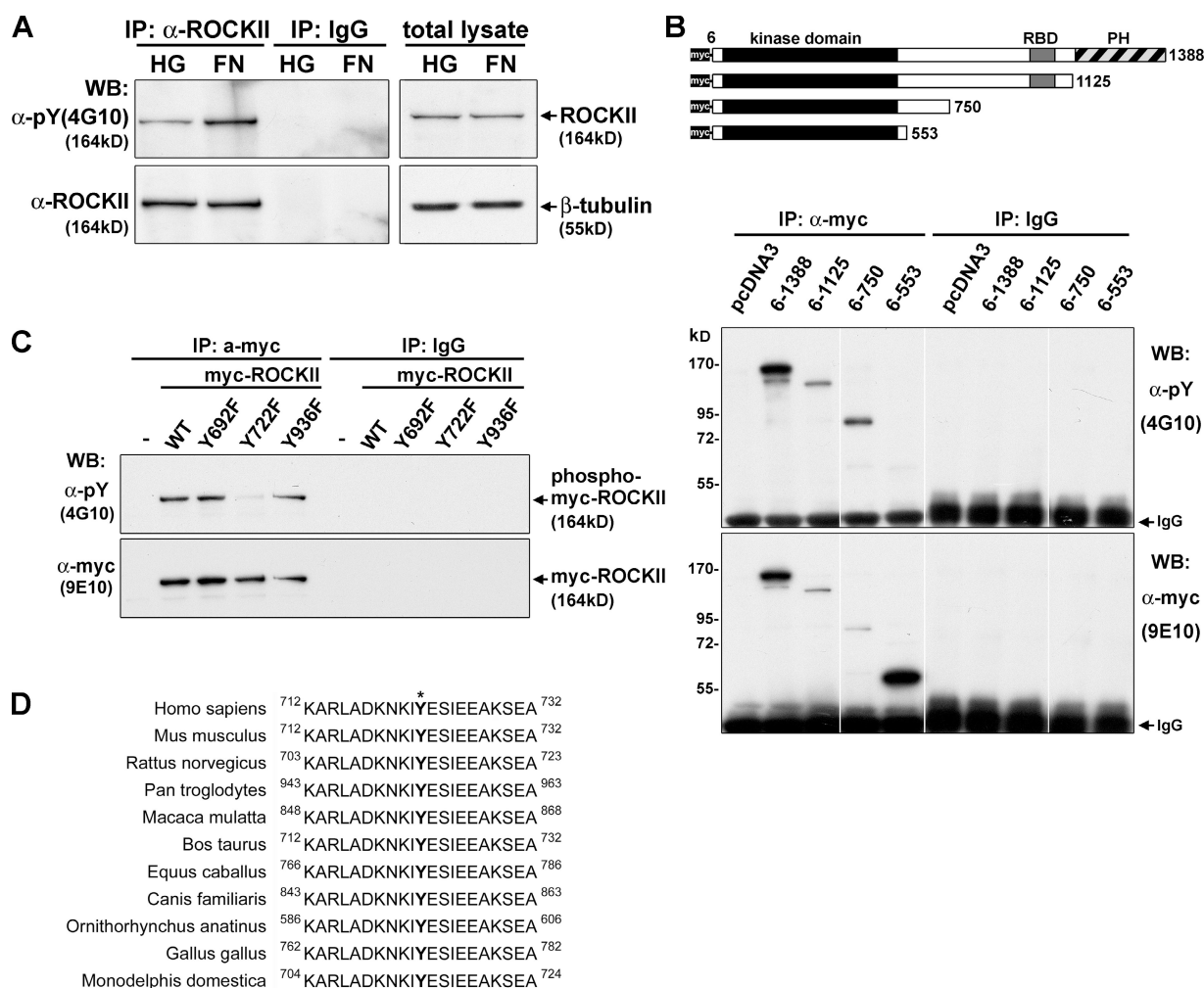


Figure 2. Tyrosine phosphorylation of ROCKII. (A) D2 cells were plated on hydrogel (HG) or FN-coated dishes in serum-containing medium for 2 h. Cells were treated with 50 μ M pervanadate for 20 min and harvested for immunoprecipitation with anti-ROCKII (C-20) antibody or normal IgG. Western blots of the immunoprecipitates with anti-phosphotyrosine (4G10) antibody followed by reprobing with anti-ROCKII (H-85) antibody are shown. (B and C) HEK293T cells were transiently transfected with control vector or the expression plasmids of wild-type and various mutants of myc-ROCKII as indicated. Cells were treated with 100 μ M pervanadate for 30 min before harvest for immunoprecipitation with anti-myc antibody or normal IgG. The immunoprecipitates were analyzed by Western blot as described in A. (D) Alignment of the vertebrate ROCKII sequence corresponding to the residues 712–732 of human ROCKII. Residues corresponding to Y722 of human ROCKII are indicated by asterisk and bold letters.

lower levels of motility with slower spreading rate (Yu et al., 1998). Experimental evidences have suggested that Shp2 regulates integrin function by dephosphorylating FAK for focal adhesion turnover during migration (Manes et al., 1999; von Wichert et al., 2003). Relevantly, data from this study showed that Shp2 is also involved in ROCKII dephosphorylation and participates in promoting activation of RhoA/ROCKII for cell detachment.

Results

Protein tyrosine dephosphorylation is required for RhoA/ROCK-mediated deadhesion

After plating suspended myeloid D2 cells in serum-free medium onto the culture dish, cells attached and exhibited spreading morphology. Upon serum stimulation, these spreading cells contracted and became rounding, whereas the presence of Y27632, a ROCK

inhibitor, or vanadate, a general tyrosine phosphatase inhibitor, prevented serum-responsive rounding (Fig. 1 A). Consistent with the change in the cell rounding, MLC phosphorylation was increased by serum stimulation, and this serum-responsive alteration was abolished by vanadate or Y27632 treatment (Fig. 1 B). Serum stimulation, as expected, increased endogenous RhoA activity; however, neither vanadate nor Y27632 treatment inhibited serum-responsive RhoA activation (Fig. 1 C). In fact, these two treatments increased RhoA activity. Treatment of cells attached to FN-coated dish in serum-free medium with TAT-RhoAV14 protein, which is a cell-permeable dominant-active form of RhoA, also induced cell rounding and detachment (Fig. 1 D). Again, vanadate or Y27632 treatment prohibited TAT-RhoAV14-induced cell detachment and MLC phosphorylation (Fig. 1 E). Together, all these results point out that biochemical events regulated by tyrosine dephosphorylation are required for RhoA/ROCK-mediated cell deadhesion.

