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ERK1/2 phosphorylate GEF-H1 to enhance its guanine nucleotide exchange activity toward RhoA

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Abstract

Rho GTPases play an essential role in the regulation of many cellular processes. Although various guanine nucleotide exchange factors (GEFs) are involved in the activation of Rho GTPases, the precise mechanism regulating such activity remains unclear. We have examined whether ERK1/2 are involved in the phosphorylation of GEF-H1, a GEF toward RhoA, to modulate its activity. Expression of GEF-H1 in HT1080 cells with constitutive ERK1/2 activation induced its phosphorylation at Thr^{678}, which was totally abolished by treating the cells with PD184352, an ERK pathway inhibitor. Stimulation of HeLa S3 cells with 12-*O*-tetradecanoyl-phorbol-13-acetate induced the phosphorylation of GEF-H1 in an ERK-dependent manner. ERK1/2-mediated Thr^{678}-phosphorylation enhanced the guanine nucleotide exchange activity of GEF-H1 toward RhoA. These results suggest that the ERK pathway, by enhancing the GEF-H1 activity, contributes to the activation of RhoA to regulate the actin assembly, a necessary event for the induction of cellular responses including proliferation and motility.

Keywords: Rho GTPase; RhoA; Guanine nucleotide exchange factor; GEF-H1; ERK1/2; PD184352

Rho GTPases play an important role in the regulation of many cellular processes, including cytoskeletal dynamics and cell motility, gene expression, and cell cycle progression [1,2]. Their activity is controlled by regulatory proteins such as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [3–5]. GEFs catalyze the exchange of GTP for GDP, thereby switching Rho GTPases to an active conformation [3]. GAPs enhance the intrinsic GTPase activity of Rho GTPases, thus leading to GTP hydrolysis to induce their inactivation [5]. The spatial and temporal control of signaling by Rho GTPases is thought to be determined by regulating the localization and activation of those regulatory proteins at specific subcellular sites, but our knowledge about these processes is still limited.

GEF-H1 was originally identified as a microtubule-associated GEF with an activity toward RhoA [6]. The GEF activity of GEF-H1 is inhibited by the interaction with microtubules, which increases upon release from microtubules. The amino- and carboxy-terminal regions of GEF-H1 are involved in the co-localization of GEF-H1 with microtubules. The deletion and/or mutation of these regions results in the loss of microtubule co-localization and the up-regulation of GEF-H1 activity [7]. Recently, modulation of GEF-H1 activity by protein kinase-mediated phosphorylation has been suggested [8,9]. For example, phosphorylation of GEF-H1 at Ser^{885} by p21-activated kinase (PAK)-1 has been shown to induce its recruitment to microtubules through binding to 14-3-3, thus resulting in the suppression of GEF-H1 activity [8]. Ser^{885} has been identified as the principal phospho-acceptor site for Aurora A and Ser^{959} as the primary Cyclin-dependent kinase (CDK) 1/Cyclin B phosphorylation site,

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both of which induce the down-regulation of GEF-H1 activity [9].

GEF-H1 contains the Ser/Thr-Pro motif at nine sites (Fig. 1A), which represents the consensus phospho-acceptor site for mitogen-activated protein kinases (MAPKs) and CDKs [10–12]. We have now examined whether ERK-MAPK 1 and 2 (ERK1/2) are involved in the phosphorylation of GEF-H1 to modulate its activity. Our results suggest that ERK1/2 phosphorylate GEF-H1 at Thr^{678}, which results in the enhanced activation of GEF-H1 activity toward RhoA.

Materials and methods

Materials. Rabbit polyclonal antibodies to GEF-H1 were generated in response to a peptide corresponding to residues 714–727 (CRADSDSSQRDRNG) of the human protein. Other antibodies used included those to phosphorylated ERK1/2 (M8159) from Sigma–Aldrich, RhoA (sc-418), HA-probe (sc-7392) or ERK1/2 (sc-94) from Santa Cruz Biotechnology, phospho-(Thr) MAPK/CDK substrates (#2321) or phospho-(Ser) MAPK/CDK substrates (PXSP or SPXR/K) (#2325) from Cell Signaling Technology, green fluorescent protein (GFP) (GF090R) from Nacalai Tesque (Kyoto, Japan). PD184352, a specific inhibitor of MAPK/ERK kinase 1 and 2 (MEK1/2), was synthesized as described previously [13].

