Sprouty-4 negatively regulates cell spreading by inhibiting the kinase activity of testicular protein kinase

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TESK1 (testicular protein kinase 1) is a serine/threonine kinase that phosphorylates cofilin and plays a critical role in integrin-mediated actin cytoskeletal reorganization and cell spreading. We previously showed that TESK1 interacts with Sprouty-4 (referred to as Spry4), an inhibitor of growth factor-induced Ras/MAP (mitogen-activated protein) kinase signalling, but the functional role of this interaction has remained unknown. In the present study, we show that Spry4 inhibits the kinase activity of TESK1 by binding to it through the C-terminal cysteine-rich region. Expression of Spry4 in cultured cells suppressed integrin-mediated cell spreading, and TESK1 reversed the inhibitory effect of Spry4 on cell spreading. Furthermore, Spry4 suppressed integrin- and TESK1-mediated cofilin phosphorylation during the spreading of cells on laminin. These findings suggest that Spry4 suppresses cell spreading by inhibiting the kinase activity of TESK1. Although tyrosine phosphorylation is required for the inhibitory activity of Spry4 on a Ras/MAP kinase pathway, mutation of the corresponding tyrosine residue (Tyr-75 in human Spry4) to an alanine had no apparent effect on its inhibitory actions on TESK1 activity and cell spreading, which suggests a novel cellular function of Spry to regulate the actin cytoskeleton, independent of its inhibitory activity on the Ras/MAP kinase signalling.

Key words: cell spreading, cofilin, laminin, mitogen-activated protein kinase (MAP kinase), Sprouty, testicular protein kinase (TESK).

INTRODUCTION

TESK1 (testicular protein kinase 1) is a serine/threonine kinase, with the structure composed of an N-terminal protein kinase domain and a C-terminal proline-rich region [1]. TESK1 was named after its higher expression in the testis [1,2], but we later found that it is expressed in various tissues and cell lines, albeit at a relatively low level [3,4]. Thus TESK1 seems to have general cellular functions rather than a specific function in the testis. The protein kinase domain of TESK1 is closely related to those of LIM kinases (LIM motif-containing protein kinases, where LIM is an acronym of the three gene products Lin-11, Isl-1 and Mec-3), although their domain structures differ [1,5]. Similar to LIM kinases, TESK1 phosphorylates cofilin, an actin-binding protein capable of stimulating depolymerization and severance of actin filaments [6,7], specifically at Ser-3, and thereby inhibits its actin-disassembling activity [8–11]. Overexpression of TESK1 in cultured cells induced cofilin phosphorylation and actin cytoskeletal reorganization, including the formation of focal adhesions and stress fibres [10]. Expression of a kinase-inactive TESK1 mutant suppressed focal adhesion, stress fibre formation and cell spreading in cells plated on fibronectin, thus indicating that TESK1 plays an important role in integrin-mediated actin remodelling and cell spreading [10,12]. LIM kinases are activated by the Rho family GTPases Rac, Rho and Cdc42 through their downstream protein kinases, such as ROCK (Rho-associated kinase) and PAK (p21-activated kinase) [13–15], whereas TESK1 was not activated by these kinases [10]. Although 14-3-3 was shown to regulate the activity and localization of TESK1 [12], the signalling mechanism regulating TESK1 activity remains largely uncharacterized.

Using yeast two-hybrid screening, TESK1 was found to interact with Sprouty-4 (referred to as Spry4) [16]. Spry was originally identified in *Drosophila* as a negative regulator of FGF (fibroblast growth factor) signalling during tracheal development [17] and then regarded as a general inhibitor of the growth factor-induced RTK (receptor tyrosine kinase) signalling pathways involved in *Drosophila* development and organogenesis [18–20]. In mammals, four Spry orthologues (Spry1–Spry4) have been identified [21,22]. Mammalian Sprys similarly inhibit growth factor-induced cell responses by inhibiting the RTK-dependent Ras/MAP (mitogen-activated protein) kinase signalling pathway [23–30]. Several mechanisms for Spry inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including the avoidance of Grb2–Sos (Son of Sevenless) recruitment [18,29] or the inhibition of Raf [28,30]. The structure of a Spry is composed of a highly conserved cysteine-rich region in the C-terminus and a variable N-terminal region [22]. Spry mutant, in which a highly conserved tyrosine residue (Tyr-75 for human Spry4) in the N-terminal region is replaced by a non-phosphorylatable residue, acts as a dominant-negative form that prevents Spry from inhibiting FGF-induced MAP kinase activation, which suggests that phosphorylation of this tyrosine residue is essential for the inhibitory activity of Spry on MAP kinase activation [26,29].