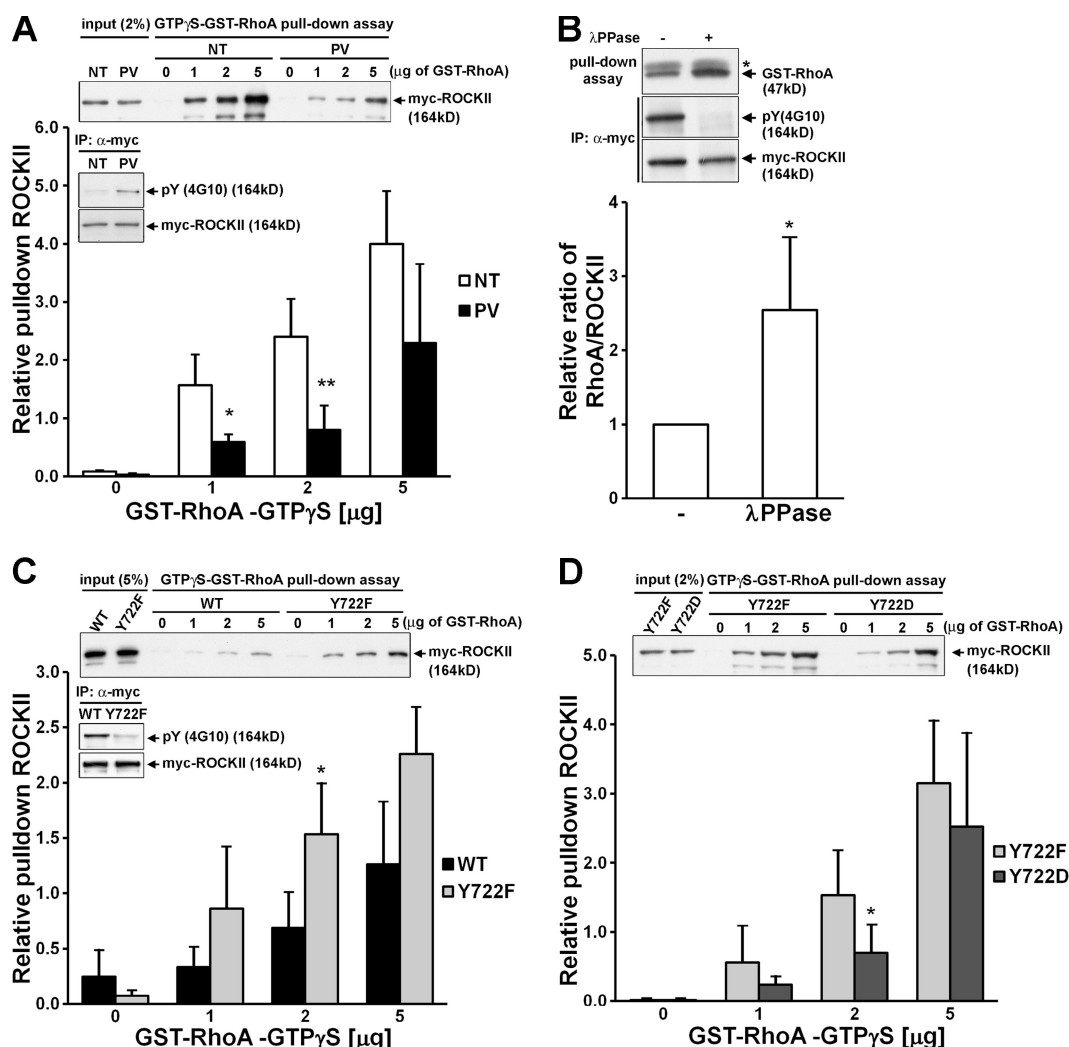


Figure 3. Y722 Phosphorylation reduces the level of ROCKII binding to GTP-RhoA. (A) HEK293T cells expressing myc-ROCKII were treated with or without 100 μ M pervanadate (PV) for 30 min. Equal amounts of cell lysates were incubated with the indicated amount of GTP γ S-loaded GST-RhoA protein followed by glutathione-Sepharose beads pull-down. A representative Western blot probed by myc antibody of the pull-down samples is shown at the top. Quantification of amounts of myc-ROCKII in the pull-down materials relative to the total input from three independent experiments is shown at the bottom. The inset indicates the phosphorylation status of myc-ROCKII by 4G10 antibody. (B) Myc-ROCKII immunoprecipitates from pervanadate-treated cells were incubated with or without λ protein phosphatase at 30°C for 20 min before incubation with 2 μ g of GTP γ S-loaded GST-RhoA protein for 30 min. After extensive wash, GST-RhoA protein pulled down by myc-ROCKII protein beads was detected by Western blotting with anti-RhoA antibody (top). *, heavy chain of IgG. Relative amount of RhoA pulled down by myc-ROCKII beads from three independent experiments is also shown (bottom). (C) Wild-type or Y722F mutant of myc-ROCKII from pervanadate-treated cells were used for the GTP γ S-GST-RhoA pull-down assay. (D) Y722F and Y722D myc-ROCKII from cells without pervanadate treatment were used for GTP γ S-GST-RhoA pull-down assay. All data are represented as mean \pm SD (*, $P < 0.1$; **, $P < 0.01$; $n \geq 3$).

ROCKII is tyrosine phosphorylated

We then examined the possible existence of tyrosine-phosphorylated forms of ROCKs in suspended and adherent D2 cells. These cells were treated with another phosphatase inhibitor, pervanadate, for 20 min before immunoprecipitation followed by Western blot analysis, using phosphotyrosine-specific monoclonal antibody 4G10. The result showed that endogenous ROCKII was indeed tyrosine phosphorylated. Interestingly, the tyrosine phosphorylation signal was increased with FN-mediated adhesion (Fig. 2 A). Because a small amount of endogenous ROCKI was immunoprecipitated by antibody against ROCKI in D2 cell extracts, we then focused on tyrosine phosphorylation of ROCKII for the rest of our study. To locate the region of the tyrosine phosphorylation site on ROCKII, we generated several deleted expression constructs of myc-ROCKII

and examined their phosphorylation statuses when expressed in HEK293T cells. We found that the tyrosine phosphorylation signal was retained in the wild-type myc-ROCKII (6–1,388), myc-ROCKII (6–1,125), and myc-ROCKII (6–750) but not in myc-ROCKII (6–553; Fig. 2 B). Our data indicated that the tyrosine phosphorylation site is located within the coiled-coil region between aa 554 and 750 of ROCKII. Two tyrosine residues (Y692 and Y722) within this region were each mutated to phenylalanine. We found that Y722F mutation markedly diminished the tyrosine phosphorylation signal, whereas Y692F and Y936F mutations had no effect (Fig. 2 C). Accordingly, we conclude that ROCKII is phosphorylated at the tyrosine 722 site. Sequence analysis showed that the surrounding sequence covering Y722 of ROCKII is highly conserved among different species (Fig. 2 D).

Y722 phosphorylation decreases the binding capacity of ROCKII to GTP-bound RhoA

Given that pretreatment of cells with tyrosine phosphatase inhibitor abolished RhoA14-mediated cell detachment, it is possible that tyrosine dephosphorylation of ROCKII affects RhoA-dependent activation of ROCKII. We then tested whether the status of ROCKII tyrosine phosphorylation affects its interaction with active RhoA. To this end, HEK293T cells were transfected by the expression vector of myc-ROCKII and treated with or without pervanadate for 30 min before harvesting for GTP γ S-loaded GST-RhoA binding assay. This RhoA binding activity assay was validated by the correlated amount of myc-ROCKII in GTP γ S-loaded GST-RhoA (active state) pulldown with the total input from untreated cells and the lack of ROCKII in the pulldown materials by GDP-loaded GST-RhoA (inactive state) beads (unpublished data). As shown in Fig. 3 A, the GTP γ S-RhoA binding activity was reduced for ROCKII from pervanadate-treated cells in which ROCKII was phosphorylated. Similarly, ROCKI binding to RhoA-GTP was also sensitive to pervanadate pretreatment (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>). Removing tyrosine phosphorylation of myc-ROCKII immunoprecipitates from pervanadate-treated cells by λ protein phosphatase was capable of increasing the amount of GTP γ S-RhoA binding to ROCKII, indicating the negative effect by tyrosine phosphorylation in RhoA binding (Fig. 3 B). We also compared GTP γ S-RhoA binding of wild-type and Y722F myc-ROCKII expressed in cells treated with pervanadate. Equal amounts of cell lysates were incubated with increasing amounts of GTP γ S-loaded GST-RhoA protein for pulldown assays. The results showed that more nonphosphorylatable Y722F myc-ROCKII was pulled down by GTP γ S-RhoA than wild-type myc-ROCKII at a different dosage of GTP γ S-RhoA protein (Fig. 3 C). Moreover, the amount of phosphomimetic Y722D mutant pulled down by GTP γ S-RhoA at the lower dosage was also significantly less than that of nonphosphorylatable Y722F mutant (Fig. 3 D).

RhoA-dependent ROCKII activation is sensitive to increased Y722 phosphorylation

We further compared the kinase activities of wild-type and Y722F ROCKII in response to GTP γ S-RhoA addition and pervanadate treatment. Cells expressing wild-type and Y722F ROCKII were treated with or without pervanadate before harvesting for immunoprecipitation. The ROCKII immunoprecipitates were incubated with different amounts of GTP γ S-GST-RhoA for kinase activity assay. Without pervanadate treatment to retain tyrosine phosphorylation, kinase activity of wild-type ROCKII was increased up to 2.5-fold by RhoA as compared with little induction of kinase activity by RhoA for ROCKII with tyrosine phosphorylation maintenance by pervanadate treatment. In contrast, kinase activity of Y722F ROCKII was increased by RhoA regardless of the pervanadate treatment (Fig. 4 A). As compared with Y722F mutant, Y722D mutant showed less RhoA responsiveness in kinase activation (Fig. 4 B). Collectively, these data demonstrate that RhoA-dependent kinase activation of ROCKII is inversely correlated with its Y722 phosphorylation status.