Cell culture. Human tumor cell lines HT1080 (fibrosarcoma), HeLa S3 (cervical adenocarcinoma) and LK2 (squamous cell carcinoma) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum [14].

Plasmids and cell transfection. To generate an expression plasmid encoding either EGFP-tagged GEF-H1 or its deletion mutant GEF-H1(ΔC), cDNA fragments were amplified from human TIG-3 diploid fibroblast cDNA by the polymerase chain reaction (PCR) with the primers 5′-CGGAATTCCATGTCTCGGATCGAATCC-3′ (forward), with the underlined sequence corresponding to an EcoRI site, and either 5′-CCGCTCGAGTTAGCTCTCGGAGGCTAC-3′ (reverse) for GEF-H1 or 5′-CCGCTCGAGTTAGCGCACGCTCTGCTGAAT-3′ (reverse) for GEF-H1(ΔC), with the underlined sequences corresponding to a XhoI site. The PCR products were digested with EcoRI and XhoI, and the resulting fragments were cloned into the EcoRI- and SalI-digested pEGFP-C1 expression vector (Clontech) and verified by sequencing. The mutagenesis of EGFP-GEF-H1 Thr^{678} to Ala (T678A) was performed using the QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions with the following mutagenic primers: 5′-GGAACTGCTCTTGGCACCCCGAGAGCCAGCC-3′ and 5′-GGCTGGCTCTCGGGGGTGCCAAGAGCAGTTCC-3′. HA-tagged ERK2 cDNA (a kind gift from Dr. Michael J. Weber, University of Virginia Health Science Center) was subcloned into the EcoRI site of the pcDNA3 expression vector (Invitrogen). The expression plasmid for ΔRaf-1:ER (pBabepuro3ΔRaf-1:ER) [15] was kindly provided by Dr. Martin McMahon (University of California, SF). Transfection of cells with these expression plasmids was performed with the use of Lipofectamine 2000 reagent (Invitrogen). For the establishment of LK2 cells stably expressing ΔRaf-1:ER, the cells were transfected with NotI-digested pBabepuro3ΔRaf-1:ER, subjected to selection in the presence of puromycin (6 μg/ml), and the individual resistant colonies isolated were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and puromycin (6 μg/ml).

Cell lysis and immunoblot analysis. Cells were scraped off plates into a hypotonic lysis buffer [25 mM Tri–HCl (pH 7.4), 25 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM *p*-nitrophenyl phosphate, 20 nM okadaic acid, 0.2 mM sodium molybdate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml)] and lysed by sonication for 60 s. Lysates were fractionated by SDS–PAGE and subjected to immunoblot analysis as described [16,17]. Immune complexes were visualized with the enhanced chemiluminescence system (GE Healthcare Bio-Sciences).

Immunoprecipitation. Cells were lysed in IP lysis buffer [25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM *p*-nitrophenyl phosphate, 20 nM okadaic acid, 0.2 mM sodium molybdate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml)], and cell lysates were incubated and gently rocked for 6 h at 4 °C with respective antibodies. Immune complexes were precipitated with Protein A/G Plus-Agarose (Santa Cruz Biotechnology) [18], and then subjected to immunoblot analysis.

RhoA pull-down assay. To determine Rho activation, the EZ-Detect™ Rho Activation Kit (Pierce) was used. In brief, cells on 100-mm dishes were transfected with the expression plasmid encoding EGFP, EGFP-GEF-H1 or EGFP-GEF-H1 (T678A) for 24 h, after which the cells were lysed in RhoA buffer [25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 5% Glycerol, 5 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), and aprotinin (10 μg/ml)]. Cell lysates (400 μg) were incubated with GST-Rhotekin-Rho-binding domain (RBD) in the presence of SwellGel Immobilized Glutathione Disc for 1 h with gentle rocking. The pulled-down fractions were subjected to immunoblot analysis with antibody to RhoA.

