The mammalian formin FHOD1 is activated through phosphorylation by ROCK and mediates thrombin-induced stress fibre formation in endothelial cells

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Formin-family proteins, in the active state, form actin-based structures such as stress fibres. Their activation mechanisms, however, are largely unknown except that mDia and its closely related formins can be activated by direct binding of the small GTPase Rho or Cdc42. Here we show that the Rho-dependent protein kinase ROCK phosphorylates the C-terminal residues Ser1131, Ser1137, and Thr1141 of formin homology domain protein 1 (FHOD1), a major endothelial formin that is normally autoinhibited by intramolecular interaction between the N- and C-terminal regions. Phosphorylation of FHOD1 at the three residues fully disrupts the autoinhibitory interaction, which culminates in formation of stress fibres. We also demonstrate that, in vascular endothelial cells, thrombin, a vasoactive substance leading to Rho activation, elicits both FHOD1 phosphorylation and stress fibre formation in a ROCK-dependent manner, and that FHOD1 depletion by RNA interference impairs thrombin-induced stress fibre formation. Based on these findings we propose a novel mechanism for activation of formin-family proteins: ROCK, activated by G protein-coupled receptor ligands such as thrombin, directly phosphorylates FHOD1 at the C-terminal region, which renders this formin in the active form, leading to stress fibre formation.

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Introduction

Formin-family proteins, which regulate actin cytoskeletal dynamics, are structurally characterized by the presence of two conserved regions, the FH 1 (formin homology 1) and FH2 domains (Zigmond, 2004; Higgs, 2005; Faix and Grosse, 2006; Kovar, 2006; Goode and Eck, 2007). The FH1 region,

composed of stretches of poly-proline residues, appears to serve as a target of the actin monomer-binding protein profilin. The FH2 domain, located C-terminally to the FH1 domain, is the most conserved region among the formin family and possesses actin nucleation and polymerization activities, which are accelerated by FH1-mediated recruitment of the profilin-actin dimer. Through cooperation of the core modules FH1 and FH2, formins construct actin-based structures comprising linear unbranched filaments that are used in stress fibres, actin cables, microspikes, and contractile rings (Watanabe *et al*, 1999; Evangelista *et al*, 2002; Pelham and Chang, 2002; Schirenbeck *et al*, 2005).

Consistent with the dynamic nature of the actin-based structures, the activities of formins are regulated in a sophisticated manner. In the resting state, several mammalian formins, including mDia1, FRL, and formin homology domain protein 1 (FHOD1) (also known as Fhos1), as well as the yeast formin Bni1p, are likely to be inhibited through an intramolecular interaction between the C-terminal Dia autoregulatory domain (DAD) and its recognition region at the N-terminus: the interaction is considered to mask the FH1 and FH2 domains (Watanabe *et al*, 1999; Zigmond, 2004; Higgs, 2005; Kovar, 2006). The DAD of FHOD1 is composed of the core motif and its C-terminally flanking region abundant in basic residues, both of which are required for the autoregulatory interaction (Takeya and Sumimoto, 2003; Schönichen *et al*, 2006); this is in contrast to the finding that the corresponding polybasic region is dispensable for autoregulation of mDia (Lammers *et al*, 2005). In mDia1, the small GTPase RhoA, in the GTP-bound form, binds to the N-terminal region and thereby relieves the autoinhibitory interaction, leading to activation of this formin-family protein (Watanabe *et al*, 1999; Li and Higgs, 2003; Otomo *et al*, 2005; Rose *et al*, 2005). Similarly, GTP-dependent binding of Rho3p and Rho4p to Bni1p and interaction of GTP-bound Cdc42 with FRL seem to activate respective formins (Dong *et al*, 2003; Seth *et al*, 2006).

However, little has been known about other regulatory mechanisms for relieving the autoinhibition. It has been also largely unknown how formins participate in cellular functions mediated by intrinsic regulators. For instance, although vasoactive substances such as thrombin induces stress fibre formation in endothelial cells, which contributes to inflammatory response or pathological conditions (Mehta and Malik, 2006), the role of formins in this process has remained to be elucidated.

Results and discussion

The triple replacement of Ser1131, Ser1137, and Thr1141 by aspartate mimicking a phosphorylated residue renders FHOD1 in an active state

FHOD1, harbouring a DAD in the C-terminus (Figure 1A), normally occurs in an inactive form through a DAD-mediated

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Figure 1 Triple replacement of Ser1131, Ser1137, and Thr1141 in FHOD1-DAD by aspartates is sufficient for release of autoinhibition. (A) Domain arrangement of human FHOD1. (B) Amino-acid sequences of the DAD regions of various FHOD1 proteins: Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*. Conserved residues are shown in red. (C) Effect of Ser/Thr replacement on the interaction of FHOD1-DAD with the N-terminal region. His-tagged FHOD1-N was incubated with GST-fused mutant FHOD1 DAD domains carrying the indicated amino-acid substitution: 3 x D, the S1131D/S1137D/T1141D substitution; 3 x A, S1131A/S1137A/T1141A. Proteins were pulled down with glutathione-Sepharose-4B and the precipitants were subjected to SDS-PAGE followed by CBB staining. (D) Bead co-pelleting assay for the interaction between FHOD1-DAD and FHOD1-N. His-tagged FHOD1-N was incubated with indicated concentrations of GST-FHOD1-DAD or DAD-3 x D bound to glutathione beads. The mixtures were centrifuged for 5 min at 12 000 g and the supernatants were subjected to SDS-PAGE and stained with CBB for quantification. Fractions of bound FHOD1-N were determined as total minus the fraction remaining in the supernatant. The graph represents the mean ± s.e.m. of data from four independent experiments. (E) Effect of the S1131D/S1137D/T1141D (3 x D) substitution on stress fibre formation. HeLa cells were transfected with a plasmid encoding GFP-FHOD1-3 x D. Cells were fixed followed by visualization by GFP fluorescence (green) or phalloidin staining (red). Scale bar, 20 μm. (F) Quantitative analysis of cell elongation by FHOD1-3 x D. The lengths of the long and short axes were measured on immunofluorescent images. Box-and-whisker plots in this and subsequent figures indicate mean (open circle), 25th percentile (bottom line), median (middle line), 75th percentile (top line), nearest observations within 1.5 times the interquartile range (whiskers), and outliers (closed circle). **P* < 0.0001 (Welch's *t*-test). (G) Cells showing parallel actin fibres aligned with the long axis throughout the cell (as in D) were counted and the percentages from three independent transfections are expressed as the mean ± s.d.