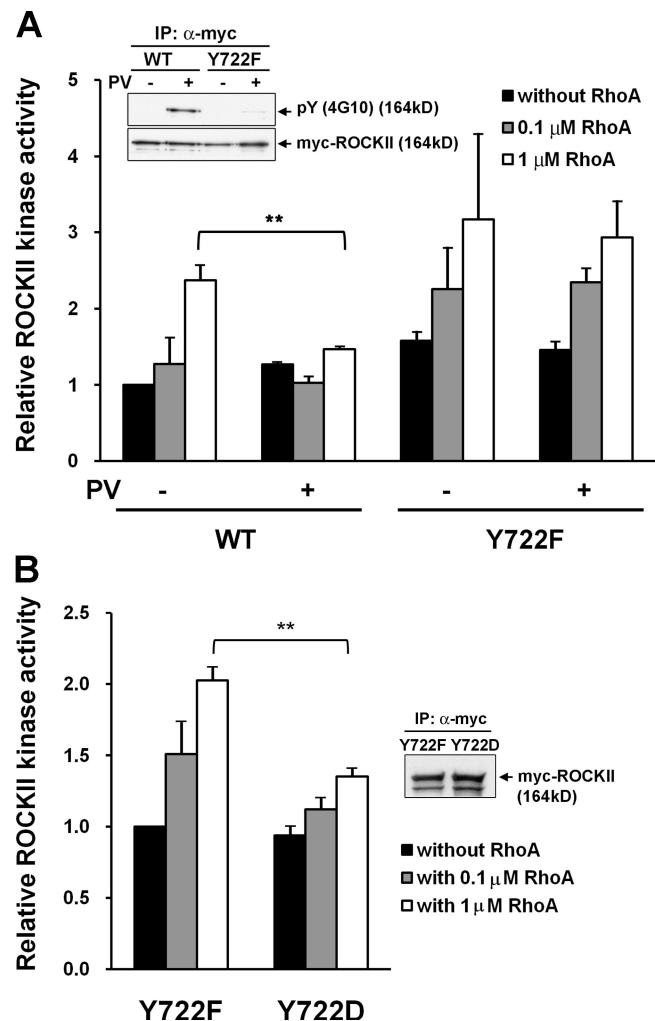
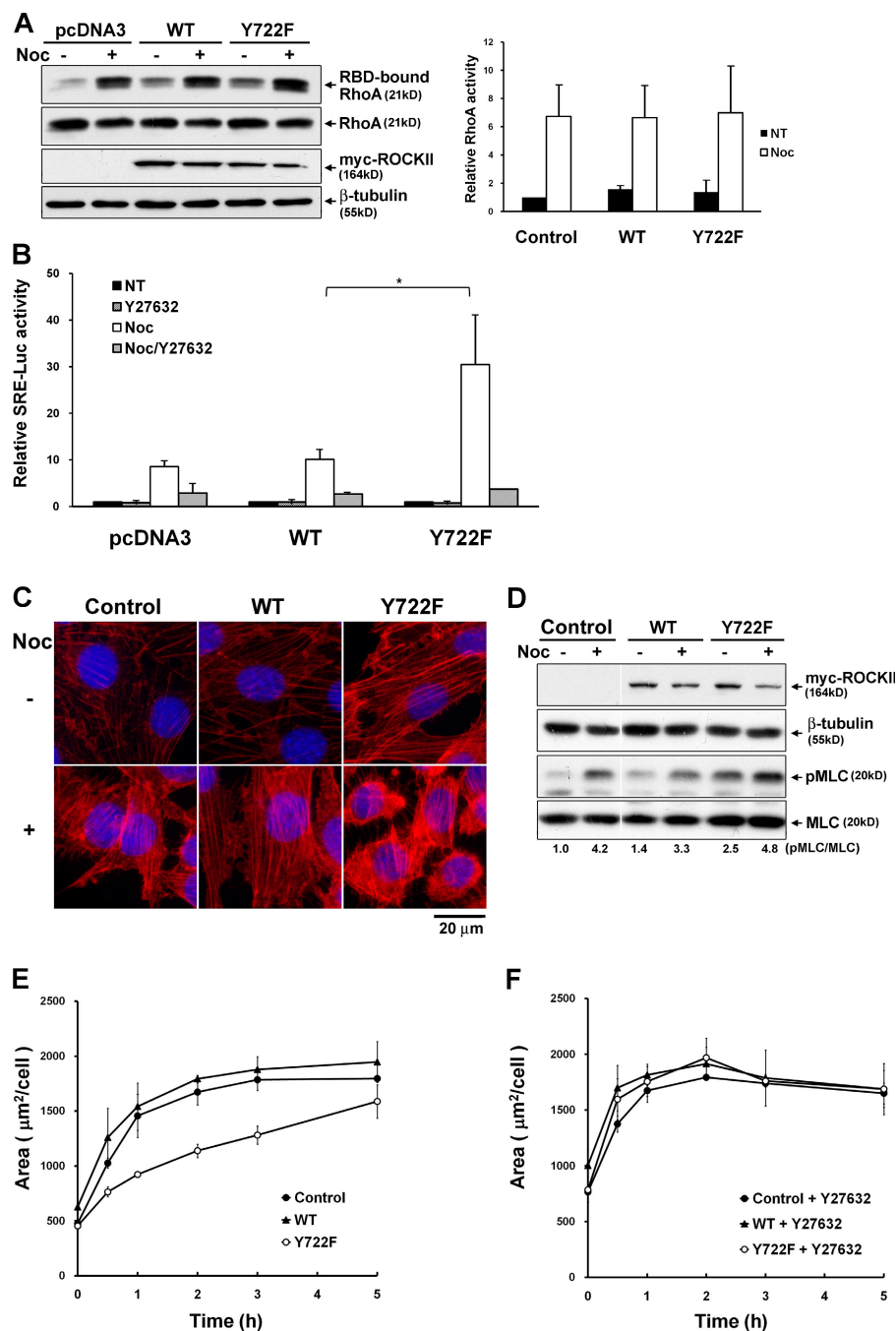


Figure 4. RhoA-dependent ROCKII activation is sensitive to Y722 dephosphorylation. (A) In vitro kinase assay in the absence or presence of 0.1 or 1 μ M GTP γ S-GST-RhoA protein with wild-type or Y722F myc-ROCKII immunoprecipitates from transfected cells treated with or without pervanadate. Inset indicates the phosphorylation status of ROCKII by Western blotting with anti-pY (4G10) antibody. (B) Comparison of RhoA-dependent kinase activation of Y722F and Y722D myc-ROCKII. Data are expressed as fold increase relative to wild-type myc-ROCKII without GTP-RhoA stimulation (mean \pm SD; **, $P < 0.05$).

Disrupting Y722 phosphorylation of ROCKII augments RhoA-dependent ROCK activation in cells

It has been shown that RhoA/ROCK signaling increases serum response factor (SRF)-dependent gene transcription (Chihara et al., 1997). To ascertain that Y722 phosphorylation negatively regulates the RhoA-dependent ROCKII activation in vivo, we then compared serum-responsive element (SRE)-dependent transcriptional activities in cells expressing wild-type and Y722F ROCKII. In serum-starved fibroblasts treated with nocodazole, RhoA is activated and stimulates focal adhesion and stress fiber formation (Bershadsky et al., 1996; Liu et al., 1998). Endogenous RhoA can be activated because of the release of GEF-H1 from microtubules (Krendel et al., 2002; Chang et al., 2006). We then used this experimental system to evaluate the functional effect of disrupting Y722 phosphorylation on the extent of ROCKII

Figure 5. Disrupting Y722 phosphorylation of ROCKII augments RhoA-dependent ROCK activation in cells. (A and B) NIH3T3 cells were cotransfected with pSRE-Luc, pCMV-HRC, and wild type, Y722F myc-ROCKII, or pcDNA3. After transfection for 24 h, cells were serum starved and treated with or without 10 μ M of nocodazole for 4 h. (A) Endogenous RhoA activities in the cells were measured by GST-RBD pulldown assay. A representative Western blot for the assay is shown on the left. Relative endogenous RhoA activity from three independent experiments is shown on the right. (B) Transfected cells were pretreated with or without 20 μ M of Y27632 before nocodazole stimulation. The reporter activity was determined and expressed as induction folds relative to the nontreated cells. Values are mean \pm SD of three independent experiments. (*, $P < 0.1$). (C) NIH3T3 cells stably expressing wild-type or Y722F myc-ROCKII were selected and these cells were serum starved and treated with or without 10 μ M of nocodazole for 1 h, fixed, and stained with rhodamine-phalloidin and HOECHST 33342. Bar, 20 μ m. (D) A parallel set of cells were harvested for Western blot analysis. The relative ratio of phospho-MLC to total MLC is shown below. (E) Cells were trypsinized and replated to culture dishes and monitored for 5 h by time-lapse microscopy. Areas of spreading were measured using MetaMorph software and expressed as mean \pm SD. (F) A parallel set of cells were treated with Y27632 for the spreading assay. Videos 1 and 2 (available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>) correspond to E and F, respectively.