Colony formation assay. HT1080 cells were transiently transfected for 24 h with the expression plasmid encoding EGFP, EGFP-GEF-H1 or EGFP-GEF-H1(T678A). The detached cells floating in the culture medium were collected, plated into Matrigel-coated dishes (BD Biosciences) in the presence of 200 μg/ml Geneticin (Invitrogen) and then allowed to form colonies by incubation for 7 days. The resulting colonies were fixed with 70% ethanol and stained with 0.5% crystal violet. Colonies consisting of >50 cells were counted.

Results and discussion

ERK1/2 phosphorylate GEF-H1 at Thr^{678}

To determine whether or not GEF-H1 is phosphorylated by ERK1/2, HT1080 fibrosarcoma cells with constitutive ERK1/2 activation [14] were transfected with an expression plasmid for either EGFP-GEF-H1 or GEF-H1(ΔC) and treated with 10 μM PD184352. Immunoprecipitation with anti-GFP antibody and immunoblotting with anti-phospho-Thr (MAPK/CDK substrates) antibody revealed that the threonine residue of GEF-H1, but not of GEF-H1(ΔC) was phosphorylated in an ERK1/2-dependent manner (Fig. 1B). Although immunoblotting with anti-phospho-Ser (MAPK/CDK substrates) antibody showed the serine phosphorylation of GEF-H1 in HT1080 cells, it was not affected by the treatment of the cells with PD184352. These results indicate that the observed serine phosphorylation of GEF-H1 is induced in an ERK1/2-independent manner, but it could possibly represent phosphorylation by CDK1/Cyclin B at Ser^{959} [9].

When HeLa S3 cells, in which the ERK pathway is not activated, were transfected with those plasmids and stimulated with 12-*O*-tetradecanoyl-phorbol-13-acetate (PMA) (25 ng/ml), threonine phosphorylation of GEF-H1, but not of GEF-H1(ΔC), was induced. PD184352-treatment of the cells totally abolished PMA-induced ERK1/2 activation as well as the threonine phosphorylation of GEF-H1. Stimulation of such transfected cells with epidermal growth

