Rac1 Regulation of Surface Expression of Protease-Activated Receptor-1 and Responsiveness to Thrombin in Vascular Smooth Muscle Cells

Tatsuya Yufu, Katsuya Hirano, Dan Bi, Mayumi Hirano, Junji Nishimura, Yukihide Iwamoto, Hideo Kanaide

Objective—Protease-activated receptor-1 (PAR1) mediates the thrombin-induced proliferation and hypertrophy of vascular smooth muscle cells. A role of Rac1 in the regulation of PAR1 expression was investigated.

Methods and Results—Treatment with simvastatin, a hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor, for 24 hours attenuated the transient [Ca\(^{2+}\)] elevation induced by thrombin. Immunofluorescence staining revealed that simvastatin decreased the surface expression of PAR1 in a manner dependent on protein geranylgeranylation. Introduction of a Rac1/Cdc42 inhibitory fragment but not a RhoA inhibitory fragment using a cell-penetrating peptide also attenuated the response to thrombin and decreased the surface expression of PAR1. Finally, downregulation of Rac1, but not RhoA, using an RNA interference technique attenuated the thrombin-induced [Ca\(^{2+}\)] elevation. However, the level of PAR1 mRNA and the total amount of PAR1 protein remained unchanged.

Conclusions—Here, we provide for the first time 3 lines of evidence that Rac1 plays a critical role in maintaining the surface expression of PAR1 and the responsiveness to thrombin in vascular smooth muscle cells. Rac1 is suggested to regulate the constitutive trafficking of PAR1 and thereby regulate the surface expression of PAR1. (Arterioscler Thromb Vasc Biol. 2005;25:1506-1511.)

Key Words: expression ■ protease-activated receptor ■ Rac1 ■ smooth muscle ■ thrombin

Protease-activated receptor-1 (PAR1) belongs to a family of G-protein–coupled receptors (GPCRs), and it is known to mediate the cellular effects of thrombin in various types of cells.\(^1\)–\(^5\) In vascular smooth muscle cells, PAR1 regulates the contraction, proliferation, and hypertrophy.\(^1\)–\(^4\)\(^,\)\(^6\) The expression of PARs has been reported to either increase or decrease under various pathological conditions, including atherosclerosis, balloon injury of arteries, cerebral ischemia, and neuroinflammatory and neurodegenerative disorders.\(^7\)–\(^12\) Therefore, elucidating the mechanism regulating the expression of PARs is important to understand the pathophysiology of such diseases and also to establish new therapeutic strategies. The nascent PAR1 is considered to be targeted first to the plasma membrane, and then subjected to constitutive internalization, thus resulting in the formation of an intracellular pool.\(^13\),\(^14\) PAR1 then cycles between the plasma membrane and the intracellular pool under resting conditions. The steady-state level of PAR1 on the cell surface is thus determined by a balance among the rate of de novo synthesis, the receptor internalization, and the recruitment from the intracellular pool.\(^14\),\(^15\) However, the molecular mechanism regulating the expression of PAR1 remains to be elucidated.

The small G-protein is known to regulate the intracellular vesicle trafficking.\(^16\) The Rab family is the most studied small G-protein that regulates GPCR trafficking.\(^17\) Rab5a has been shown to be required for the agonist-triggered internalization of PAR2, whereas Rab11a has been shown to contribute to the transport of PAR2 from the Golgi apparatus to the plasma membrane.\(^18\) On the other hand, the Rho family of small G-proteins has been suggested recently to play an important role in the internalization of membrane proteins such as thromboxane A\(_2\) receptor TP\(_{\beta}\), epidermal growth factor (EGF) receptor, and E-cadherin, and in the endocytosis of fibroblast growth factor 2.\(^19\)–\(^22\) However, the role of the Rho families in the regulation of PAR1 expression has yet to be elucidated.