activation when endogenous RhoA activation was induced by microtubule disassembly. Expression vectors of wild-type and Y722F ROCKII were individually cotransfected with pSRE-Luc reporter in NIH3T3 fibroblasts to quantitatively compare the magnitude of RhoA-mediated ROCK activation. Endogenous RhoA activities in cells expressing wild-type and Y722F ROCKII were similarly elevated by nocodazole treatment for 4 h (Fig. 5 A). However, RhoA-responsive SRE-dependent transcription activity was increased 30-fold by nocodazole treatment in cells expressing Y722F ROCKII, as compared with a 10-fold increase observed in control and wild-type ROCKII cells (Fig. 5 B). Inhibition of ROCK by incubating cells with Y27632 abolished nocodazole-induced SRE-dependent transcription, confirming

the contribution of ROCK signaling in SRE-dependent transcription. We also compared the effect of serum stimulation on SRF activation in cells expressing wild-type and Y722F ROCKII. ROCKII-dependent activation of SRF activation, as judged by the reporter activity sensitive to Y27632, after serum stimulation was also significantly higher in cells expressing Y722F mutant than in wild-type and control cells (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>). Thus, Y722F mutation renders ROCKII more active in stimulating SRF-mediated transcription in response to the RhoA signal.

To substantiate these observations, we further established NIH3T3 cells stably expressing wild-type and Y722F ROCKII. As expected, the steady-state level of MLC phosphorylation

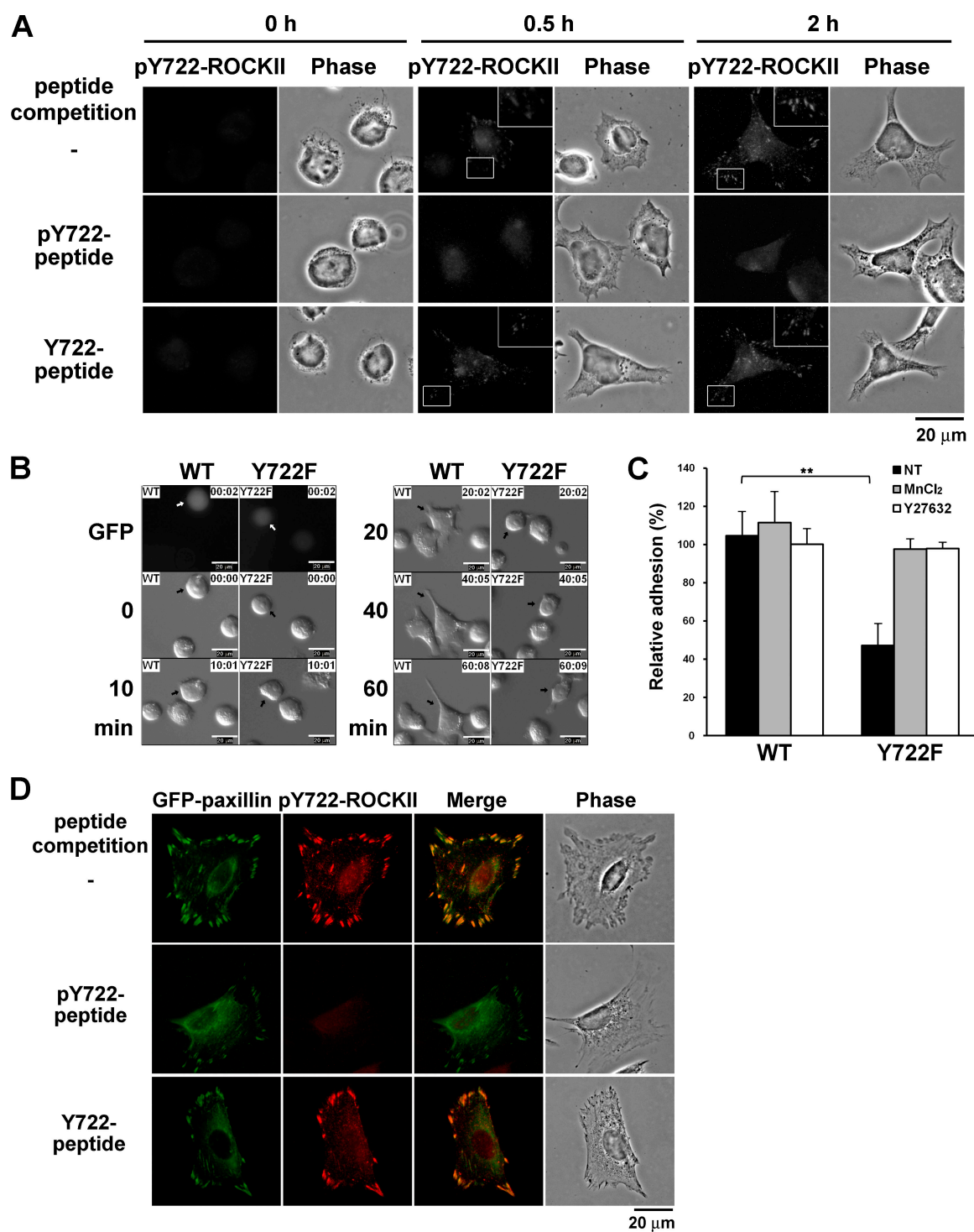


Figure 6. Y722 phosphorylation of ROCKII is associated with cell adhesion. (A) D2 cells in suspension and plated onto FN-coated coverslips for 0.5 and 2 h were fixed and stained with anti-pY722ROCKII antibody, which had been preincubated with or without 1 μ g/ml of Y722 peptide or pY722 peptide. Insets show enlargements of the boxed areas. Bar, 20 μ m. (B) D2 cells were transfected with the pEGFP and wild-type or Y722F myc-ROCKII (1:5 ratio). Transfected cells were plated on FN-coated dishes and monitored by time-lapse microscopy for 60 min after plating. Arrows indicate the GFP-positive transfected cells. Video 3 (available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>) corresponds to this figure. Bars, 20 μ m. (C) Transfected cells, as described in B, were preincubated with or without 2 mM MnCl₂ or 20 μ M Y27632 for 30 min and then assayed for their adhesion to FN-coated plates within 30 min as described in Materials and methods. Data are represented as mean \pm SD (**, $P < 0.05$). (D) NIH3T3 cells were transfected with the expression vector of GFP-paxillin. After serum starvation, cells were treated with 10 μ M nocodazole for 1 h and fixed for immunofluorescence staining of pY722-ROCKII. Bar, 20 μ m.

was increased in Y722F cells with a stronger stress fiber formation (Fig. 5, C and D). These cells were then treated with nocodazole to stimulate endogenous RhoA. If ROCKII is readily

activated by RhoA, these fibroblasts would become rounding in response to microtubule disassembly. In fact, fibroblasts expressing wild-type ROCKII continued to spread after nocodazole

treatment, indicating that the level of ROCKII activation by this RhoA signaling does not generate enough contraction force to dissociate molecular linkage for adhesion. In contrast, Y722F cells become rounding after nocodazole treatment, which is an indication of more contractility generated from RhoA/ROCKII activation (Fig. 5 C). To know whether loss of Y722 phosphorylation affects cell spreading, we measured the spreading rate of NIH3T3 cells stably expressing wild-type and Y722F ROCKII during adhesion. When plated onto culture dishes, Y722F fibroblasts spread slower than wild-type cells. With Y27632 pretreatment, these cells spread at a similar rate (Fig. 5, E and F; and Videos 1 and 2, available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>). It is likely that Y722 phosphorylation of ROCKII plays a role in the spreading process.