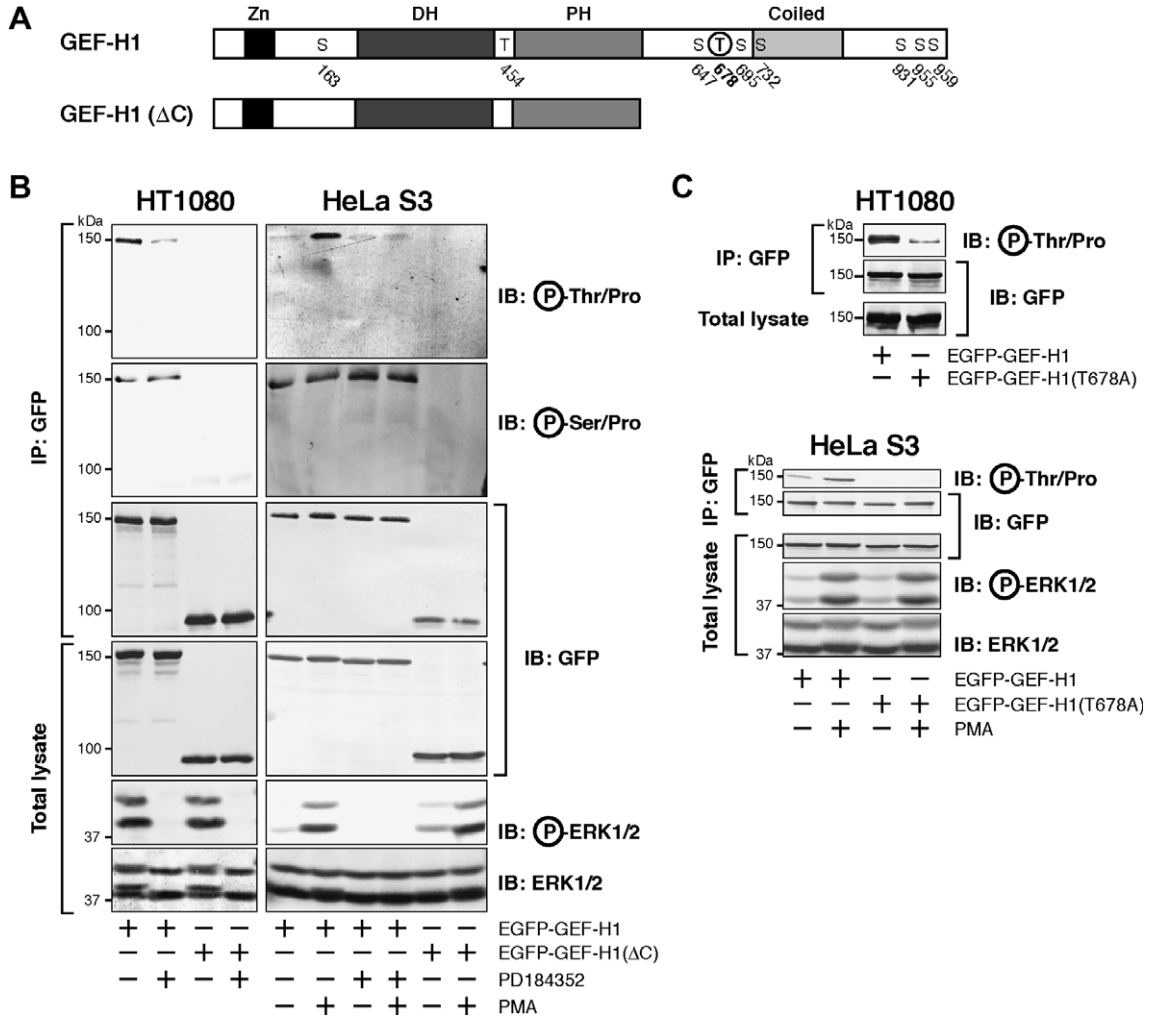

Fig. 1. ERK1/2 phosphorylate GEF-H1 at Thr^{678}. (A) Domain structures of GEF-H1 and GEF-H1(ΔC). Zn, zinc finger-like motif; DH, Dbl-homologous domain; PH, pleckstrin homology domain; Coiled, coiled-coil region. S/T, Ser/Thr-Pro motif. (B) HT1080 cells or HeLa S3 cells were transfected with the expression plasmid for EGFP-GEF-H1 or EGFP-GEF-H1(ΔC) for 24 h, and then treated with 10 μM PD184352 for 1 h (HT1080 cells) or treated with 25 ng/ml PMA for 1 h in the presence or absence of 10 μM PD184352 (HeLa S3 cells). Cell lysates (200 μg of protein) were subjected to immunoprecipitation (IP) with anti-GFP antibody. The resulting precipitates, as well as the original cell lysates (20 μg of protein), were subjected to immunoblot analysis (IB) using the indicated antibodies. (C) HT1080 cells or HeLa S3 cells were transfected with the expression plasmid for EGFP-GEF-H1 or EGFP-GEF-H1(T678A). HeLa S3 cells were treated with 25 ng/ml PMA for 1 h. Cell lysates (200 μg of protein) were subjected to immunoprecipitation with anti-GFP antibody, and then the resulting precipitates, as well as the original cell lysates (20 μg of protein), were subjected to immunoblot analysis using the indicated antibodies. Data are representative of three separate experiments.

factor (10 ng/ml) showed essentially the same results (data not shown). Although the serine phosphorylation of GEF-H1 was observed in HeLa S3 cells, it was not affected by either PMA-stimulation or PD184352-treatment of the cells. These results suggest that ERK1/2 phosphorylate the threonine residue of GEF-H1 in its carboxy-terminal fragment, most probably Thr^{678}.

In order to confirm the possibility that Thr^{678} is phosphorylated by ERK1/2, those cells were transfected with the expression plasmid for a GEF-H1 mutant in which Thr^{678} is replaced with Ala [GEF-H1(T678A)]. The mutation of Thr^{678} to Ala markedly reduced the phosphorylation of GEF-H1 in HT1080 cells as well as in PMA-stimulated HeLa S3 cells (Fig. 1C), thus clearly indicating that ERK1/2 phosphorylate GEF-H1 at Thr^{678}.