In the present study, we investigated the role of Rho proteins in the regulation of expression of PAR1 on the cell surface in vascular smooth muscle cells. We first used hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) to inhibit the protein isoprenylation such as geranylgeranylation and farnesylation, which is essential for small G-proteins to be functional.\(^16\),\(^23\)–\(^25\) Second, we introduced the inhibitory proteins of Rho proteins using...
the cell-penetrating peptide of Tat protein. The RhoA-binding domain (RB) of Rho kinase and the Rac1/Cdc42-binding domain (PBD) of p21-activated protein kinase-1 was used as inhibitory proteins of Rho proteins, as described previously. Finally, we downregulated RhoA and Rac1 by RNA interference. As a result, the present study provides 3 lines of evidence supporting the critical role that Rac1 plays in the maintenance of PAR1 expression and the responsiveness to thrombin in cultured vascular smooth muscle cells.

Materials and Methods
The human coronary artery smooth muscle cells (HCASMCs) and the rat aortic smooth muscle cells (RASMCs) in primary culture were used. Cells were treated with HMG-CoA reductase inhibitors (statins), inhibitor proteins of RhoA, and Rac1/Cdc42 tagged with a cell-penetrating peptide of Tat protein or small interfering RNA targeted to RhoA and Rac1. Then, the responsiveness to thrombin and the surface expression of PAR1 were evaluated by fura-2 fluorometry and immunofluorescent staining, respectively. The total expression of PAR1, Rac1, RhoA, and β-actin mRNA was evaluated by RT-PCR. The total expression of PAR1 protein was evaluated by an immunoblot analysis.

Expanded information for Materials and Methods can be found in the online supplement, available at http://atvb.ahajournals.org.

Results
Long-Term Treatment With Simvastatin Attenuated the Thrombin-Induced $[Ca^{2+}]_i$

Elevation in Vascular Smooth Muscle Cells
In HCASMCs, thrombin concentration-dependently induced a transient $[Ca^{2+}]_i$ elevation (EC$_{50}$: 0.17 U/mL), with the maximum response at 1 U/mL (Figure 1A). PAR1-activating peptide (100 μmol/L TFLLR-NH$_2$) induced a $[Ca^{2+}]_i$ elevation (47.5±4.5%; n=4), which is comparable to that obtained with 1 U/mL thrombin, whereas PAR4-activating peptide (100 μmol/L GYPGKF-NH$_2$) induced only a small $[Ca^{2+}]_i$ elevation (1.0±1.6%; n=4; data not shown). PAR1 is thus suggested to mediate most of the response to thrombin in HCASMCs. The 24-hour treatment with simvastatin concentration-dependently attenuated the $[Ca^{2+}]_i$ elevation induced by 1 U/mL thrombin, with a significant inhibition seen at 100 nmol/L and higher concentrations (Figure 1C). Such effective concentrations are similar to the plasma concentrations of simvastatin after the oral administration.

However, application of 1 μmol/L simvastatin 10 minutes before and during stimulation with 1 U/mL thrombin had no effect on the thrombin-induced $[Ca^{2+}]_i$ elevation (data not shown). The similar attenuation of the thrombin-induced $[Ca^{2+}]_i$ elevation was observed with fluvastatin at a similar concentration range (data not shown). On the other hand, a hydrophilic statin, pravastatin, at a concentration of up to 1 μmol/L, had no significant effect on the $[Ca^{2+}]_i$ elevation induced by 1 U/mL thrombin in HCASMCs (Figure 1A and 1C). A similar attenuation of the response to thrombin by simvastatin was observed in RASMCs (Figure 1B and 1D). In RASMCs, thrombin induced $[Ca^{2+}]_i$ elevations with EC$_{50}$ of 0.11 U/mL, but slightly higher concentrations of simvastatin were required to induce a significant inhibition of the thrombin-induced $[Ca^{2+}]_i$ elevations.
Downregulation of the Surface Expression of PAR1 by Simvastatin in Vascular Smooth Muscle Cells