Although the mechanisms by which Sprys inhibit the RTK/Ras/MAP kinase signalling have been extensively studied [17–30], the biological significance of the interaction between Spry4 and TESK1 has remained unknown. We report in the present study that Spry4 negatively regulates the kinase activity of TESK1 by associating with it through the C-terminal cysteine-rich region of Spry4. We also provide evidence that Spry4 has the potential to inhibit integrin-mediated cofilin phosphorylation and cell spreading by repressing the kinase activity of TESK1. Phosphorylation of the conserved tyrosine residue is not required for the inhibitory actions of Spry4 on TESK1 activity and cell spreading. These

Abbreviations used: CFP, cyan fluorescent protein; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor; GST, glutathione S-transferase; HA, haemagglutinin; MAP, mitogen-activated protein; P-cofilin, Ser-3-phosphorylated cofilin; P-ERK, phosphorylated ERK; PTP-1B, protein tyrosine phosphatase-1B; RTK, receptor tyrosine kinase; Spry, Sprouty; TESK1, testicular protein kinase 1; YFP, yellow fluorescent protein.

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findings suggest a novel cellular function of Spry on the actin cytoskeletal reorganization, independent of its inhibitory activity on the RTK/Ras/MAP kinase signalling pathway.

EXPERIMENTAL

Plasmids

Plasmids coding for Myc epitope-tagged human TESK1 [wild-type and kinase-inactive D170A ($Asp^{170} \rightarrow Ala$)] and HA (haemagglutinin)-tagged human Spry4 were constructed as described in [1,16]. Expression plasmids for truncated mutants of Myc–TESK1 (Myc–TESK1-N and Myc–TESK1-C) and HA–Spry4 (HA–Spry4-N and HA–Spry4-C) were constructed by inserting PCR-amplified fragments into pCAG-Myc and pcDNA3.1-HA expression vectors [16] respectively. The plasmid for Spry4-(Y75A), in which Tyr-75 was replaced by an alanine residue, was constructed by PCR-based mutagenesis. Plasmids for YFP (yellow fluorescent protein)- and CFP (cyan fluorescent protein)-tagged proteins were constructed by inserting the corresponding cDNAs into pEYFP-C1 and pECFP-C1 vectors (ClonTech, Palo Alto, CA, U.S.A.) respectively. Plasmids coding for GST (glutathione S-transferase)-fusion proteins were constructed by inserting PCR-amplified Spry4 cDNA fragments into the pGEX-2T vector (Amersham Biosciences).

Antibodies

Monoclonal antibodies against Myc (9E10) and HA (12CA5) were purchased from Roche Diagnostics (Tokyo, Japan). Anti-ERK-1/2 and anti-P-ERK-1/2 polyclonal antibodies (where ERK stands for extracellular-signal-regulated kinase and P-ERK for phosphorylated ERK) were purchased from Sigma. Anti-GFP antibody (where GFP stands for green fluorescent protein) was from Invitrogen. Polyclonal antibodies against cofilin and P-cofilin (Ser-3-phosphorylated cofilin) were prepared as described in [10]. Anti-HA rabbit polyclonal antibody was provided by Dr Y. Fujiki (Kyushu University, Fukuoka, Japan).

Cell culture and transfection

Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum. Cells were transfected with plasmids using LIPOFECTAMINE™ (Life Technologies, Gaithersburg, MD, U.S.A.).

Co-precipitation assay

COS-7 cells co-transfected with Myc–TESK1 and HA–Spry4 or its mutants were suspended in lysis buffer [20 mM Hepes, pH 7.8, 150 mM NaCl, 10 % (v/v) glycerol, 1 % Nonidet P40, 1 % sodium deoxycholate, 0.1 % SDS, 1 mM PMSF, 1 mM dithiothreitol and 10 μg/ml leupeptin] and then incubated on ice for 30 min. After centrifugation, lysates were precleared with Protein A–Sepharose and the supernatants were subjected to immunoprecipitation as described in [10]. Immunoprecipitates were separated by SDS/PAGE and analysed by immunoblotting with an anti-Myc or an anti-HA antibody, as described in [10,16].