Y722 phosphorylation of ROCKII in adhesion

Next, we generated antibody specifically recognizing phosphorylated Y722 peptide. Fig. S3 (available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>) shows the characterization of this antibody, which was then used for immunofluorescence staining of D2 cell transition from suspension to adhering onto FN-coated plates. The immunofluorescence staining revealed a clear punctate pattern in the peripheral membrane protrusion areas of D2 cells that were adhering to FN-coated dishes for 0.5 or 2 h (Fig. 6 A). The staining signal was neutralized by phosphorylated Y722 peptide but not by unphosphorylated peptide, indicating the specificity of this antibody in detecting Y722-phosphorylated ROCKII. The intensity of Y722-phosphorylated ROCKII was increased in D2 cells during transition from suspension to adhesion (Fig. 6 A). A similar punctate staining pattern was also observed in HEK293T cells, and ROCKII depletion by siRNA significantly diminished this staining pattern, confirming the specificity of this antibody in detecting phosphorylated Y722 ROCKII in the peripheral membrane protrusion areas (Fig. S3 D). Some residual staining in ROCKII-depleted cells could be caused by either incomplete knockdown or a low level of nonspecific reactivity. Having found that Y722 phosphorylation is increased with cell adhesion without pervanadate stimulation, we then assessed whether Y722 dephosphorylation of ROCKII affects deadhesion and adhesion. D2 cells were transfected with wild-type and Y722F ROCKII expression vectors and plated onto FN-coated dishes in the presence of serum for time-lapse video recording. The successfully transfected cells were indicated by green fluorescence from cotransfected pEGFP. By 20 min, cells expressing wild-type ROCKII readily adhered and spread onto FN matrix, whereas Y722F cells remained floating and did not adhere to spread until 60 min of plating (Fig. 6 B and Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>). Quantitative analysis of the cell number adhering to FN-coated dishes after seeding for 30 min showed that expression of Y722F mutant significantly delayed D2 cell adhesion onto the FN matrix (Fig. 6 C). Previously, we used antibody blocking β 1-integrin and found that FN-mediated adhesion for D2 cells is β 1-integrin-dependent (unpublished data). Considering the possibility that expression of Y722F ROCKII might directly affect integrin function of D2 cells, we then pretreated the transfected cells with $MnCl_2$ which

locks β 1-integrin in active conformation (Gailit and Ruoslahti, 1988). $MnCl_2$ pretreatment restored the adhesion ability of D2 cells expressing Y722F ROCKII, indicating that the decrease of adhesion is not caused by a functional defect of β 1-integrin. Y27632 treatment had a similar effect to $MnCl_2$, demonstrating that gain of ROCKII function by Y722F mutation makes cells less susceptible for FN-mediated integrin activation (Fig. 6 C). We also transfected NIH3T3 cells with expression vector of GFP-paxillin, an indicator of focal adhesion contact sites, followed by immunofluorescence staining using antibody against phosphorylated Y722 ROCKII. The result showed colocalization of Y722-phosphorylated ROCKII with GFP-paxillin, suggesting that Y722 phosphorylation of ROCKII is specified in the focal adhesion contact sites (Fig. 6 D).

Shp2 is essential for ROCK-dependent deadhesion in response to RhoA activation

Because RhoA-dependent activation of ROCKII is interfered with by inhibition of phosphatase, we further addressed the question of which phosphatase is involved in its activation during deadhesion. It is well established that Shp2 is a tyrosine phosphatase involved in regulation of focal adhesion turnover and cell motility. We then tested whether Shp2 is involved in the RhoA V14-induced cell detachment. Cells expressing GFP-RhoA V14 showed rounding morphology, whereas coexpression of catalytic-inactive Shp2(C/S) prevented GFP-positive cells from rounding. In contrast, coexpression of another catalytic-dead tyrosine phosphatase, PTP-1B (C/S), had no effect on inhibiting RhoA V14-induced rounding. (Fig. 7, A and B). Expression of the constitutive-active form of ROCKII (ROCKII(CAT)) induced cell rounding regardless of Shp2(C/S) expression (Fig. 7 C), suggesting that Shp2 is an upstream regulator for ROCK activation in RhoA V14-induced cell rounding. Shp2(C/S) expression or Shp2 knockdown by siRNA in HEK293T cells also impaired RhoA V14-induced cell detachment and stimulation of MLC phosphorylation (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>), confirming the functional involvement of Shp2 in RhoA-dependent ROCK activation. Furthermore, expression of gain-of-function Shp2 (E76G) mutant in D2 cells also reduced cell adhesion onto FN-coated dishes in a Y27632-sensitive manner, indicating that active Shp2 induction of cell deadhesion is dependent on ROCK activity (Fig. 7 D).

We also tested the inhibitory effect of Shp2(C/S) expression on serum-induced rounding in D2 cells transfected with wild-type and Y722F ROCKII. The transfected cells were plated onto the culture dishes in serum-free medium to allow cells spreading. Transfected cells indicated by green fluorescence positivity were counted before a 20% serum addition to induce cell rounding. Thereafter, spreading and rounding green fluorescence-positive cells were counted. Expression of Shp2(C/S) significantly prevented cells expressing wild-type ROCKII from serum-induced rounding. In contrast, cells expressing Y722F ROCKII were insensitive to Shp2(C/S) expression and underwent serum-induced rounding (Fig. 7, E and F). Accordingly, Y722F represents a gain-of-function mutation for ROCKII, which dispenses the requirement of Shp2-mediated dephosphorylation for its activation.