intramolecular interaction with the N-terminal region (Gasteier *et al*, 2003; Koka *et al*, 2003; Takeya and Sumimoto, 2003). The autoinhibitory interaction can be disrupted by deletion of the N- or C-terminal region. The disruption renders the FH1-FH2 domain in an active state, thereby leading to formation of actin stress fibres (Gasteier

et al, 2003; Koka *et al*, 2003; Takeya and Sumimoto, 2003), which contains non-muscle myosin II in a sarcomeric pattern (Supplementary Figure S1). The mechanism for FHOD1 activation under physiological conditions, however, has remained to be elucidated. The small GTPase Rac can bind to FHOD1 but in a GTP-independent manner (Westendorf, 2001;

Gasteier *et al*, 2003; and R Takeya and H Sumimoto, unpublished observations). The independence suggests that Rac binding by itself does not have an important function in a signal-dependent activation of FHOD1, although additional signals could function together in activation of FHOD1 as proposed in that of mDia1 (Faix and Grosse, 2006; Goode and Eck, 2007). The DAD of FHOD1 comprises the core motif and polybasic region (Takeya and Sumimoto, 2003; Schönichen *et al*, 2006), each containing conserved Ser/Thr residues (Figure 1B). This raises the possibility that phosphorylation of these regions may contribute to regulation of the auto-inhibition. Among these residues, Ser1131 in the polybasic region is reported to be phosphorylated (Wang *et al*, 2004), but its role has remained unknown. To know the role of phosphorylation, we replaced the conserved serine/threonine residues in FHOD1-DAD by aspartate, a residue mimicking a phosphorylated one, and tested their effects on the interaction with the FHOD1 N-terminal region. As shown in Figure 1C, the interaction was attenuated by replacement of Ser1131, Ser1137, or Thr1141, whereas substitution for Ser1114 or Thr1116 in the DAD core motif did not affect the interaction (data not shown). The interaction was further attenuated by double replacement (Ser1131/Ser1137 or Ser1137/Thr1141) in the polybasic region, and almost completely abrogated by triple replacement (Ser1131/Ser1137/Thr1141) (Figure 1C). On the other hand, triple replacement of these Ser/Thr residues with Ala had no effect. To quantitatively estimate the effect of aspartate replacement, we performed a glutathione bead co-pelleting assay (Lee *et al*, 1999). In the assay, after being precipitated with glutathione-S-transferase (GST)-DAD-wt immobilized on glutathione beads, the amounts of FHOD1-N remaining in the supernatant were quantified (Figure 1D, blots). The fraction of FHOD1-N bound to GST-DAD-wt was calculated by subtracting the fraction remaining in the supernatant from the total protein added, and the values obtained were plotted against the concentration of the GST-fusion protein immobilized on beads (Figure 1D, right panel). FHOD1-N bound to GST-DAD in a concentration-dependent manner with an estimated K_d value of about 25 μ M. On the other hand, FHOD1-N interacted with GST-DAD-3 $\times$ D to an only slightly more extent than to GST alone. Thus, the simultaneous substitution of aspartate for Ser1131, Ser1137, and Thr1141 appears to be sufficient for disruption of the intramolecular interaction in FHOD1. Furthermore, expression of a full-length FHOD1 carrying the triple substitution of aspartate for Ser1131, Ser1137, and Thr1141 in HeLa cells led to cell elongation (Figure 1E and F) and formation of actin stress fibres aligned with the long axis of the cells (Figure 1E and G). This phenotype is similar to that induced by FHOD1- Δ C, a constitutively active mutant that lacks the DAD region (Takeya and Sumimoto, 2003). Thus, the triple replacement of Ser1131, Ser1137, and Thr1141 by aspartate mimicking a phosphorylated residue renders FHOD1 in an active state.

ROCK phosphorylates Ser1131, Ser1137, and Thr1141 of FHOD1 downstream of RhoA

To test whether FHOD1 is indeed phosphorylated, we prepared rabbit polyclonal antibodies that recognize phospho-Ser1131, phospho-Ser1137, or phospho-Thr1141, designated as the anti-pS1131, anti-pS1137, or anti-pT1141 antibodies, respectively (for details, see Materials and methods). The