Purification of GST-fusion proteins

GST-fusion proteins were expressed in *Escherichia coli* and purified on a glutathione–Sepharose column (Amersham Biosciences), as described in [12].

In vitro pull-down assay

Lysates of COS-7 cells transfected with plasmids for Myc–TESK1 or its mutants were precleared with glutathione–Sepharose and incubated with GST–Spry4 and glutathione–Sepharose, as described in [12]. The beads were subjected to SDS/PAGE and analysed by immunoblotting.

MAP kinase assay

293T cells transfected with plasmids for YFP or YFP-fusion proteins were serum-starved for 8 h and then stimulated with 10 ng/ml basic FGF for 30 min. Cell lysates were subjected to SDS/PAGE and analysed by immunoblotting with anti-ERK and anti-P-ERK antibodies.

In vitro kinase assay

Lysates of COS-7 cells expressing Myc–TESK1 were immunoprecipitated with 9E10 anti-Myc antibody. Immunoprecipitates were washed twice with kinase reaction buffer (50 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM dithiothreitol, 1 mM NaF, 0.1 mM sodium vanadate, 5 mM $MnCl_2$ and 5 mM $MgCl_2$) and incubated on ice for 30 min with GST, GST–Spry4 or its mutants in the kinase reaction buffer and then for 30 min at 30 °C in a buffer containing 10 μM ATP, 185 kBq of [γ^{32}-P]ATP (110 TBq/mmol; Amersham Biosciences) and 50 μg/ml His_6-tagged cofilin. The reaction mixture was solubilized in SDS sample buffer (50 mM Tris/HCl, pH 6.8, 10 % glycerol, 1 mM dithiothreitol, 1 % SDS and 0.002 % Bromophenol Blue) for 5 min at 95 °C and subjected to SDS/PAGE. Proteins were transferred on to a PVDF membrane and ^{32}P incorporation into cofilin was visualized by autoradiography, using a BAS 1800 Bio-Image analyser (Fuji Film, Tokyo, Japan). The kinase activity was normalized by dividing the radioactivity incorporated into cofilin by the immunoreactive density of TESK1 estimated by a densitometer.

Cell staining

Cultured HeLa and C2C12 cells were washed twice with PBS, fixed in 4 % (w/v) formaldehyde in phosphate buffer (80 mM K_2HPO_4 and 20 mM KH_2PO_4, pH 7.4) and stained with 9E10 anti-Myc antibody and anti-HA polyclonal antibody, as described in [10]. Cells were also stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR, U.S.A.) for F-actin.

Cell spreading assay

For the cell spreading assay, coverglasses were coated overnight at 37 °C with 8.0 μg/ml laminin in PBS. C2C12 cells transfected with plasmids coding for YFP- or CFP-fusion proteins were cultured for 18 h and serum-starved for 8 h. Cells were then suspended and replated on to laminin-coated coverglasses. After incubation for 30 min at 37 °C, cells were washed twice with PBS, fixed in 4 % formaldehyde in phosphate buffer and stained with rhodamine–phalloidin for F-actin. To obtain quantitative data of the extent of cell spreading, the cell areas were calculated using IPLab image analysis software (Scanalytics, Fairfax, VA, U.S.A.), and cells were categorized into three classes: cell area $< 800\ \mu m^2$ (class 1), $800\ \mu m^2 <$ cell area $< 1600\ \mu m^2$ (class 2) and cell area $> 1600\ \mu m^2$ (class 3). For time-lapse analysis, YFP fluorescence images were recorded every 5 min for 30 min after replating cells on laminin, using a Zeiss LSM510 laser scanning confocal microscope. To analyse the changes in the level of P-cofilin, cells were lysed by hot-SDS buffer [2 % (w/v) SDS, 1 mM dithiothreitol and 50 mM Tris/HCl, pH 6.8] at 95 °C. Lysates were boiled for 15 min at 95 °C and sonicated. After centrifugation, lysates were subjected to SDS/PAGE and analysed by immunoblotting with anti-P-cofilin and anti-cofilin antibodies.