The surface expression of PAR1 was evaluated by immunofluorescence staining with a monoclonal antibody WEDE15 without fixation or permeabilization of the cells, as described previously. The untreated control HCASMCs exhibited a spotted pattern of fluorescence mainly on the cell periphery under fluorescence microscopy (Figure 3A). The fluorescence intensity at the peak of the fluorescence distribution was 856.7 ± 290.1 arbitrary units (n = 3) in control cells (Figure 3B). The treatment with simvastatin reduced the fluorescence staining and shifted the fluorescence distribution to the left (Figure 3A). The fluorescence intensity at the peak distribution (386.7 ± 75.7 arbitrary units; n = 3) was significantly lower than the control (Figure 3B). We reported previously that trypsin removes the epitope of WEDE15 (residues 51 to 64) of PAR1. Treatment with trypsin removed most of the staining in control and the simvastatin-treated cells and caused a leftward shift of the fluorescence distribution, thus resulting in a similar peak intensity (55.0 ± 17.3 arbitrary units for control; 55.3 ± 13.1 arbitrary units for simvastatin-treated cells; n = 3; Figure 3A and 3B).

Effect of Rac1 Inhibition on the Expression of PAR1 mRNA and Protein in Vascular Smooth Muscle Cells

An RT-PCR analysis revealed that the level of PAR1 mRNA did not change after the 24-hour treatment of RASMCs with 10 μmol/L simvastatin, either in the presence or absence of 10 μmol/L GGPP (Figure 3C). On the other hand, a Western blot analysis revealed that the level of PAR1 protein did not

Figure 3. The effect of simvastatin and TATHA-PBD on the surface expression of PAR1 and the total cellular expression of PAR1 mRNA and protein in vascular smooth muscle cells. A, The representative microscopic images of immunofluorescence staining with phycoerythrine-conjugated anti-PAR1 antibody and the representative data of flow cytometry obtained with HCASMCs either untreated (control) or treated with 1 μmol/L simvastatin for 24 hours. Cells were stained before (intact) and after trypsin treatment (trypsin). The dashed lines in the histogram indicate the peak of the fluorescence distribution. Bar = 50 μm. B, Summary of the fluorescence intensity at the peak of the fluorescence distribution in the cells untreated (control) and treated with 1 μmol/L simvastatin before and after trypsin treatment. Data are the mean ± SEM (n = 3). C, RT-PCR analysis of PAR1 mRNA expression in RASMCs untreated (control) and treated with 10 μmol/L simvastatin with and without 10 μmol/L GGPP for 24 hours. D, Immunoblot detection of the total amount of PAR1 in HCASMCs untreated (control) and treated with 1 μmol/L simvastatin in the presence and absence of 1 μmol/L GGPP, 3 μmol/L TATHA-PBD and 3 μmol/L (His)6-PBD. A densitometric analysis of electrophoresis was shown below the representative photos.
The Effect of Inhibition of Rho Signaling on the Response to Thrombin and the Expression of PAR1

The 24-hour treatment with TATHA-PBD concentration-dependently (EC_{50}, 0.85 μmol/L) inhibited the [Ca^{2+}]\text{e} elevation induced by 1 U/mL thrombin (Figure 4A and 4B). The inhibition seen with ≥3 μmol/L concentrations of TATHA-PBD was similar to that seen with 1 μmol/L simvastatin (Figure 4B). However, the application of 3 μmol/L TATHA-PBD 10 minutes before and during stimulation with thrombin had no significant effect on the [Ca^{2+}]\text{e} elevation induced by thrombin (data not shown). The removal of the cell-penetrating peptide (His)_6-PBD abolished the inhibitory effect of TATHA-PBD (Figure 4A and 4B). On the other hand, TATHA-RB, a RhoA inhibitory protein, had no effect on the response to thrombin (Figure 4A and 4B). In line with this, 24-hour treatment with 1 μmol/L Y27632, a Rho kinase inhibitor, also had no significant effect on the response to thrombin (Figure 4B). The protein transduction was confirmed by an immunoblot analysis as described previously. The extract of the cells exposed to the recombinant protein for 24 hours was subjected to the immunoblot detection with an anti-(His)\_6 antibody. This antibody detected all recombinant proteins (Figure 4C). However, PBD and RB were detected in the cell extract only when they were conjugated with Tat peptide (Figure 4C).