specificity of these antibodies was examined by immunoblot analysis (Figure 2A and B). HeLa-FHOD1 cells, stably expressing Flag-tagged FHOD1, were treated with okadaic acid (OA), a protein phosphatase inhibitor. OA treatment induced an upward shift in the mobility of FHOD1 on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): this mobility shift appears to be due to phosphorylation, as the shift was not observed after treatment of Flag-FHOD1 with bacterial alkaline phosphatase (Figure 2A). The anti-pS1131 antibody specifically recognized the mobility-shifted FHOD1, but not the phosphatase-treated FHOD1. Similarly, both anti-pS1137 and anti-pT1141 antibodies solely recognized phosphorylated FHOD1 (Figure 2B). Using the three anti-pFHOD1 antibodies, we examined the effect of serum depletion on FHOD1 phosphorylation, as FHOD1 were substantially phosphorylated in OA-untreated cells cultured in a serum-containing medium (Figure 2A). As shown in Figure 2C, FHOD1 phosphorylation at Ser1131, Ser1137, and Thr1141 was decreased in cells cultured in a medium containing 0.5% serum. As it is well known that serum components such as lysophosphatidic acid induce ROCK activation through a RhoA-mediated signalling pathway (Maekawa *et al*, 1999), we tested the effect of Y-27632, a specific inhibitor of ROCK (Uehata *et al*, 1997), on FHOD1 phosphorylation. Treatment of HeLa cells with Y-27632 abolished phosphorylation of FHOD1 at Ser1131, Ser1137, and Thr1141 (Figure 2C), suggesting the involvement of ROCK.

To know whether ROCK directly phosphorylates FHOD1, we performed an *in vitro* kinase assay. When GST-fused FHOD1-DAD was treated with recombinant ROCK in the presence of [γ - 32 P]ATP, FHOD1-DAD was efficiently phosphorylated by ROCK (Figure 2D). Experiments using mutant FHOD1-DAD proteins with replacement of serine/threonine by alanine, a kinase-insensitive residue, revealed that ROCK directly phosphorylates Ser1131, Ser1137, and Thr1141. Direct phosphorylation of these three residues by ROCK was confirmed by immunoblot analysis using the phosphospecific antibodies (Figure 2E and F; Supplementary Figure S2A). We next tested the effect of the intramolecular interaction of FHOD1 on phosphorylation by ROCK. FHOD1-N inhibited phosphorylation of DAD-wt at Ser1131 in a dose-dependent manner (Supplementary Figure S2B), suggesting that ROCK is inaccessible to Ser1131 of FHOD1 in the presence of the intramolecular interaction. In contrast, phosphorylation at Ser1137 and Thr1141 of the wild type (Supplementary Figure S2B) or mutants carrying substitution for serine/threonine residues (Supplementary Figure S2C) was not affected by excess amounts of FHOD1-N. Thus, ROCK is likely capable of phosphorylating Ser1137 and Thr1141 independently of the intramolecular interaction in FHOD1.

Furthermore, transfection of HeLa-FHOD1 cells stably expressing Flag-FHOD1 with cDNA for the myc-tagged wild-type ROCK resulted in a drastic increase in phosphorylation at Ser1131, Ser1137, and Thr1141 (Figure 2G). Active forms of ROCK, ROCK- Δ 1 and ROCK- Δ 3, induced FHOD1 phosphorylation more strongly, whereas the dominant-negative form of ROCK (ROCK-KDIA) (Ishizaki *et al*, 1997) had little activity. Thus, the three residues of FHOD1 are phosphorylated by ROCK *in vivo*. In addition, expression of the constitutively active form of RhoA(Q63L), a direct activator of ROCK, induced FHOD1 phosphorylation at Ser1131, Ser1137, and Thr1141 (Figure 2H). The RhoA-dependent phosphorylation

Figure 2 ROCK phosphorylates FHOD1 at Ser1131, Ser1137, and Thr1141 both *in vivo* and *in vitro*. (A, B) Specificity of antibodies raised against a region containing the phospho-Ser1131, phospho-Ser1137, or phospho-Thr1141 in FHOD1. HeLa cells expressing Flag-FHOD1 were treated with 1 μ M OA for 1 h. Proteins of cell lysates were immunoprecipitated with the anti-Flag antibody and the precipitants were treated with the bacterial alkaline phosphatase for the indicated time (A) or 4 h (B) at 37°C, and subjected to SDS-PAGE followed by immunoblot analysis with the anti-pS1131 (A), anti-pS1137 (B), or anti-pT1141 (B) antibodies. (C) Effect of serum on FHOD1 phosphorylation. HeLa cells expressing Flag-FHOD1 were cultured with 0.5 or 10% serum for 15 h, and treated in the presence or absence of 20 μ M Y-27632 for 1 h. Proteins in cell lysates were immunoprecipitated with the anti-Flag antibody and subjected to SDS-PAGE followed by immunoblot analysis with the anti-pS1131, anti-pS1137, or anti-pT1141 antibodies. (D–F) *In vitro* phosphorylation of GST-FHOD1-DAD by ROCK. GST-FHOD1-DAD-wt or mutant proteins carrying the indicated substitutions were phosphorylated by recombinant ROCK in the presence of [γ - 32 P]ATP, and subjected to SDS-PAGE followed by autoradiography (D). The intensity of each band on the autoradiogram was expressed as a percent of that of DAD-wt. CBB indicates Coomassie Brilliant Blue. ROCK-phosphorylated GST-FHOD1-DAD were analysed by immunoblot analysis with the anti-pS1131 (E), anti-pS1137 (F), or anti-pT1141 (F) antibodies. (G) *In vivo* phosphorylation of FHOD1 by ROCK. HeLa cells stably expressing Flag-FHOD1 were transfected with a plasmid encoding the indicated mutant proteins of ROCK. Proteins of cell lysates were immunoprecipitated with the anti-Flag antibody and subjected to SDS-PAGE followed by immunoblot with the anti-pS1131, anti-pS1137, or anti-pT1141 antibodies. (H) Effect of RhoA expression on FHOD1 phosphorylation. HeLa cells stably expressing Flag-FHOD1 were transfected with a plasmid encoding RhoA(Q63L). After incubation for 15 h, cells were treated with or without 20 μ M Y-27632 for 1 h. Proteins of cell lysates were immunoprecipitated with the anti-Flag antibody and the precipitants were subjected to SDS-PAGE followed by immunoblotting with the anti-pS1131, anti-pS1137, or anti-pT1141 antibodies.

of FHOD1 was completely inhibited by treatment with Y-27632. Thus, ROCK, activated by RhoA, appears to directly phosphorylate FHOD1 at Ser1131, Ser1137, and Thr1141.