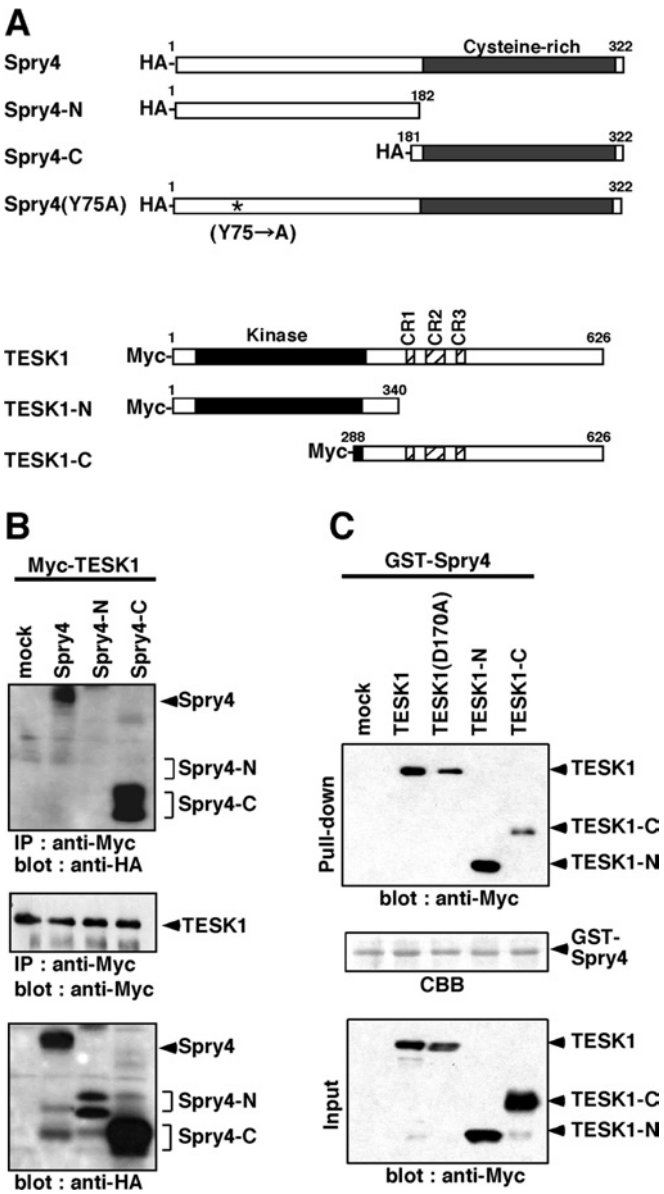

Figure 1 TESK1 interacts with Spry4 through the C-terminal cysteine-rich region

(**A**) Schematic representation of HA–Spry4, Myc–TESK1 and their mutants. Numbers above the boxes indicate amino acid residue numbers. CR1–CR3 indicate the highly conserved regions between TESK1 and TESK2 [34]. (**B**) Co-precipitation assay. Myc–TESK1 and HA–Spry4 mutants were co-expressed in COS-7 cells. Cell lysates were immunoprecipitated with anti-Myc antibody and analysed by immunoblotting with anti-HA antibody (top panel) or anti-Myc antibody (middle panel). Expression of HA–Spry4 and its mutants was analysed by immunoblotting cell lysates with anti-HA antibody (bottom panel). IP, immunoprecipitation; blot, immunoblotting. (**C**) *In vitro* pull-down assay. COS-7 cell lysates expressing Myc–TESK1 or its mutants were incubated with GST–Spry4 bound to glutathione–Sepharose, and precipitates were analysed by immunoblotting with anti-Myc antibody (top panel) and Coomassie Brilliant Blue (CBB) staining (middle panel). Expression of Myc–TESK1 or its mutants was analysed by immunoblotting cell lysates with an anti-Myc antibody (bottom panel).