The effect of the transduction of TATHA-PBD on the expression of PAR1 was investigated by immunofluorescence staining with phycoerythrine-conjugated anti-PAR1 antibody in the cells untreated (control) and treated with 1 μmol/L simvastatin, 3 μmol/L TATHA-PBD, 3 μmol/L (His)_6-PBD, TATHA-RB, and (His)_6-RB (lanes C). The purified proteins (100 ng) were loaded as a positive control (lanes P). Actin was detected with naphtol blue black staining after immunodetection to validate the equal loading of the cell extracts.

Effect of Downregulation of RhoA and Rac1 on the Response to Thrombin

RhoA and Rac1 were downregulated by an RNA interference technique. The transfection of control small interfering RNA had no effect on the responsiveness to thrombin in HCASMCs (Figure 6). On the other hand, downregulation of Rac1 but not RhoA significantly attenuated the [Ca^{2+}]\text{e} elevation induced by 1 U/mL thrombin.
The mechanism regarding how Rac1 regulates the expression of PAR1 on the cell surface remains to be elucidated. The downregulation of the surface expression of PAR1 by simvastatin was not associated with the downregulation of PAR1 mRNA. The change in the transcription of the PAR1 gene or the stability of PAR1 mRNA is thus not suggested to play a major role in the downregulation of PAR1 induced by inactivation of Rac1. Treatment with simvastatin or inhibition of Rac1 signaling had little effect on the total amount of PAR1 protein. It is thus likely that the alteration of the trafficking of PAR1 plays a critical role in the downregulation of PAR1 induced by the inactivation of Rac1.

PAR1 exhibits both constitutive and agonist-triggered internalization. The 2 modes of PAR1 internalization have been demonstrated to require the distinct residues in C-terminal region of PAR1, and they are thus suggested to be differentially regulated by distinct mechanisms. The agonist-activated PAR1 has been shown to be internalized via a clathrin- and dynamin-dependent but arrestin-independent pathway, and then it mainly targets to lysosome degradation. On the other hand, the constitutive internalization takes place under resting conditions without receptor stimulation, and it has been reported to be necessary for the formation and the maintenance of the intracellular PAR1 pool. Therefore, the resting level of the surface expression of PAR1 is suggested to be dynamically maintained by continuous cycling between the cell surface and the intracellular pool. Our finding suggests that Rac1 regulates such constitutive trafficking of PAR1. The inhibition of Rac1 activity may either increase the constitutive internalization or inhibit the membrane targeting, thereby decreasing the level of PAR1 expressed on the cell surface in cultured smooth muscle cells.

PAR1 has been reported to be upregulated in smooth muscle cells seen in the human atherosclerotic lesions and in a rat carotid artery balloon injury model. Growth factors such as platelet-derived growth factor, EGF or insulin, phorbol ester, and some GPCR agonists such as bombesin and lysophosphatidic acid were shown to activate Rac1. Rac1 is thus speculated to contribute to the upregulation of PAR1 in the vascular lesions. Our observations suggest that cultured vascular smooth muscle cells have some basal activity of Rac1, which contributes to the maintenance of surface expression of PAR1 under resting conditions. In the present study, we cultured and maintained the cells in the media containing 5% serum, 0.5 ng/mL EGF, 2 ng/mL fibroblast growth factor-B and 5 μg/mL insulin. Thus, it is conceivable that some growth factors in the media contributed to the basal activity of Rac1 in the cultured smooth muscle cells. Thus, our observations may be relevant to situations that are related to atherosclerotic lesions. However, such a possibility remains to be evaluated.