GEF-H1 associates with ERK1/2 in the cells

To determine whether or not GEF-H1 is associated with ERK1/2 to make an enzyme/substrate complex in the cells, HT1080 or HeLa S3 cells were transfected with the expression plasmids for EGFP-GEF-H1/GEF-H1(ΔC) and HA-ERK2. Immunoprecipitation with anti-HA/anti-GFP antibody and immunoblotting with anti-GFP/anti-HA antibody showed that ERK2 interacted with GEF-H1, but not with GEF-H 1(ΔC), in these cells (Fig. 2, and data not shown). Furthermore, PMA-stimulation of HeLa S3 cells to induce the activation of ERK1/2 increased the association of GEF-H1 with ERK2. These results suggest that GEF-H1 associates with ERK2 via its C-terminal fragment, enforcing the idea that ERK1/2 directly phosphory-

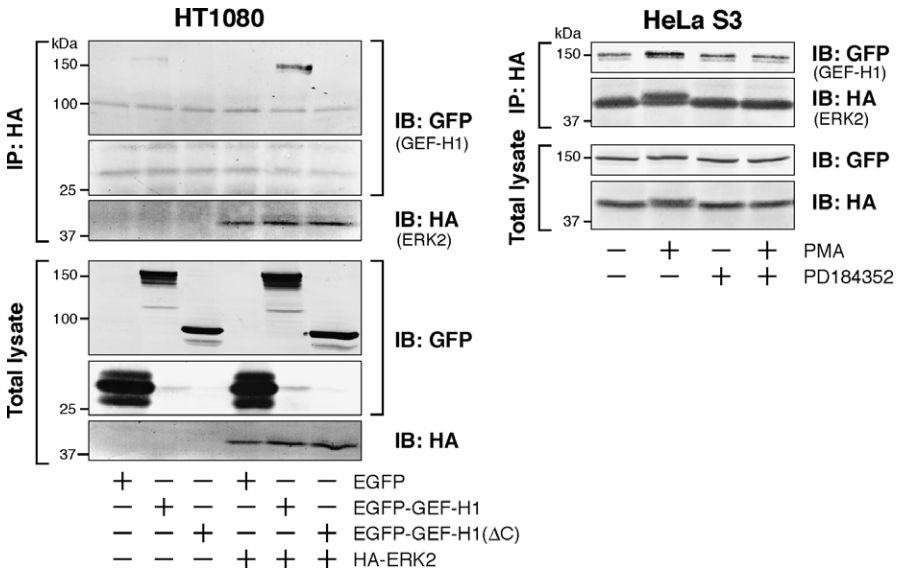

Fig. 2. GEF-H1 associates with ERK2 in the cells. HT1080 cells or HeLa S3 cells were co-transfected with expression plasmids for HA-ERK2 and EGFP, EGFP-GEF-H1 or EGFP-GEF-H1(ΔC). After 24 h, HeLa S3 cells were treated with 25 ng/ml PMA for 1 h in the presence or absence of 10 μM PD184352. Cell lysates (200 μg of protein) were subjected to immunoprecipitation with anti-HA antibody, and then the resulting precipitates, as well as the original cell lysates (20 μg of protein), were subjected to immunoblot analysis with antibodies to GFP or to HA. Data are representative of three separate experiments.

late GEF-H1 in the cells. The precise region through which GEF-H1 is associated with ERK1/2 remains to be determined.

ERK1/2 phosphorylate GEF-H1 under physiological conditions

We attempted to determine whether ERK1/2 phosphorylate GEF-H1 under physiological conditions. To this end, HeLa S3 cells were stimulated with PMA for 1 h in the presence or absence of PD184352. Immunoprecipitation with anti-GEF-H1 antibody and immunoblotting with the anti-phospho-Thr antibody revealed that PMA-stimulation of the cells induces the phosphorylation of GEF-H1 at threonine residue, which was totally abolished in the presence of PD184352 (Fig. 3). Although immunoblot analysis of the immunoprecipitates with the anti-phospho-Ser antibody showed the serine phosphorylation of GEF-H1, it was not affected by either PMA-stimulation or PD184352-treatment of the cells. These results suggest that ERK1/2 target endogenous GEF-H1 for phosphorylation most probably at Thr^{678} in response to PMA-stimulation of the cells. At present, the possibility that some serine residue(s) of GEF-H1 is phosphorylated by ERK1/2 cannot be completely ruled out because the anti-phospho-Ser antibody used in this study preferentially detects phosphoserine in a PXS*P or S*PXR/K motif.