RESULTS

TESK1 interacts with Spry4 through the C-terminal cysteine-rich region

We previously showed that TESK1 interacts with Spry4 [16]. To determine the TESK1-binding region of Spry4, we constructed plasmids coding for the N- and C-terminal fragments of Spry4,

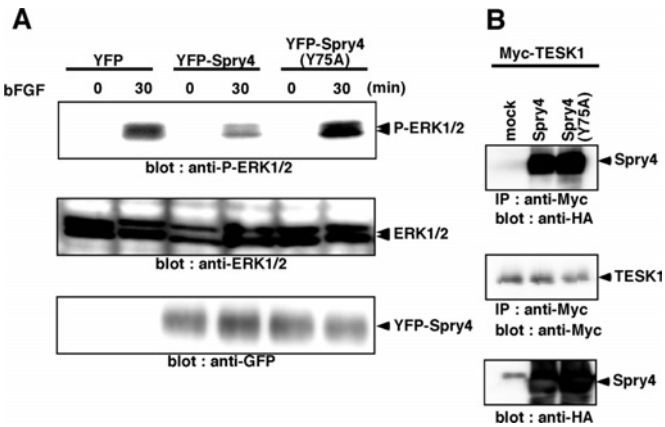

Figure 2 Phosphorylation of Spry4 at Tyr-75 is required for the inhibitory activity on FGF-induced ERK activation, but not for TESK1 binding

(**A**) Effects of expression of Spry4 or Spry4(Y75A) on FGF-induced ERK activation. 293T cells expressing YFP, YFP–Spry4 or YFP–Spry4(Y75A) were serum-starved and then stimulated with basic FGF (bFGF) for 30 min. Cell lysates were subjected to SDS/PAGE and analysed by immunoblotting with anti-P-ERK1/2 antibody to detect ERK1/2 activation (top panel), anti-ERK1/2 antibody (middle panel) and anti-GFP antibody (bottom panel). (**B**) Co-precipitation of Spry4(Y75A) with TESK1. COS-7 cells were co-expressed with Myc–TESK1 and HA–Spry4 or HA–Spry4(Y75A). Cell lysates were immunoprecipitated with anti-Myc antibody and analysed by immunoblotting with anti-HA antibody (top panel) or anti-Myc antibody (middle panel). Expressions of HA–Spry4 and HA–Spry4(Y75A) were analysed by immunoblotting cell lysates with anti-HA antibody (bottom panel).

Spry4-N (amino acids 1–182) and Spry4-C (amino acids 181–322; Figure 1A). When Myc–TESK1 was co-expressed with HA–Spry4 or its truncated mutants in COS-7 cells and immunoprecipitated with anti-Myc antibody, it was found that HA–Spry4 and HA–Spry4-C, but not HA–Spry4-N, co-precipitated with Myc–TESK1 (Figure 1B). Thus TESK1 interacts with Spry4 through the C-terminal cysteine-rich region of Spry4.

We also examined the Spry4-binding region of TESK1. TESK1 is composed of an N-terminal protein kinase domain and a C-terminal non-catalytic domain (Figure 1A). Myc–TESK1 and its N- and C-terminal fragments, TESK1-N (amino acids 1–340) and TESK1-C (amino acids 288–626), were expressed in COS-7 cells and subjected to *in vitro* pull-down assays using GST–Spry4. Myc–TESK1 was pulled down with GST–Spry4 (Figure 1C), but not with control GST (results not shown). Similarly, both TESK1-N and -C were pulled down with GST–Spry4 (Figure 1C). Although the C-terminal fragment (amino acids 459–626) of TESK1 was originally identified as the Spry4-binding fragment in a yeast two-hybrid screen [16], TESK1-C bound to GST–Spry4 more weakly when compared with TESK1-N. Thus Spry4 interacts with TESK1 through both the N- and C-terminal regions of TESK1. In addition, a kinase-inactive mutant, TESK1(D170A), bound to GST–Spry4 (Figure 1C), indicating that the kinase activity of TESK1 is not necessary for Spry4 binding.

Tyrosine phosphorylation is not required for TESK1-binding activity of Spry4

Sprys contain a conserved tyrosine residue (Tyr-75 in human Spry4) in a short sequence motif, N(D/E)YX(D/E)XP, in the N-terminal region [22]. Phosphorylation of the corresponding tyrosine residue (Tyr-53 in mouse Spry4 or Tyr-55 in mouse Spry2) is required for the inhibitor activity of Sprys on FGF-induced MAP kinase activation [26,29]. To determine whether phosphorylation of this tyrosine residue is related to the TESK1-binding activity of Spry4, we constructed a human Spry4(Y75A) mutant, in which Tyr-75 was replaced by an alanine residue. Similar to the cases of other Sprys [26,29], expression of human