The preventive effect of FPP differed between HCASMCs and RASMCs. GGPP and FPP prevented the simvastatin-induced attenuation of the thrombin response in RASMCs, whereas only GGPP was effective in HCASMCs. Because GTTI but not FTI mimicked the effect of simvastatin in
RASMCs, the findings suggested that geranylgeranylation but not farnesylation was involved in the effect of simvastatin in HCASMCs and RASMCs. We speculate that FPP was converted to GGPP in RASMCs, thereby exerting its preventive effect. GGPP is synthesized by a condensation of FPP and isopentenyl pyrophosphate. The residual amount of isopentenyl pyrophosphate after the inhibition of HMG-CoA reductase by simvastatin may be high enough to convert the exogenously added FPP to GGPP and to restore protein geranylgeranylation in RASMCs. However, this was not the case in HCASMCs, in which the amount of isopentenyl pyrophosphate was not sufficient to convert FPP to GGPP. The degree of inhibition of HMG-CoA reductase by statins or metabolism of isopentenyl pyrophosphate may differ between HCASMCs and RASMCs.

In conclusion, the present study demonstrated for the first time that Rac1 plays a critical role in the maintenance of the surface expression of PAR1 and the responsiveness to thrombin in the cultured vascular smooth muscle cells. The inactivation of Rac1 by HMG-CoA reductase inhibitor, the introduction of the inhibitor protein and downregulation by RNA interference attenuated the responsiveness to thrombin by reducing the level of the surface expression of PAR1. Because the level of PAR1 mRNA and the total amount of PAR1 protein remained unchanged, Rac1 is suggested to regulate the constitutive trafficking of PAR1.

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Expanded information for Methods

Materials
Thrombin (bovine plasma, 1880 NIH units/mg protein) was purchased from Sigma (St. Louis, MO, U.S.A.). Simvastatin, fluvastatin and pravastatin were supplied by Merck & Co. (Rahway, N.J., U.S.A.), Novartis Pharma Inc. (Tokyo, Japan) and Sankyo Co. (Tokyo, Japan), respectively. TFLLR-NH₂ (PAR1-activating peptide) and GYPGKF-NH₂ (PAR4-activating peptide) were purchased from Bachem (Bubendorf, Switzerland). Geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were purchased from Sigma (St. Louis, MO, U.S.A.). Geranylgeranyl transferase inhibitor (GGTI-298) and farnesyl transferase inhibitor (FTI-277) were purchased from Calbiochem (San Diego, U.S.A.). The antibodies and their source were as follows: phycoerythrine-conjugated anti-PAR1 antibody WEDE15 (Immunotech, Marseille, France), anti-(His)₆ antibody (Qiagen, Hilden, Germany), Anti-PAR1 antibody (sc-5605, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), horse radish peroxidase-conjugated anti-mouse IgG (Sigma) and horse radish peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.). Oligonucleotides was synthesized by Hokkaido System Sciences (Sapporo, Japan).

Cell culture
The human coronary artery smooth muscle cells (HCASMCs) were purchased from Kurabo (Osaka, Japan), and cultured in HuMedia-SG2 (Kurabo) as described in
manufacturer’s instruction. The rat aortic smooth muscle cells (RASMCs) in primary culture were enzymatically dispersed from the aortic media of male Wistar rats and cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and antibiotics, as previously described. The growth medium was renewed every 2-3 days. The cells were used in all experiments on culture days 4-6 and before confluence.

**Measurement of [Ca^{2+}] in smooth muscle cells**
The cells were loaded with fura-2 by incubating them in Dulbecco’s modified Eagle medium containing 10 μmol/L fura-2 acetoxyethyl ester for 1 h at 37 °C as previously described. After loading with fura-2, the cells were washed and equilibrated in HEPES-buffered saline (HBS; 10 mmol/L Hepes, pH 7.4, 135 mmol/L NaCl, 5 mmol/L KCl, 1.0 mmol/L CaCl₂, 1.0 mmol/L MgCl₂ and 5.5 mmol/L D-glucose) for at least 30 min at room temperature before starting the measurements. The changes in [Ca^{2+}], were monitored using a front-surface fluorometer as previously described. Fluorometry was performed at 25 °C to prevent any leakage of fura-2. The 500 nm fluorescence intensities at 340 nm and 380 nm excitation were continuously monitored. The fluorescence ratio data were expressed as a percentage, while assigning the values at rest and at the peak [Ca^{2+}] elevation induced by ionomycin (50 μmol/L for HCASMC and 25 μmol/L for RASMC) to be 0% and 100%, respectively. All data were collected using a computerized data acquisition system MacLab (Analog Digital Instruments, Australia).