ROCK-catalysed phosphorylation activates FHOD1 through disruption of the intramolecular interaction

The present findings that FHOD1 may be activated by simultaneous phosphorylation of Ser1131, Ser1137, and Thr1141 (Figure 1) and that ROCK phosphorylates FHOD1 at these three residues both *in vivo* and *in vitro* (Figure 2), suggest the involvement of ROCK-catalysed phosphorylation in FHOD1 activation. To address this issue, we tested the effect of phosphorylation of FHOD1-DAD on its interaction with the His-tagged N-terminal region. When GST-fused FHOD1-DAD was phosphorylated by recombinant ROCK, the

intramolecular interaction was abrogated; on the other hand, ROCK-catalysed phosphorylation was not observed when the mutant FHOD1-DAD-3 $\times$ A carrying replacement of the three residues by alanine was used instead of the wild-type FHOD1-DAD (Figure 3A and B). Thus, ROCK-catalysed phosphorylation likely disrupts the autoinhibitory interaction in FHOD1. We next investigated whether ROCK can activate FHOD1 *in vivo*. Ectopic expression of the full-length FHOD1 did not induce the formation of actin stress fibre in HeLa cells, consistent with our previous observation (Takeya and Sumimoto, 2003; Figure 3C), and expression of the full-length ROCK in HeLa cells induced the formation of a low number of actin fibres sparsely distributed, as previously described (Ishizaki *et al*, 1997). When the full-length FHOD1 and ROCK were coexpressed in HeLa cells, cells were elongated

Figure 3 Phosphorylation by ROCK disrupts the autoinhibition. (A, B) Effect of phosphorylation of FHOD1-DAD on the intramolecular interaction with the N-terminal region. Untreated or ROCK phosphorylated GST-FHOD1-DAD was incubated with His-tagged FHOD1-N. Interactions were analysed by the pull-down assay as in Figure 1C. Precipitated FHOD1-N was detected by CBB staining (A) or immunoblotting with anti-His antibody (B). (C) HeLa cells were cotransfected with plasmids encoding GFP-FHOD1 and myc-ROCK. After treatment with or without 20 μ M Y-27632 for 2 h, cells were fixed and visualized by GFP fluorescence (green), myc-immunostaining (blue), and phalloidin staining (red). Scale bar, 20 μ m. (D) Quantitative analysis of cell elongation by coexpression of the full-length FHOD1 and ROCK. The lengths of the long and short axes were measured on immunofluorescent images. ***Significant differences from all other groups ($P < 0.0001$, Tukey's HSD test). (E) HeLa-FHOD1 cells, stably expressing Flag-FHOD1, or parental HeLa cells were transfected with a plasmid encoding myc-ROCK. Cells were fixed followed by visualization by myc-immunostaining (green) and phalloidin staining (red). (F) Cells showing parallel actin fibres aligned with the long axis throughout the cell (as in E) were counted and the percentages from three independent transfections are expressed as the mean $\pm$ s.d.

with actin fibres parallel to the long axis, which distributed throughout the cells (Figure 3C and D). This phenotype is essentially the same as that induced by active forms of FHOD1 such as FHOD1-3 $\times$ D (Figure 1) and FHOD1- Δ C (Takeya and Sumimoto, 2003). In the presence of Y-27632, cell elongation with stress fibres was completely abolished (Figure 3C and D). Thus, ROCK is likely capable of activating FHOD1 *in vivo*. Furthermore, FHOD1-3 $\times$ A, a kinase-

insensitive mutant, was less effective in inducing cell elongation than the wild type (Figure 3D), indicating an essential role of FHOD1 phosphorylation at the three residues. Under the conditions, FHOD1-3 $\times$ A did not inhibit ROCK-wt-induced stress fibre formation (data not shown), suggesting that the inactive FHOD1 mutant does not function as an inhibitor for ROCK signalling. In addition, we tested the effect of ROCK expression in HeLa-FHOD1 cells, which stably

express Flag-tagged FHOD1 (Figure 3E). Parallel and dense actin fibres aligned with the long axis were observed in over 70% of ROCK-transfected HeLa-FHOD1 cells, but in about 15% of parental HeLa cells transfected with the ROCK cDNA (Figure 3F). The ROCK-dependent stress fibre formation appears to be directly mediated through FHOD1, as an inactive mutant FHOD1-E765Q failed to induce stress fibre formation (Supplementary Figure S3): Glu765 in the FHOD1 FH2 domain corresponds to Asp1151 of the yeast formin Bni1p, a residue that has an important function in actin assembly mediated by the Bni1p FH2 domain (Evangelista *et al*, 2002; Kadota *et al*, 2004; Yoshiuchi *et al*, 2006). Thus, the present findings indicate that ROCK-catalysed phosphor-

ylation activates FHOD1 through disruption of the intramolecular interaction.