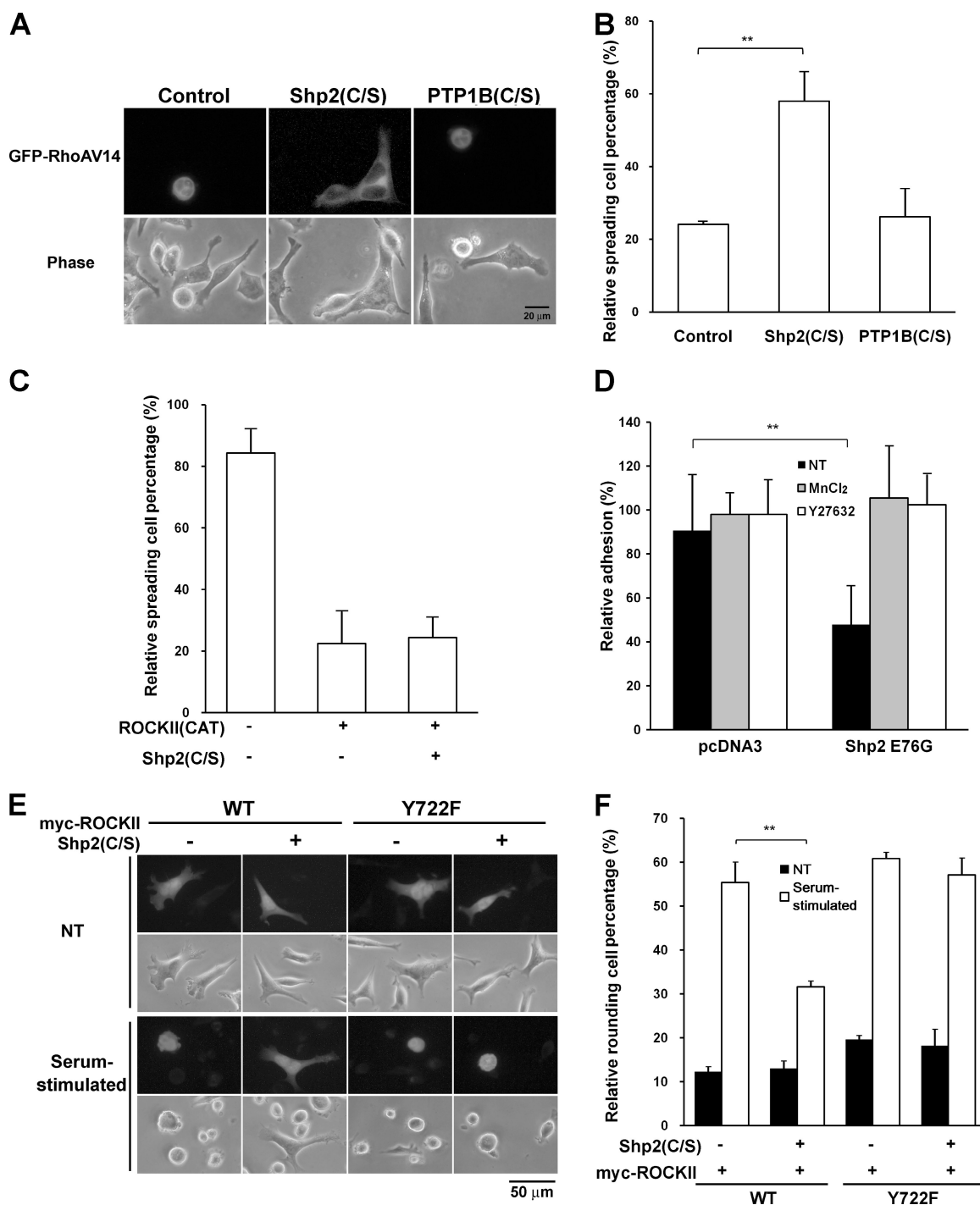


Figure 7. Shp2 is essential for ROCK-dependent rounding in response to RhoA activation. (A) Fluorescent and phase-contrast image of D2 cells transfected with expression vector of pGFP-RhoA V14 and flag-Shp2(C/S) or PTP1B(C/S) (amount of DNA ratio at 1:5) as indicated and plated onto FN-coated coverslip for 30 min. Bar, 20 μ m. (B) The percentages of GFP(+) cells spreading on coverslips in total GFP(+) cells were counted and calculated. (C) D2 cells transfected by pEGFP, pMyc-ROCKII(CAT) with or without pflag-Shp2(C/S) (amount of DNA ratio at 1:3:12) were analyzed for spreading on FN-coated coverslips. (D) D2 cells were cotransfected with the expression vectors of GFP and Shp2 E76G or pcDNA3 and were treated with MnCl₂ or Y27632, as indicated, before being plated onto FN-coated coverslips. Relative number of GFP(+) cells spreading on FN-coated coverslips within 30 min was determined. (E) Cells transfected by pEGFP, wild-type, or Y722F pMyc-ROCKII with or without pflag-Shp2(C/S) (DNA ratio at 1:2:4) were plated onto dish in serum-free medium. After 6 h of incubation, cells were stimulated with or without 20% of serum for 10 min. Cells were imaged by fluorescence microscopy. Bar, 50 μ m. (F) The rounding percentage of total GFP(+) cells was calculated. All data are represented as mean \pm SD (**, $P < 0.05$).

ROCKII is a substrate of Shp2 that mediates Y722 dephosphorylation

Next, we assessed whether ROCKs would be the direct substrates of Shp2. On the basis of the transient interaction nature

between a phosphatase and its substrate, Shp2 trapping mutant, which carries D425A/C459S mutation, was used to test for its interaction with ROCKII. We isolated flag-tagged wild-type and trapping mutant Shp2 proteins expressed in HEK293T cells

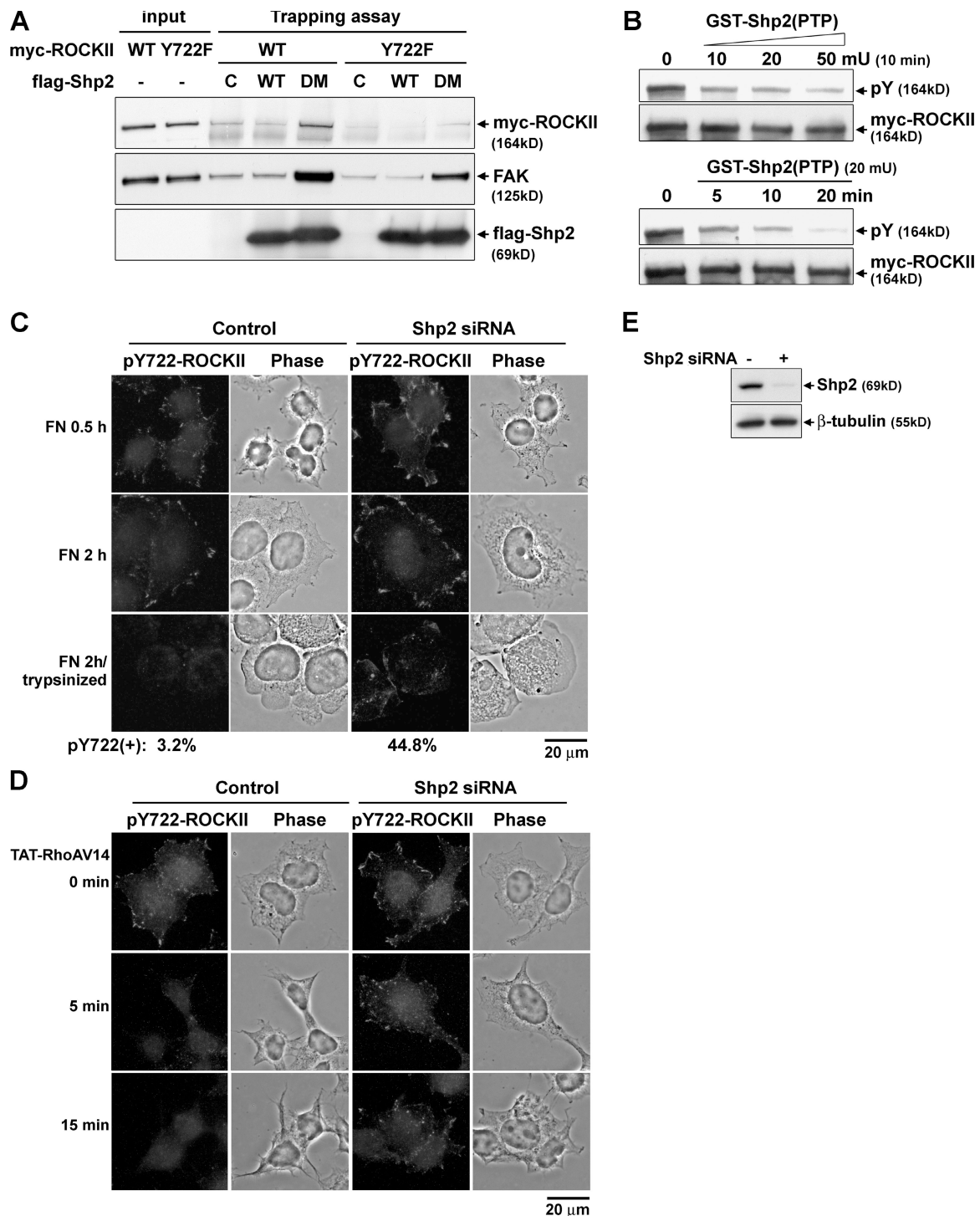


Figure 8. **ROCKII is a substrate of Shp2 that mediates Y722 dephosphorylation.** (A) In vitro substrate trapping assay. Wild-type (WT), substrate trapping mutant (DM) flag-Shp2, and control beads, as indicated, were incubated with lysates from pervanadate-treated cells expressing wild-type or Y722F myc-ROCKII. The pull-down proteins were analyzed by Western blotting. (B) Shp2 dephosphorylates ROCKII in vitro. Immunoprecipitated myc-ROCKII protein from pervanadate-treated cells was incubated with increasing amounts of GST-N-del Shp2(PTP) at 30°C for 0–20 min and analyzed by Western blotting. (C) HEK293T cells were transfected with control or Shp2 siRNA for 2 d and replated to FN-coated coverslips for 0.5 or 2 h or trypsinized after 2 h of FN-mediated adhesion. Cells were fixed and subjected to pY722-ROCKII immunofluorescence staining. The percentage of trypsinized cells that were positive in pY722-ROCKII staining is shown below. Bar, 20 μm. (D) Immunofluorescence images of pY722 ROCKII in siRNA transfected HEK293T cells on FN-coated coverslips with TAT-RhoAV14 treatment at the indicated time. (E) Western blot of siRNA transfected cells.

by M2 agarose beads, separately. Each set of protein beads were incubated with equal amounts of cell lysates from HEK293T cells expressing wild-type or Y722F ROCKII, which had been

stimulated by pervanadate treatment. Trapping mutant, but not wild-type, Shp2 was able to pull down wild-type ROCKII. This approach also allowed FAK, a known substrate of Shp2

(Tsutsumi et al., 2006), in the lysates to be pulled down by trapping mutant but not by wild-type Shp2, indicating the specific interacting relationship between the phosphosubstrate and phosphatase at the active site rather than an Shp2-containing complex formation. Very little amount of Y722F mutant was pulled down by the trapping mutant Shp2 protein, indicating the specific interaction between phosphorylated Y722 with the Shp2 catalytic site (Fig. 8 A). Interestingly, ROCKI was similarly pulled down by trapping Shp2 mutant protein (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>). We further incubated immunoprecipitated phosphorylated ROCKII with recombinant N-deleted Shp2 protein to test whether ROCKII can be a direct *in vitro* substrate of Shp2. The results showed an increase of ROCKII dephosphorylation by N-del Shp2 in a dose- and time-dependent manner (Fig. 8 B).