**Immunofluorescent staining of PAR1**
HCASMCs were either untreated or treated with simvastatin for 24 h, and then harvested by incubating in PBS containing 1 mmol/L EDTA, 5 mmol/L EGTA at 37 °C for 15 min, and scraping them off from the culture dishes as previously described. The cells were washed and suspended in PBS, and then were incubated with phycoerythrin-labeled anti-PAR1 antibodies, WEDE15, in PBS containing 1% bovine serum albumin at room temperature for 30 min. The cells were then washed 3 times in PBS and subjected to flow cytometric analysis with a flow cytometer FACSCalibur (Beckton Dickenson, San Jose, CA, U.S.A.) as previously described. The peak fluorescence
intensity of the cell population was determined on a histogram presentation of the fluorescence data. The fluorescence image was observed under a laser scanning confocal fluorescence microscope LSM GB200 (Olympus, Tokyo, Japan), using a 60x objective lens, 488 nm excitation and a 500-530 band pass emission filter. The fluorescence images were obtained at the nuclear level.

*Expression and purification of inhibitor proteins of RhoA and Rac1/Cdc42*

The RhoA-binding domain (RB) of Rho-kinase and the Rac1/Cdc42-binding domain (PBD) of p21-activated protein kinase-1 were used as inhibitors of RhoA and Rac1/Cdc42, respectively. RB and PBD were expressed in *E. coli* as (His)_6-tagged proteins with and without a cell-penetrating peptide of human immunodeficiency viral Tat protein (TAT) and a hemagglutinin tag (HA), as previously described. The recombinant proteins were expressed and purified as previously described. In brief, the bacterial pellet was homogenized in buffer A (6 mol/L guanidine-HCl, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris-HCl, pH 8.0) and clarified by centrifugation at 43,000 g, 4 °C, 15 min, and then was loaded onto the Ni²⁺-loaded Hi-Trap chelating column (Pharmacia Biotech, Tokyo, Japan). The column was washed with buffer A and buffer B (6 mol/L urea, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris-HCl, 50 mmol/L imidazole, pH 6.3). The recombinant proteins were then eluted in buffer C (6 mol/L urea, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris-HCl, 500 mmol/L imidazole, pH 6.3) and dialyzed against PBS (136.9 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na₂HPO₄, 1.47 mmol/L KH₂PO₄). The protein concentration was determined using the Coomassie protein assay kit (Pierce, Rockford, IL, U.S.A.) and bovine serum albumin as the standard.

*RhoA and Rac1 knock-down by RNA interference*

The cellular expression of RhoA and Rac1 was specifically down-regulated by using RNA interference technique. The single-stranded sense and anti-sense RNAs corresponding to the coding region 143-163 of human RhoA, their inverted sequences and the sense and anti-sense RNAs corresponding to the coding region 13-33 of human Rac1 were synthesized to contain a 3' TT overhang. The sequences of synthesized RNAs were as follows: 5'- GUG GAU GGA AAG CAG GUA GAG TT- 3' (RhoA sense), 5'- CUC UAC CUG CUU UCC AUC AUC CAC TT -3' (RhoA anti-sense), 5'- GAG
AUG GAC GAA AGG UAG GUG TT -3’ (RhoA inverted sense), 5’- CAC CUA CCU UUC GUC CAU CUC TT -3’ (RhoA inverted anti-sense), 5’- AAG UGU GUG GUG GUG GGA GAC TT -3’ (Rac1 sense), 5’- GUC UCC CAC CAC ACA CUU TT -3’ (Rac1 anti-sense). The sense and anti-sense RNAs (at 20 µmol/L) were annealed by incubating in annealing buffer (30 mmol/L HEPES, pH 7.4, 100 mmol/L potassium acetate, 2 mmol/L magnesium acetate) for 1 min at 90 °C, and then 1 min at 70 °C, followed by gradual cool-down to 37 °C for 1 h, as previously described 8,9. RNA were precipitated in ethanol and then dissolved in annealing buffer at the concentration of 20 µmol/L. The cells were plated at the density of 5x10^4 per 35 mm culture dish for fura-2 fluorometry. On the next day, the cells were exposed to 2 mL Opti-MEM1 (Invitrogen, Carlsbad, CA, U.S.A.) containing 25 nmol/L RNA and 15 nmol/L oligofectamine (Invitrogen) for 40-45 h.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of PAR1, Rac1 and RhoA mRNA**