In addition to the function as an activator, ROCK may also act downstream of FHOD1, as stress fibre formation induced by an active FHOD1 was reported to be inhibited by Y-27632 in NIH3T3 cells (Gasteier *et al*, 2003; Koka *et al*, 2003). A positive feedback loop between Dia1 and RhoA also has been recently proposed: the formin mDia facilitates ROCK activation by stimulating a guanine nucleotide exchanger for RhoA, an activator of ROCK (Kitzing *et al*, 2007). To address this issue, we examined the effect of ROCK-KDIA, a dominant-negative form of ROCK, when coexpressed with an active FHOD1 in HeLa cells (Figure 4A). Even in the presence of

Figure 4 (A) Effect of coexpression of a dominant-negative ROCK and an active FHOD1 on stress fibre formation. HeLa cells were cotransfected with a plasmid for a dominant-negative form of ROCK (myc-ROCK-KDIA) and plasmid for GFP-FHOD1-wt, GFP-FHOD1-3 × D, or GFP-FHOD1-ΔNΔC. Cells were fixed followed by visualization by GFP fluorescence (green), myc immunostaining (blue), and phalloidin staining (red). Scale bar, 20 μm. (B) Quantitative analysis of cell elongation by coexpression of a dominant-negative ROCK and an active FHOD1. The lengths of the long and short axes were measured on immunofluorescent images. **P* < 0.0001 (Welch's *t*-test).

ROCK-KDIA, active forms of FHOD1 (FHOD1-3 × D and FHOD1-ΔNΔC) were capable of inducing stress fibre formation (Figure 4A) and cell elongation (Figure 4B). In addition, we also tested whether FHOD1 is able to elicit phosphorylation of myosin light chain (MLC), an event that occurs downstream of ROCK (Riento and Ridley, 2003). MLC in human pulmonary arterial endothelial cells (HPAECs) was efficiently phosphorylated by the expression of active ROCK (ROCK-Δ1 and ROCK-Δ3) but not by that of an active form of FHOD1 (FHOD1-3 × D) (Supplementary Figure S4), suggesting that FHOD1 does not participate in ROCK activation. These findings support the present idea that ROCK functions upstream of FHOD1 through direct phosphorylation.

It is known that activation of myosin by phosphorylation of MLC is involved in stress fibre formation (Katoh *et al*, 1998). As shown above, expression of FHOD1-3 × D efficiently induced stress fibre formation, but did not seem to increase the phosphorylation level of MLC (Supplementary Figure S4). This may be because a basal level of MLC phosphorylation is sufficient for FHOD1-induced stress fibre formation. It has been reported that endogenous MLC is substantially phosphorylated in various types of cells without any stimulants added (Matsumura *et al*, 1998). Indeed, FHOD1-3 × D-induced stress fibre formation is totally abolished by blebbistatin, an inhibitor of non-muscle myosin II (data not shown). Thus, FHOD1 appears to induce stress fibre formation in collaboration with activated myosin.

FHOD1 depletion by RNA interference impairs thrombin-induced stress fibre formation in vascular endothelial cells

To explore the physiological significance of the ROCK-dependent FHOD1 activation, we used vascular endothelial cells where FHOD1 is abundantly expressed (Wang *et al*, 2004). The Rho-ROCK pathway is considered to have an important function in stress fibre formation in vascular endothelial cells, as the formation induced by vasoactive mediators such as thrombin is inhibited by Y-27632 (van Nieuw Amerongen *et al*, 2000; Wojciak-Stothard *et al*, 2001; Mehta and Malik, 2006). To investigate the role of FHOD1 in this process, we knocked down FHOD1 in HPAECs using two distinct double-stranded small interfering RNAs (siRNAs). Transfection of HPAECs with FHOD1 siRNA, #1 or #2, led to a significant decrease in FHOD1 at the protein level (Figure 5A). The specific knock down of FHOD1 with FHOD1 siRNA blocked thrombin-induced stress fibre formation in HPAECs (Figure 5B and C). A similar blockade was also observed when FHOD1 was knocked down in human aortic endothelial cells (HAECs) (Supplementary Figure 5A). Similarly, FHOD1 was required for stress fibre formation induced by histamine (Supplementary Figure 5B), indicating that FHOD1 is a key regulator of vasoactive substance-induced stress fibre formation in endothelial cells.

Thrombin induces FHOD1 phosphorylation in a ROCK-dependent manner

We finally examined whether thrombin elicits FHOD1 phosphorylation in vascular endothelial cells. As shown in Figure 6A, thrombin treatment of HPAEC led to phosphorylation of endogenous FHOD1 in HPAECs at Ser1131, Ser1137, and Thr1141. Thrombin-elicited phosphorylation of FHOD1 occurred in a dose-dependent manner (Figure 6B). This likely

Figure 5 FHOD1 is required for thrombin-induced actin reorganization in vascular endothelial cells. (A) RNAi for FHOD1. HPAECs were transfected with siRNAs for FHOD1 (#1 and #2) and were cultured for 72 h. The protein level of FHOD1 was determined by immunoblot analysis. (B) HPAECs were transfected with siRNAs for FHOD1 and cultured for 72 h. Cells were stimulated with 100 nM thrombin for 1 h, and fixed, followed by visualization by GFP fluorescence or phalloidin staining. Scale bar, 20 μm. (C) The percentages of cells showing stress fibre formation throughout the cell (as in B) were calculated and the data from three independent transfections are expressed as the mean ± s.d.

involves ROCK, as it was dose-dependently inhibited by Y-27632 (Figure 6C). Furthermore, ROCK depletion by RNA interference (RNAi) blocked FHOD1 phosphorylation (Figure 6D), confirming the involvement of endogenous ROCK in thrombin-induced FHOD1 phosphorylation. FHOD1 also underwent phosphorylation in response to histamine, another vasoactive substance (Figure 6E), which is also capable of inducing stress fibre formation in endothelial