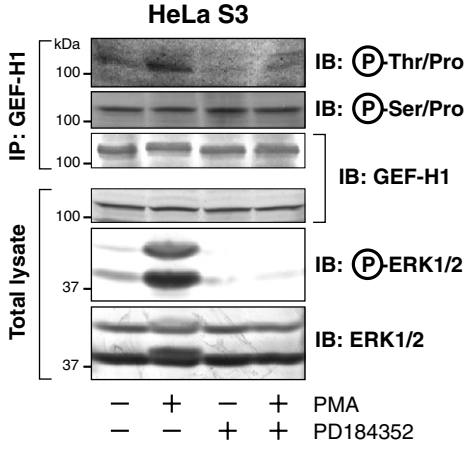

Fig. 3. ERK1/2 phosphorylate GEF-H1 under physiological conditions. HeLa S3 cells were treated with 25 ng/ml PMA for 1 h in the presence or absence of 10 μM PD184352. Cell lysates (500 μg of protein) were subjected to immunoprecipitation with anti-GEF-H1 antibody. The resulting precipitates, as well as the original cell lysates (20 μg of protein), were subjected to immunoblot analysis with the indicated antibodies. Data are representative of three separate experiments.

ERK1/2-mediated phosphorylation at Thr^{678} enhances the guanine nucleotide exchange activity of GEF-H1 toward RhoA

To determine whether the ERK1/2-mediated phosphorylation of GEF-H1 at Thr^{678} could modulate its guanine nucleotide exchange activity toward RhoA, HT1080 cells were transfected with the expression plasmid for EGFP-GEF-H1 or its non-phosphorylatable mutant [EGFP-GEF-H1(T678A)], and the ability of these GEF-H1 constructs to activate RhoA was examined by performing a pull-down assay of RhoA-GTP with the use of GST-Rhotekin-RBD. Expression of GEF-H1(T678A) resulted in the activation of RhoA to some extent (Fig. 4A). However, expression of GEF-H1 apparently induced a more