Total RNA was isolated and subjected to RT-PCR analysis, using 1 µg total RNA in 20 µl RT reaction, as previously described 1. One µl RT product was then subjected to 10 µl PCR reaction. The primer used in the RT reaction of rat PAR1 was 5’-AGT AAG AGG ATG GAT ACC TGC-3’ (rat PAR1 coding region 1243-1262), and the primers used in PCR amplification of rat PAR1 were 5’-TGA CAG TCA TAA GCA TTG AC-3’ (the sense primer: 605-634) and 5’-CCT TGA TTT ACT ACT A TG CC-3’ (the antisense primer: 1129-1149). The PCR reaction for rat PAR1 mRNA was performed as previously described 11. The primers and conditions for the RT-PCR analysis of rat β-actin mRNA were as previously described 1. The primer used in the RT reaction of human Rac1 was 5’-TTA CAA CAG CAG GCA TTT TC -3’ (human Rac1 coding region 748-767), and the primers used in PCR amplification of human Rac1 were 5’-CTA TCC TTA ACT TCC TAT CCG CAA ACA GA -3’ (the sense primer: 396-415) and 5’-CTC TTC TTC TTC TTC ACG GG-3’ (the antisense primer: 729-748). The primer used in the RT reaction of human RhoA was 5’-TTG GGT CTT TGC TGA ACA CT -3’ (human RhoA coding region 624-643), and the primers used in PCR amplification of human RhoA were 5’-CCA AGT TCC CAG GCA TTG ACT TC -3’ (the sense primer: 234-255) and 5’-TTG GGT CTT TGC TGA ACA CT -3’ (the antisense primer: 455-477). The primers for human β-actin was purchased from Toyobo (Tokyo, Japan). The PCR reaction for RhoA and Rac1
consists of the initial 2 min denaturation at 94 °C, followed by 30-cycle amplification step consisting of 30 sec. denaturation at 94 °C, 90 sec. annealing at 55 °C and 30 sec extension at 72 °C (RhoA), and 35-cycle amplification step consisting of 30 sec. denaturation at 94 °C, 90 sec. annealing at 50 °C and 30 sec extension at 72 °C (RhoA). The PCR products were separated on 3 % agarose gel electrophoresis containing 0.5 μg/mL ethidium bromide. The density of bands was determined with Gel Plotting Macros of the NIH image.

Immunoblot analysis

The cell extracts were prepared in RIPA buffer (50 mmol/L Tris-HCl, pH 7.2, 0.5 mol/L NaCl, 10 mmol/L MgCl₂, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % TritonX-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μmol/L 4-aminidophenylmethane sulfonfyl fluoride), and then were separated with SDS-PAGE on 7.5-20 % gradient polyacrylamide gel, followed by transfer to polyvinylidene difluoride membrane (BioRad, Hercules, CA, USA). Anti-(His)₆ antibody and anti-PAR1 antibody were used as the primary antibody at x100 and x250 dilution, respectively. Immune complex was detected using horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence technique (ECL plus; Amersham, Buckinghamshire, U.K.), and light emission was detected by X-OMAT AR film (Kodak, Rochester, NY, U.S.A.).
References for Online supplementary information