Figure 6 Stimulus-induced phosphorylation of FHOD1 in vascular endothelial cells. (A–C) Thrombin-induced phosphorylation of FHOD1 *in vivo*. HPAECs were pretreated with or without 20 μM Y-27632 for 1 h and stimulated with 100 nM thrombin for 5 min. Proteins of cell lysates were immunoprecipitated with anti-FHOD1 (DAD) antibody, and the precipitates were subjected to SDS–PAGE followed by immunoblot with the anti-pS1131, anti-pS1137, anti-pT1141, or anti-FHOD1 (C-20) antibodies (A). Lysates from HPAECs stimulated with the indicated concentrations of thrombin for 5 min (B) or HPAECs stimulated with 100 nM thrombin for 5 min after pretreatment with indicated concentrations of Y-27632 for 1 h (C), were subjected to SDS–PAGE followed by immunoblot with the anti-pT1141 or anti-FHOD1 (C-20) antibodies. (D) HPAECs were transfected with a mixture of ROCK-I- and ROCK-II-specific siRNAs, and cultured for 72 h. Cells were stimulated with 100 nM thrombin for 5 min and lysed, and the lysate was subjected to SDS–PAGE followed by immunoblot with the anti-pS1141, anti-FHOD1 (C-20), anti-ROCK-I, or anti-ROCK-II antibodies. (E) FHOD1 phosphorylation induced by vasoactive substances. HPAECs were treated for 5 min with 100 nM thrombin or 10 μM histamine in the presence or absence of 20 μM Y-27632. Cell lysates were subjected to SDS–PAGE followed by immunoblot analysis with the anti-pT1141 antibodies. (F) Expression of the mutant FHOD1 (S1131D/S1137D/T1141D) induces stress fibres in HPAECs. HPAECs were transfected with a plasmid encoding the wild-type or mutant GFP-FHOD1-3 × D (S1131D/S1137D/T1141D). Cells were fixed followed by visualization by GFP fluorescence (green) or phalloidin staining (red). Scale bar, 20 μm. (G) Quantitative analysis of cell elongation by FHOD1-3 × D. The lengths of the long and short axes were measured on immunofluorescent images. **P* < 0.0001 (Welch's *t*-test). (H) The percentages of cells showing stress fibre formation throughout the cell (as in F) were calculated and the data from three independent transfections are expressed as the mean + s.d.

cells. In addition, expression of the active mutant FHOD1-3 × D, mimicking a phosphorylated form, led to cell elongation and formation of actin stress fibres in HPAECs (Figure 6F–H). Thus, ROCK-catalysed phosphorylation of FHOD1 at Ser1131, Ser1137, and Thr1141 appears to be critical for vasoactive substance-induced stress fibre formation in endothelial cells.

Conclusions

The present study shows that ROCK phosphorylates FHOD1 at Ser1131, Ser1137, and Thr1141 both *in vivo* and *in vitro*, and that ROCK-catalysed phosphorylation is sufficient for disruption of the intramolecular interaction in FHOD1, which renders this protein in an active form. To our knowledge, this is the first example that the autoinhibition in formin-family proteins is relieved by phosphorylation. In mDia and its closely related formins FRL and Bni1p, the intramolecular interaction is known to be disrupted by binding of a Rho-family GTPase (Watanabe *et al*, 1999; Dong *et al*, 2003; Seth *et al*, 2006). A phosphorylation-mediated regulation, similar to that in FHOD1, may be

involved in the relief of the autoinhibition in other formins. It has been reported that Bni1p becomes phosphorylated during cortical actin cable assembly in response to pheromone (Matheos *et al*, 2004), although the mechanism whereby the phosphorylation activates Bni1p remains to be elucidated.

Here we also demonstrate that FHOD1 has an important function in stress fibre formation in vascular endothelial cells. Vasoactive mediators such as thrombin and histamine are known to elicit stress fibre formation in endothelial cells, which participates in regulation of their barrier function (Mehta and Malik, 2006). In this process, the Rho effector ROCK has an important function, which is considered to be mediated through the following two mechanisms (van Nieuw Amerongen *et al*, 2000; Wojciak-Stothard *et al*, 2001; Mehta and Malik, 2006). First, ROCK inactivates MLC phosphatase and also directly phosphorylates MLC, resulting in increased phosphorylation of MLC; this enhances the binding of myosin to actin, promoting the bundle of actin filaments to stress fibres. Second, ROCK phosphorylates the protein kinase LIMK, which in turn phosphorylates and inactivates cofilin,

Figure 7 A proposed model for the role of FHOD1 in thrombin-induced actin reorganization in vascular endothelial cells. GPCR, G protein-coupled receptor; MLC-P, MLC phosphorylated; LIMK-P, LIM kinase phosphorylated.

an actin-depolymerizing protein (Maekawa *et al*, 1999). The present findings identify FHOD1 as a novel ROCK target that contributes to vasoactivator-induced stress fibre formation in endothelial cells (Figure 7). The vasoactivator thrombin binds to G protein-coupled receptors, leading to RhoA-dependent ROCK activation. Activated ROCK phosphorylates FHOD1 at the C-terminal DAD, which disrupts the auto-inhibitory interaction between the DAD and its target region. The relief from the inhibition activates FHOD1, thereby inducing stress fibre formation. As thrombin-mediated reorganization of actin cytoskeleton is involved in various pathological events caused by inadequate endothelial permeability, such as inflammation, oedema, and vascular leakage (Mehta and Malik, 2006), the present findings may implicate FHOD1 as a potential therapeutic target to control endothelial permeability.

Materials and methods

Chemicals

OA was purchased from Wako Pure Chemical (Osaka, Japan); thrombin and histamine were from Sigma; and Y-27632 was from Calbiochem.