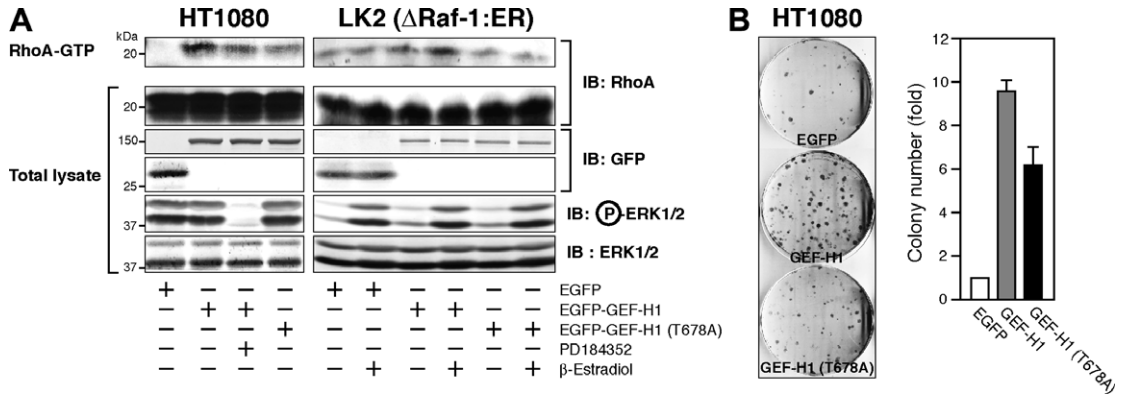

Fig. 4. ERK1/2-mediated phosphorylation at Thr^{678} enhances the guanine nucleotide exchange activity of GEF-H1 toward RhoA. (A) HT1080 cells or LK2 cells stably expressing ΔRaf-1:ER (LK2(ΔRaf-1:ER)) were transfected with the expression plasmid for EGFP, EGFP-GEF-H1 or EGFP-GEF-H1(T678A) for 24 h, and then were treated with 10 μM PD184352 (HT1080 cells) or 1 μM β-estradiol (LK2(ΔRaf-1:ER) cells) for 1 h. Cell lysates (400 μg of protein) were subjected to RhoA pull-down assay. The pulled-down fractions, as well as the original cell lysates (40 μg of protein) were subjected to immunoblot analysis with the indicated antibodies. (B) HT1080 cells were transfected with the expression plasmid for EGFP, EGFP-GEF-H1 or EGFP-GEF-H1(T678A) for 24 h. The detached cells floating in the culture medium were collected, plated into Matrigel-coated dishes in the presence of Geneticin, and allowed to form colonies by incubation for 7 days (left). Colonies consisting of >50 cells were counted. The colony number is expressed as the fold increase over that of EGFP-transfected cells (mean ± SD of values from three separate experiments) (right).

enhanced RhoA activation, which markedly decreased when the cells were treated with PD184352.

Many Dbl family proteins other than GEF-H1 also function as GEFs toward RhoA. Furthermore, the guanine nucleotide exchange activity of these Dbl family proteins could be regulated by diverse extracellular stimuli via various signaling pathways [4]. In order to more precisely examine the involvement of ERK1/2-mediated Thr^{678}-phosphorylation in the regulation of GEF-H1 activity toward RhoA, LK2 cells stably expressing ΔRaf-1:ER were utilized for further analyses: the addition of β-estradiol induces the selective activation of the Raf-1-MEK1/2-ERK1/2 pathway in cells [15]. Although expression of GEF-H1(T678A) resulted in the activation of RhoA to some extent, it was not affected by β-estradiol-treatment to induce the ERK1/2 activation in the cells. In contrast, β-estradiol apparently induced a prominent activation of RhoA in the cells in which wild-type GEF-H1 was expressed.

Activation of RhoA has been shown to increase contractile activity through a Rho/Rho-kinase pathway, which leads to mitosis-associated detachment of cells from epithelial sheets [19]. In order to confirm that the ERK1/2-mediated phosphorylation at Thr^{678} enhances the guanine nucleotide exchange activity of GEF-H1 toward RhoA, the activation of RhoA was examined by analyzing the number of detached cells in HT1080 cells transfected with EGFP-GEF-H1 or EGFP-GEF-H1(T678A). Expression of the non-phosphorylatable GEF-H1(T678A) mutant induced a significant detachment of the cells. Expression of wild-type GEF-H1, however, induced a more pronounced detachment of the cells. All these results support the notion that the ERK1/2-mediated phosphorylation of GEF-H1 at Thr^{678} enhances the guanine nucleotide exchange activity of GEF-H1 toward RhoA.

We have herein demonstrated that ERK1/2 phosphorylate GEF-H1 at Thr^{678} in cells. Phosphorylation of GEF-H1 by other kinases such as PAK1 (Ser^{885}) [8], Aurora A (Ser^{885}), and CDK1 (Ser^{959}) [9] has been reported, all of which results in the down-regulation of GEF-H1 activity. In contrast, the ERK1/2-mediated phosphorylation enhances the guanine nucleotide exchange activity of GEF-H1 toward RhoA. ERK1/2 are the major kinases whose activation is commonly induced in a variety of growth factor-stimulated cells. In this context, stimulation of cells with growth factors has been shown to induce actin reorganization, thereby forming membrane ruffles and stress fibers; while during such processes, Rho GTPases play an essential role [20]. Taken together, it seems likely that growth factor-induced ERK1/2 activation leads to the phosphorylation of GEF-H1 at Thr^{678}, thereby enhancing its guanine nucleotide exchange activity, and thus contributing to the activation of RhoA to regulate the actin assembly, a necessary event for the induction of many cellular responses including proliferation, motility, and secretion.

Acknowledgments

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