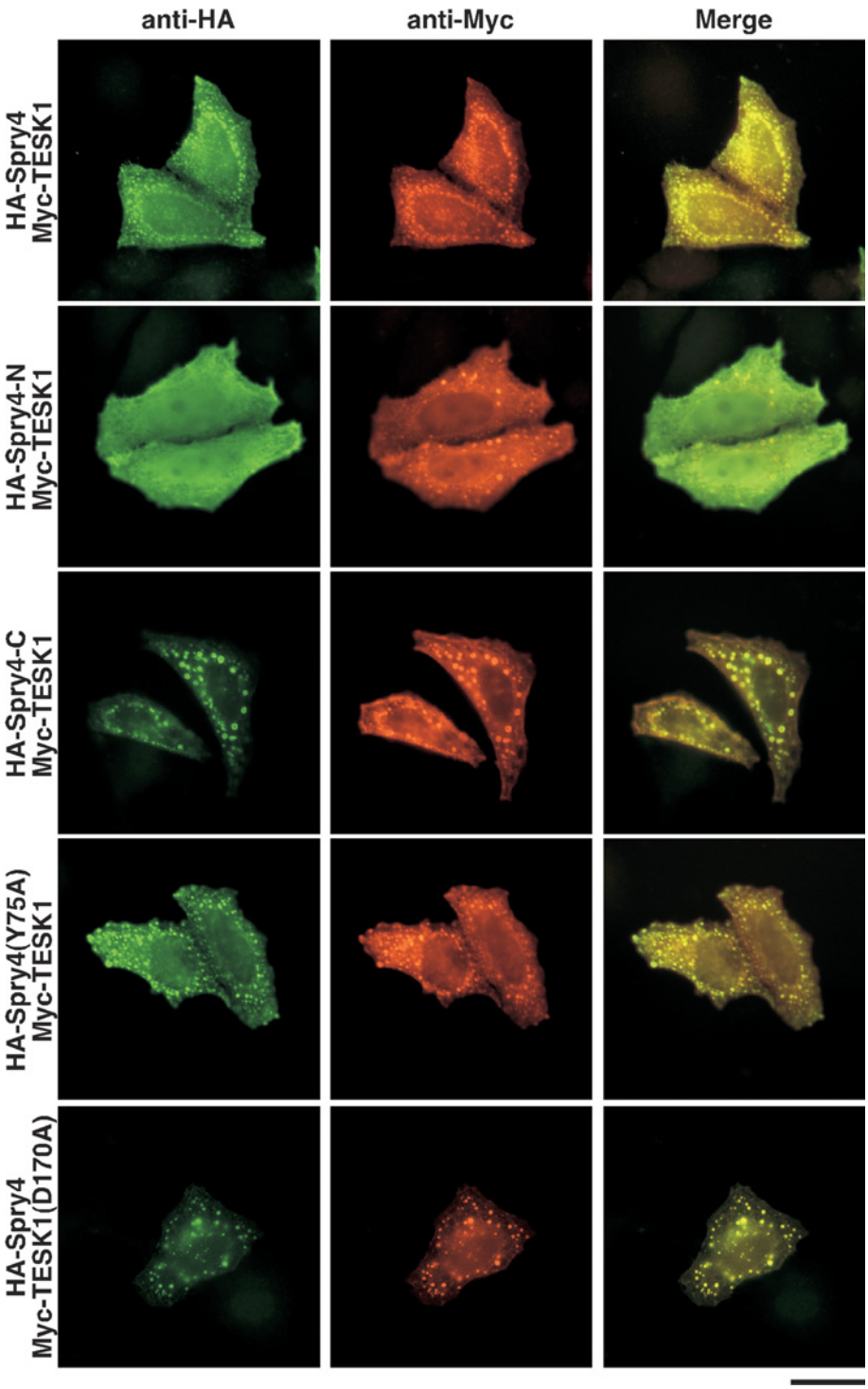

Figure 3 Co-localization of TESK1 and Spry4 in HeLa cells

HeLa cells were co-transfected with plasmids for HA–Spry4, Myc–TESK1 or their mutants, as indicated. Cells were fixed and co-immunostained with anti-HA (green) and anti-Myc (red) antibodies. In merged images, co-localization appears in yellow. Scale bar, 20 μm.