We then tested the effect of Shp2 depletion in HEK293T cells on ROCKII Y722 phosphorylation during cell spreading and rounding. When adhering to FN-coated dishes, cells depleted of Shp2 spread more with increased punctate staining of Y722-phosphorylated ROCKII in the protruding edges. Because it has been shown that RhoA/ROCK contractility is increased during cell rounding upon trypsinization (Ren et al., 1999; Maddox and Burridge, 2003), we then examined the involvement of Shp2 in ROCKII Y722 dephosphorylation in response to trypsinization. The immunofluorescence staining data showed that Y722 phosphorylation of ROCKII became diminished in the newly rounding HEK293T cells after trypsinization, whereas a significant proportion of cells depleted of Shp2 were still positive in Y722-phosphorylated ROCKII staining (Fig. 8 C). With TAT-RhoAV14 treatment to induce detachment, the staining signal of Y722-phosphorylated ROCKII was still retained in cells depleted of Shp2 but not in control cells (Fig. 8 D). Together, these results indicated that Shp2 participates in Y722 dephosphorylation of ROCKII during cell rounding.

Discussion

In this paper, we show for the first time that ROCKII activation by RhoA is negatively modulated by Y722 phosphorylation, providing an additional layer of control that the extent of ROCKII activation can be uncoupled with RhoA signaling. In addition, this paper adds ROCKII to the substrate list of Shp2, which dephosphorylates ROCKII at the Y722 site and participates in the process of RhoA-dependent ROCKII activation during cell deadhesion. In this paper, we postulate that reversible modification of ROCKII by tyrosine phosphorylation dynamically regulates RhoA/ROCK-mediated contractility, by which ROCK is fine-tuned in a spatiotemporal manner during adhesion and spreading (Fig. 9).

Because Y722 is neither located within the kinase region nor the RBD of ROCKII, an important question is how Y722 phosphorylation negatively controls the ROCKII function. To address this question, we have compared the RhoA-GTP binding activity of ROCKII, which is maintained tyrosine phosphorylated by pervanadate treatment. Our results showed that the GTP-RhoA binding activity of ROCKII was reduced by maintenance of Y722 phosphorylation. Substitution of tyrosine 722 by phenylalanine elevated RhoA-GTP binding and kinase activities

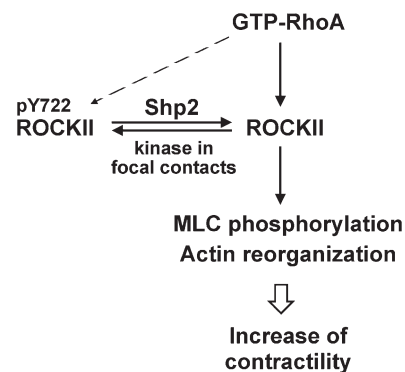


Figure 9. Model of regulation of ROCKII by reversible tyrosine phosphorylation. Y722 phosphorylation by a kinase in focal contacts decreases ROCKII binding affinity to RhoA. Dephosphorylation of ROCKII by Shp2 stimulates its RhoA binding activity, thereby increasing actomyosin contractility.

of ROCKII regardless of pervanadate treatment. The difference in the extent of RhoA-GTP binding ability correlates with the corresponding kinase activity between phosphorylated or unphosphorylated forms of ROCKII. Because the kinase activity of a phosphomimetic mutant Y722D is much less responsive to RhoA stimulation, we conclude that Y722 phosphorylation negatively modulates ROCKII binding to active form of RhoA, thereby attenuating its kinase activity. In other words, when a limiting amount of RhoA is activated, ROCKII with Y722 phosphorylation is less active as compared with unphosphorylatable Y722F mutant in its kinase activation. However, it remains an open question whether Y722 phosphorylation affects RhoA binding through an interacting protein or simply as a result of conformational change that hampers the accessibility of the RBD to RhoA. Another important question is whether ROCKI binding to GTP-RhoA is also regulated by tyrosine phosphorylation. Like ROCKII, the endogenous amount of ROCKI in HeLa cells and myc-ROCKI expressed in HEK293T cells pulled down by GST-RhoA-GTP was also sensitive to pervanadate treatment, and tyrosine phosphorylation was detected in ROCKI immunoprecipitate (Fig. S1). Together with the result showing that the substrate trapping mutant Shp2 is able to pull down ROCKI in adherent D2 cell lysates (Fig. S5), the negative control by tyrosine phosphorylation is probably also true for ROCKI regulation. Because ROCKI does not have a corresponding Y residue, its tyrosine phosphorylation site remains to be determined.

In this study, we observed a higher level of tyrosine phosphorylation of ROCKII in adherent than suspension D2 cells, implying that ROCKII is phosphorylated at the time of adhesion. Of note, Y722 phosphorylated ROCKII was mainly detected in the peripheral protruding regions in spreading cells. As indicated by colocalization with GFP-paxillin in spreading NIH3T3 fibroblasts, it is apparent that Y722 phosphorylation of ROCKII is particularly enriched in the focal adhesion contact sites. It is likely that the action by tyrosine kinase in the adhesion contact sites makes ROCKII tyrosine phosphorylated to become less active. Actomyosin-based contractility stimulated by MLC phosphorylation has been shown to be necessary for the focal adhesion formation. Treatments of cells with inhibitors of contractility,

such as BDM and MLC kinase inhibitor, cause the dissociation of periphery focal adhesions. However, inhibition of ROCK disrupts focal adhesions in the central region rather than in the periphery (Totsukawa et al., 2004), implying that ROCK-mediated contractility is not involved in the assembly of focal adhesion in the periphery region where RhoA is consistently activated for microtubule stabilization via RhoA/Dia signaling (Palazzo et al., 2004). Our data showing that ROCK Y722 phosphorylation is specified in the focal contacts in the cell periphery suggest that Y722 phosphorylation plays a role in restricting ROCK activation in a spatial manner for the maturation of focal contact formation in cell spreading. Because RhoA/Dia signaling for microtubule stabilization is essential for cell motility, it is possible that negative control by Y722 phosphorylation provides a means to have Dia preferentially activated in a spatiotemporal manner so that ROCK is uncoupled with the RhoA signal precisely. In support of this hypothesis, we found that NIH3T3 fibroblasts expressing Y722F ROCKII also spread slower, as compared with the cells expressing similar levels of wild-type ROCKII. Likewise, expression of Y722F mutant retards D2 cell adhering to FN-coated dish, indicating that the decrease in FN-mediated adhesion is closely associated with lack of Y722 phosphorylation. Both in vitro and in vivo data are consistent with the scenario that Y722 phosphorylation plays a role in optimizing ROCKII-mediated contractility for adhesion and spreading. Upon deadhesion signal, ROCKII might become dephosphorylated, thereby increasing its RhoA binding activity. As a result, ROCKII becomes fully activated to generate contraction force, disrupting the linkage between integrin and matrix to cause rounding for detachment.

Although the kinase in the adhesion complex for ROCKII phosphorylation remains to be defined, our experimental data indicate that Shp2 is required for dephosphorylation of ROCKII in response to the deadhesion signal. Probably, the kinase and Shp2 work dynamically in the adhesion contact sites so that the contraction force generated by local ROCKII activity can be precisely regulated without turning RhoA signaling off or on. Shp2-deficient fibroblasts or MCF-7 cells expressing dominant-negative Shp2(C/S) display reduced cell motility and enhanced focal adhesion (Yu et al., 1998; Manes et al., 1999; Inagaki et al., 2000), suggesting that Shp2 plays an important role in the control of cell spreading and migration. It has been demonstrated that the function of Shp2 in cell motility involves FAK dephosphorylation, which is the signal that turns off the focal adhesion contact for local detachment (von Wichert et al., 2003). Our results showing that expression of Y722F ROCKII reverses the inhibitory effect of loss of Shp2 function on serum-stimulated cell rounding suggest that the action of Shp2 on ROCKII dephosphorylation is necessary for cell rounding. Given the fact that ROCKII phosphorylation is associated with adhesion-detachment signaling pathways necessary for cell motility, Shp2 effect on cell motility might be related to the control of ROCKII dephosphorylation. Collectively, we proposed that the Shp2 might dephosphorylate ROCKII and FAK in a concerted manner to result in focal adhesion turnover for cell migration.

Shp2 deficiency causes defect in myelopoiesis and erythropoiesis in the mice model (Qu et al., 1997). Gain-of-function mutation of Shp2 is associated with several leukemias (Chan and Feng,

2007; Mohi and Neel, 2007). One study has demonstrated that expression of gain-of-function mutant Shp2 E76A can still increase SRF-mediated *c-fos* SRE reporter transcription even in the presence of dominant-active RhoA in lymphocyte after IL-2 stimulation, indicating additional up-regulation of ROCK-mediated SRF activation by Shp2 activation (Arnaud et al., 2004). In this study, we showed that expression of another gain-of-function mutant, Shp2 E76G, in D2 cells decreased cell adhesion onto FN in a Y27632-sensitive manner. Conversely, disruption of Shp2 function abolished RhoA-induced deadhesion. Because regulation of adhesion and deadhesion is also an important process for hematopoiesis, this study also provokes the question of whether Shp2-mediated regulation of ROCK is critical for myelopoiesis and erythropoiesis and is involved in leukemia development associated with gain-of-function mutation of Shp2.