Plasmid construction

The human cDNA fragments encoding FHOD1-wt (amino acids 1–1164), FHOD1-ΔC (1–1053), FHOD1-ΔNAC (415–1053), FHOD1-N (1–569), and FHOD1-DAD (1081–1145) were amplified from the human FHOD1 cDNA by PCR using specific primers (Takeya and Sumimoto, 2003). Mutations leading to the indicated amino-acid substitutions were introduced by PCR-mediated site-directed mutagenesis. The DNA fragments were ligated to pGEX-6P (GE Healthcare Bio-Sciences) or pProEX-HTb (Invitrogen) for bacterial expression as a protein fused to GST or a His-tagged protein, respectively, and to pEGFP-C1 (Clontech) or pEF-BOS-Flag for expression in mammalian cells as an N-terminally GFP-tagged or Flag-tagged protein, respectively. All the constructs were sequenced for confirmation of their identities.

For expression of ROCK, pCAG plasmids encoding myc-tagged p160ROCK-wt (1–1354), p160ROCK-Δ1 (1–1080), p160ROCK-Δ3 (1–727), and p160ROCK-KDIA (a kinase-dead mutant with the K105A/I1009A substitution) were used as previously described (Ishizaki *et al*, 1997).

An in vitro pull-down binding assay

GST- or His-tagged proteins were expressed in *Escherichia coli* strain BL21 and purified by glutathione-Sepharose-4B (GE Healthcare), or His-bind resin (Novagen), respectively, according to the manufacturer's protocol. Pull-down binding assays were performed as previously described (Takeya and Sumimoto, 2003). Briefly, a GST-fusion (10 µg) and a His-tagged protein (10 µg) were mixed in 1 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH 7.4) containing 1% Triton X-100. A slurry of glutathione-Sepharose-4B was added to the mixture followed by incubation for 30 min at 4°C. After washing three times with PBS, proteins were eluted with 10 mM glutathione. The eluates from the resin were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (CBB).

An in vitro bead co-pelleting assay

Bead co-pelleting assays were performed by the method of Lee *et al* (1999), with minor modifications. Briefly, His-tagged FHOD1-N at 0.3 µM was mixed with various concentrations (0–90 µM) of GST-FHOD1-DAD bound to glutathione-Sepharose-4B in PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH 7.4) containing 0.1% Triton X-100 and 1 mM dithiothreitol. The mixtures were centrifuged at 12000g for 5 min and the supernatants were subjected to SDS-PAGE and stained with CBB for quantification. Densitometric analysis of the CBB stain was performed using a LAS-1000 (Fuji photo film) image analyser. Bound fractions were determined as total minus fraction remaining in the supernatant.

Cells and transfection

HeLa cells were cultured in DMEM supplemented with 10% FCS. HeLa cells were transfected with plasmids using Lipofectamine (Invitrogen) and cultured for 3 h. After the addition of DMEM containing 10% FCS, cells were cultured for another 13 h followed by treatment with 20 µM Y-27632 for 1 or 2 h when indicated.

HPAECs and HAECs were purchased from Kurabo (Osaka, Japan). The cells were cultured in HuMedia EG2 (Kurabo) containing human EGF (10 ng/ml), human FGF-B (5 ng/ml), heparin (10 µg/ml), hydrocortisone (1 µg/ml), gentamycin (50 µg/ml), amphotericin B (50 ng/ml), and 2% FBS. Transfection of HPAECs and HAECs with plasmids was performed using the Amaxa Nucleofector (Amexa Biosystems, Cologne, Germany) according to the manufacturer's protocol. Briefly, HPAECs were suspended in basic nucleofector solution for primary endothelial cells at a final concentration of 5×10^5 cells per 100 µl. The cells were electroporated in the presence of 2 µg of plasmid DNAs using the Nucleofector programme M-03. After transfection, the cells were cultured for 16 h and used for the experiments.

Fixation and immunofluorescence staining

Cells were washed three times with PBS, and fixed for 15 min in 3.7% formaldehyde. Cells were subsequently permeabilized for 4 min with 0.1% Triton X-100 in PBS. After washed three times, the permeabilized cells were blocked with PBS containing 3% bovine serum albumin for 60 min (Ishizaki *et al*, 1997). Indirect immunofluorescence analysis was performed using an anti-myc primary antibody (9E10; Roche) and Alexa Fluor 350-labelled goat anti-mouse secondary antibody (Invitrogen). For F-actin staining, Texas Red-X phalloidin (Invitrogen) was used. Images were taken with a microscope (Axiovert 200; Carl Zeiss MicroImaging) coupled to a camera (AxioCam HRm; Carl Zeiss MicroImaging).

Quantification of cell elongation was carried out by measuring the length of the long and short axes using AxioVision 4.6 (Carl Zeiss MicroImaging). Statistical analysis of cell elongation data was performed using JMP software (SAS Institute). Box-and-whisker plots represent 25th percentile, median, 75th percentile, nearest observations within 1.5 times the interquartile range, and outliers. Statistical differences between two conditions were determined using Welch's *t*-test. For multiple conditions, mean values were compared by analysis of variance followed by Tukey's HSD test.

Antibodies

Anti-FHOD1 (C-20) rabbit antisera were raised against the peptide comprising the C-terminal 20 amino acids, as previously described (Takeya and Sumimoto, 2003). Anti-FHOD1 (DAD) rabbit antisera were raised against the GST-fusion protein comprising amino acids 1081–1145, and the anti-FHOD1 (DAD) antibodies were

affinity-purified by a HiTrap NHS-activated HP column (GE Healthcare) conjugated with the immunogen.