Spry4 suppressed FGF-induced ERK1/2 MAP kinase activation, as measured by phospho-ERK immunoblotting, but Spry4(Y75A) had the opposite effect, enhancing ERK activation (Figure 2A). When Myc–TESK1 and HA–Spry4(Y75A) were co-expressed in COS-7 cells, HA–Spry4(Y75A) was co-precipitated with Myc–TESK1, to an extent similar to that of HA–Spry4 (Figure 2B), thus indicating that phosphorylation of Tyr-75 is not required for the TESK1-binding activity of Spry4.

Co-localization of TESK1 and Spry4 in cultured cells

To determine whether TESK1 and Spry4 co-localize in cultured cells, Myc–TESK1 and HA–Spry4 were co-expressed in HeLa

cells and co-stained with anti-Myc and anti-HA antibodies. As shown in Figure 3, TESK1 co-localized with Spry4 on vesicular structures in the cytoplasm of HeLa cells. We also examined the subcellular localization of Spry4 mutants. When HA–Spry4 mutants were co-expressed with Myc–TESK1 in HeLa cells, Spry4-C and Spry4(Y75A), similar to wild-type Spry4, co-localized with Myc–TESK1 in vesicular spots in the cytoplasm (Figure 3). In contrast, Spry4-N was diffusely distributed in the cytoplasm and did not co-localize with the vesicular distribution of Myc–TESK1 (Figure 3). These observations agree with the results of binding assays shown in Figure 1 and suggest that TESK1 interacts with Spry4 through the C-terminal cysteine-rich region in cultured cells and that Tyr-75 phosphorylation is not necessary for this interaction. In addition, TESK1(D170A) co-localized with Spry4 in HeLa cells (Figure 3), again indicating that the kinase activity of TESK1 is not needed for the interaction with Spry4.

Spry4 inhibits the kinase activity of TESK1

We next examined the effect of Spry4 on the kinase activity of TESK1. Myc–TESK1 expressed in COS-7 cells was immunoprecipitated with anti-Myc antibody and subjected to an *in vitro* kinase assay, using recombinant His_6–cofilin as a substrate, in the presence or absence of GST–Spry4 or control GST. As shown in Figure 4(A), the kinase activity of TESK1 was inhibited by GST–Spry4 in a dose-dependent manner, but not by GST. We also tested the effects of Spry4 mutants on the kinase activity of TESK1. Similar to wild-type Spry4, Spry4-C and Spry4(Y75A) suppressed the kinase activity of TESK1, yet Spry4-N did not do so (Figure 4B). These findings suggest that Spry4 inhibits the kinase activity of TESK1 by directly associating with it through the C-terminal region of Spry4 and that Tyr-75 phosphorylation is not required for Spry4 to inhibit TESK1 activity.

Spry4 suppresses cell spreading

TESK1 is involved in integrin-mediated cell spreading [10,12]. Since the kinase activity of TESK1 is inhibited by Spry4, we assumed that the expression of Spry4 may affect integrin-mediated cell spreading. C2C12 cells were expressed with YFP–actin together with CFP–Spry4 or control CFP, then suspended and replated on laminin-coated coverglasses. Time-lapse fluorescence analyses of YFP–actin for 30 min after replating revealed that CFP–Spry4-expressing cells spread more slowly, compared with the control CFP-expressing cells (Figure 5A). To quantify the levels of cell spreading, C2C12 cells transfected with YFP or YFP–Spry4 were suspended, replated on laminin, cultured for 30 min, then fixed and stained with rhodamine–phalloidin to visualize actin filaments. On the basis of the cell area, as measured by IPLab image software, cells were categorized into three classes: cell area $> 800\ \mu m^2$ (class 1, round cells weakly adhered on to the substrate), $800\ \mu m^2 >$ cell area $< 1600\ \mu m^2$ (class 2, weakly spread cells in the course of spreading) and cell area $> 1600\ \mu m^2$ (class 3, flat and well-spread cells with extended pseudopodia; Figure 5B). At 30 min after replating, the ratio of class 1 (round) cells increased and that of class 3 (well-spreading) cells decreased in YFP–Spry4-expressing cells, compared with those in control YFP-expressing cells (Figures 5C and 5D). These findings suggest that Spry4 has the potential to regulate negatively integrin-mediated cell spreading.