Materials and methods

Plasmids

pcDNA3-myc-ROCKII (6–553), (6–750), and (6–1,125) were constructed by insertion of the corresponding cDNA fragments derived from pEF-myc-ROCKII (6–1,388, wild type; obtained from K. Kaibuchi, Nara Institute of Science and Technology, Ikoma, Japan) into pcDNA3 vector. The Y692F, Y722F, Y722D, and Y936F mutations were individually introduced to pEF-myc-ROCKII (6–1,388) using the Quick-Change site-directed mutagenesis kit (Stratagene). Expression construct of paxillin (obtained from R.H. Chen Institute of Biochemistry, Academia Sinica, Taipei, Taiwan) was subcloned into pEGFP vector. Expression constructs of wild-type and C459S Shp2 (obtained from T.C. Meng, Institute of Biochemistry, Academia Sinica, Taipei, Taiwan) were subcloned into pflag-CMV2 vector. The double mutant of Shp2 (D425A and C459S) was generated by site-directed mutagenesis. Expression construct of Shp2 E76G was obtained from J.Y. Chen (Department of Hematology, National Taiwan University Hospital, Taipei, Taiwan). The expression construct of myc-ROCKI was provided by J. Hamelin (INSERM U461, Faculté de Pharmacie, Châtenay-Malabry, France). The cDNA of RhoA (wild type) and RhoAV14 was cloned into pGEX-2T and pTAT-HA vector to generate pGEX-RhoA and pTAT-RhoAV14, respectively.

Antibodies and reagents

Y27632 was obtained from EMD; anti-phospho-MLC2 (T^{18}/S^{19}) antibody was obtained from Cell Signaling Technology; anti-MLC, anti-HA, anti- β -actin, and anti- β -tubulin antibodies and rhodamine-phalloidin were obtained from Sigma-Aldrich; anti-ROCKII and anti-RhoA antibodies were purchased from Santa Cruz Biotechnology, Inc.; anti-phosphotyrosine (clone 4G10) antibody was obtained from Millipore; and anti-myc antibody was purified from hybridoma clone 9E10. TAT-RhoAV14 protein was purified as previously reported (Chang et al., 2006). The recombinant N-del Shp2 protein was purchased from Abcam. The siRNA targeting human Shp2 was obtained from Thermo Fisher Scientific. The anti-pY722ROCKII antibody was raised using phosphopeptide Ac-LADKNKI(pY)ESIEEAC-amide and affinity purified by the phosphopeptide column with the removal of the nonphospho antibodies (Quality Controlled Biochemicals). Of note, this antibody was able to detect a drastically increased signal of Y722 phosphorylated ROCKII in cells after pervanadate treatment. In particular, the strong immunofluorescence staining of Y722-phosphorylated ROCKII was all over the membrane periphery in pervanadate-treated HEK293T cells, which was a contrast to the weak fluorescence intensity in punctate pattern seen in untreated cells (Fig. S3).

Cell culture and transient transfection

D2 cells were cultured in RPMI1640 containing 10% heat-inactivated FBS. HEK293T and NIH3T3 cells were maintained in DME containing 10% FBS or calf serum. For transient transfection experiments, HEK293T and NIH3T3 cells were transfected by Lipofectamine reagent (Invitrogen). D2 cells were transfected by MicroPorator MP-100 (Digital Bio Technology). For siRNA transfection experiment, HEK293T cells were transfected by Lipofectamine 2000 reagent.

Endogenous RhoA activity and RhoA-ROCK interaction assays

GST-RBD affinity precipitation of cellular GTP-bound RhoA was used to assay endogenous RhoA activity as described by Ren and Schwartz (2000).

The RhoA-ROCK interaction was determined by GST-RhoA-GTP γ S in vitro pull-down assay. GST-RhoA protein was purified and loaded with GTP γ S (Amano et al., 2000). Cells expressing myc-ROCKII were harvested and lysed with a buffer (0.2% NP-40, 20 mM Tris-HCl, pH 7.4, 20 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 mM NaF, 2 mM Na₃VO₄, 1 mM PMSF, 10% glycerol, and protease inhibitor cocktail). The precleared supernatants were incubated with different amounts (0–5 μ g) of GTP γ S-loaded GST-RhoA protein for 15 min and followed by glutathione beads for pull-down at 4°C. After extensive washing, myc-ROCKII pulled down by GTP γ S-GST-RhoA was detected by Western blotting with anti-myc antibody.

Immunoprecipitation and in vitro kinase assays

D2 cells were harvested in an immunoprecipitation buffer (1% Brij58, 25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 20 mM NaF, 2 mM Na₃VO₄, 10 μ M PAO, and protease and phosphatase inhibitor cocktail) and the lysates were immunoprecipitated with anti-ROCKII antibody (C-20). For in vitro kinase assay, myc-ROCKII expressed in HEK293T cells was immunoprecipitated and suspended in a kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 0.5 mM DTT, 5 mM NaF, and 0.1 mM Na₃VO₄). Upon addition of GTP γ S-GST-RhoA, kinase reaction was started by adding 2.5 μ g of myelin basic protein, 5 μ Ci of γ -[³²P]ATP, and cold ATP (20 μ M final concentration). After incubation at 30°C for 20 min, 5 μ l of reaction mixture was spotted onto P81 paper (Whatman) and washed five times with 75 mM phosphoric acid. Incorporation of ³²P into the substrate was determined by scintillation counting.

Analysis of cell adhesion and spreading

D2 cells were plated on FN-coated plate in serum-containing medium and placed in the temperature- and CO₂-controlled chamber of a microscope (Axiovert 200M; Carl Zeiss, Inc.). Images were collected at 30-s intervals over 60 min with a cooled charge-coupled device video camera (Photometrics CoolSNAP HQ2; Roper Scientific) operated by MetaMorph image software (MDS Analytical Technologies). For quantitative analysis, cells were seeded on FN-coated coverslips. After 30 min, the nonadherent cells were removed and the percentage of GFP-positive cells that remained on the FN was determined. For spreading assay, NIH3T3 cells were plated onto 6-well plates in serum-free medium for 30 min. The spreading of these rounding cells was stimulated by addition of 10% serum and then monitored for 5 h by time-lapse microscopy. Areas of spreading were measured using MetaMorph software.

Immunofluorescence analysis

Cells were fixed with 3% PFA in PBS for 30 min, followed by permeabilization with 0.3% Triton X-100/TBS for 5 min. After blocking with 5% normal goat serum/Tris-buffered saline containing 0.1% of Triton X-100 for 60 min, cells were incubated with anti-pY722ROCKII antibody (preneutralizing with or without 1 μ g/ml of Y722-peptide or pY722-peptide for 1 h) overnight at 4°C, and then incubated with TRITC-conjugated goat anti-rabbit antibody for 1 h. Cells were then washed, mounted, and examined by a fluorescence microscope (AX70; Olympus) with a 100 \times oil lens. Images were captured with a digital camera (E330; Olympus) and arranged using Photoshop software (Adobe).

In vitro dephosphorylation assay

Myc-ROCKII protein was immunoprecipitated from pervanadate-treated HEK293T cells and incubated with 25 μ l of reaction buffer containing 25 mM of Tris, pH 7, 50 mM NaCl, 2 mM EDTA, 5 mM DTT, 0.1 mg/ml of BSA, and N-del Shp2 at 30°C. After 5–20 min, the reaction was stopped by the addition of Laemmli buffer containing 2 mM Na₃VO₄ and boiled at 95°C for 10 min.

Online supplemental material

Fig. S1 shows that the maintenance of tyrosine phosphorylation decreased the GTP γ S-RhoA binding activity of ROCKI. Fig. S2 shows the effect of Y722 mutation on ROCKII-dependent SRF activation in response to serum stimulation. Fig. S3 shows the characterization of anti-pY722 ROCKII antibody. Fig. S4 shows the requirement of Shp2 on RhoA14-induced cell detachment and MLC phosphorylation. Fig. S5 shows in vitro Shp2 substrate trapping assay. Videos 1 and 2 show cell spreading of NIH3T3 fibroblasts expressing wild-type and Y722F ROCKII. Video 3 shows the effect of Y722F ROCKII expression on cell adhesion in D2 cells. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200710187/DC1>.

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