Antisera for phospho-Ser1131, phospho-Ser1137, and phospho-Thr1141 were raised against the synthetic peptides ARERKRpSRGNRKSL, RSRGNRKpSLRRTLKS, and NRKSLRRpTLKSGGLD, respectively. These sera were subjected to the respective phosphopeptide-conjugated columns, and the bound fractions were further purified using a column conjugated with a corresponding unphosphorylated peptide, ARERKRSRGNRKSL, RSRGNRKSLRRTLKS, or NRKSLRRTLKSGGLD (Asahi Techno Glass, Chiba, Japan). The flow-through fractions of the second columns were used as phosphospecific antibodies: the anti-pS1131, anti-pS1137, or anti-pT1141 antibodies.

Polyclonal antibodies against myosin II heavy chain A and phospho-MLC 2 (Ser19) were purchased from COVANCE and Cell Signaling Technology, respectively. Anti-ROCK-I and anti-ROCK-II monoclonal antibodies were purchased from BD Transduction Laboratories.

An in vitro phosphorylation assay

Phosphorylation of recombinant GST-FHOD1-DAD was carried out using 2 or 5 µg of the fusion protein in the final volume of 20 µl. The reaction mixture contained 0.1 U of ROCK (rat recombinant ROK α /ROCK-II: Upstate), 1 mM ATP, 3 µCi [γ -³²P]ATP, 1 mM dithiothreitol, 10 mM MgCl₂, 0.5 mM CaCl₂, and 50 mM Hepes, pH 7.4. Incubation was carried out for 30 min at 30°C. The phosphorylated proteins were subjected to SDS-PAGE followed by autoradiography or immunoblot with the anti-pS1131, anti-pS1137, or anti-pT1141 antibodies. Quantitative analysis of the autoradiogram was performed using a BAS-5000 (Fuji photo film) image analyser.

An in vivo phosphorylation assay

HeLa-FHOD1 cells that stably express Flag-tagged FHOD1 were established by transfection of parental HeLa cells using ViraPower™ Lentiviral Expression System (Invitrogen) according to manufacturer's instructions. HeLa-FHOD1 cells were transfected with plasmids encoding myc-RhoA, myc-ROCK-wt, myc-ROCK-Δ1, myc-ROCK-Δ3, or myc-ROCK-KDIA using Lipofectamine (Invitrogen), and cultured for 18 h in DMEM supplemented with 10% FCS. After treatment with 1 µM OA or 20 µM Y-27632 for 1 h, cells were lysed with a lysis buffer (10% glycerol, 135 mM NaCl, 5 mM EDTA, and 20 mM Hepes, pH 7.4) containing 1% NP-40. The lysate was precipitated with an anti-Flag antibody (M2; Sigma-Aldrich) in the presence of protein G-Sepharose (GE Healthcare), and washed three times with the lysis buffer. When indicated, the precipitants were treated with 0.8 U of bacterial alkaline phosphatase (*E. coli* C75; Takara) in 20 µl of a reaction mixture (1 mM MgCl₂ and 500 mM Tris, pH 8) for the indicated times at 37°C. The precipitants were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was probed with the anti-pFHOD1 polyclonal antibodies, anti-Flag monoclonal antibody, or anti-myc polyclonal antibodies (Santa Cruz).

For detection of endogenously phosphorylated FHOD1 in HPAECs, cells were treated with 100 nM thrombin or 10 µM

histamine for 5 min. When indicated, cells were pretreated with 20 µM Y-27632 for 1 h. Cells were broken with the lysis buffer containing 1% NP-40. The lysate was precipitated with the anti-FHOD1 (DAD) antibodies in the presence of protein A-Sepharose (GE Healthcare), and washed three times with the lysis buffer. The precipitants were subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was probed with the anti-pS1131, anti-pS1137, anti-pT1141, or anti-FHOD1 (C-20) antibodies.

RNAi for knockdown of FHOD1 and ROCK

Double-stranded siRNAs targeting FHOD1 and ROCK were synthesized as a 25-nucleotide modified synthetic RNA (Stealth™ RNAi; Invitrogen). The sequences were as follows: FHOD1-#1 (sense), 5'-GGAAGAGCGGCAGAGAUAUGAGGAA-3'; FHOD1-#1 (antisense), 5'-UUCUCAAUUCUUCUGCCGUCUUC-3'; FHOD1-#2 (sense), 5'-GAGAAGCUACUGACCAUGAUGCCCA-3'; FHOD1-#2 (antisense), 5'-UGGGCAUCAUGGUCAGUAGCUUC-3'; ROCK-I (sense), 5'-UUAAGCAAGCUGUGAAUUCUGACUGA-3'; ROCK-I (antisense), 5'-UCAUGCAGAAUUCACAGCUUGCUAA-3'; ROCK-II (sense), 5'-UGUUCUUUCUGUUAUAGCUGCUUC-3'; ROCK-II (antisense), 5'-GAAGCAGCUAAUUAACAGAAAGAAACA-3'. As negative control for FHOD1 siRNAs, medium-GC duplex of Stealth RNAi-negative control duplexes (Invitrogen) was used. As negative control for ROCK-I and ROCK-II siRNAs, low-GC duplex of Stealth RNAi-negative control duplexes (Invitrogen) was used. Transfection of HPAECs and HAECs with siRNA was performed using the Amaxa Nucleofector (Amaxa Biosystems), according to the manufacturer's protocol. Briefly, HPAECs were suspended in basic nucleofector solution for primary endothelial cells at a final concentration of 5 × 10⁵ cells per 100 µl. The cells were electroporated in the presence of 2 µg of siRNA duplex using the Nucleofector programme M-03. After transfection, cells were cultured for 72 h in HuMedia EG2 and used for the experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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