TESK1 rescues the inhibition of cell spreading by Spry4

We next determined whether expression of TESK1 could rescue the inhibitory effect of Spry4 on the spreading of cells on laminin. Before this experiment, we examined the effects of single expression of YFP-tagged wild-type TESK1 or kinase-dead TESK1(D170A) on the spreading of C2C12 cells on laminin. Similar to the case of HeLa cells cultured on fibronectin [10,12], expression of TESK1 in C2C12 cells cultured on laminin stimulated actin assembly and cell spreading, and the expression of TESK1(D170A) suppressed the spreading of cells to retain their round shapes (Figure 6A). The ratio of well-spreading (class 3) cells slightly increased in wild-type TESK1-expressing cells compared with control YFP-expressing cells (Figure 6B). In contrast, the ratio of class 3 cells significantly decreased and that of class 1 cells inversely increased in TESK1(D170A)-expressing cells (Figure 6B). These results further suggest that TESK1 plays a critical role for integrin-mediated cell spreading.

We next examined the effect of TESK1 expression on the inhibition of cell spreading induced by Spry4. Whereas the

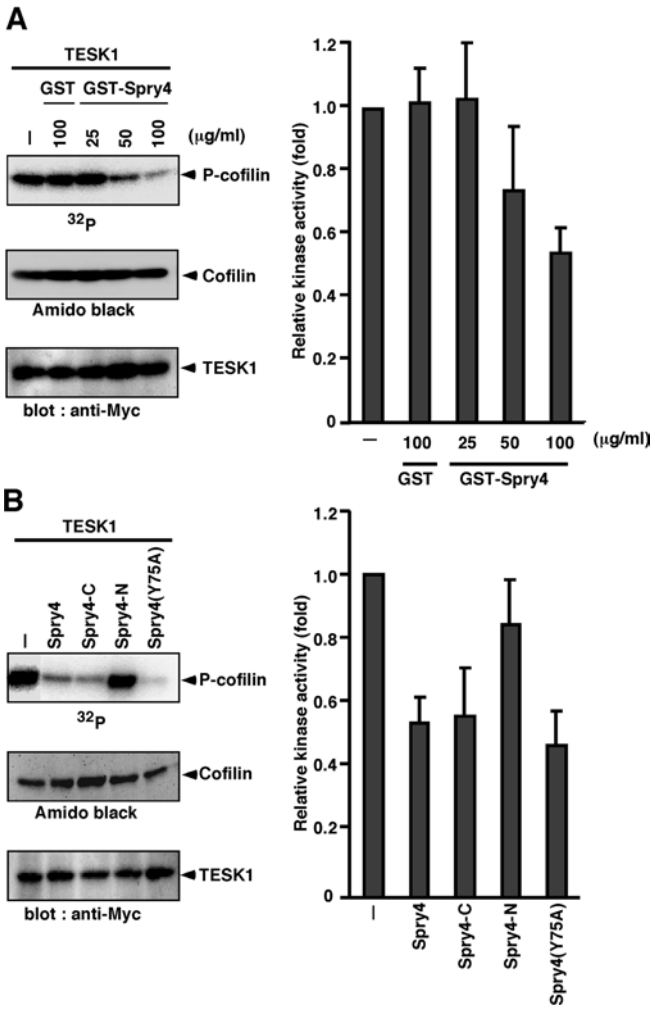

Figure 4 Spry4 inhibits the kinase activity of TESK1

Effects of GST, GST–Spry4 (**A**) or its mutants (**B**) on the kinase activity of TESK1. Myc–TESK1 expressed in COS-7 cells was immunoprecipitated with anti-Myc antibody, incubated in the absence (–) or presence of the indicated amounts of GST or GST–Spry4 (**A**) or 100 μg/ml GST–Spry4 or its mutants (**B**) and then subjected to *in vitro* kinase assay, using His_6–cofilin as a substrate. (**A**, **B**) Reaction mixtures were separated by SDS/PAGE and cofilin was analysed by autoradiography (top panels) and Amido Black staining (middle panels). Myc–TESK1 was analysed by immunoblotting with anti-Myc antibody (bottom panels). The right panels show the relative kinase activities of TESK1 expressed as means ± S.E.M. for triplicate experiments, with the activity of TESK1 in the absence of GST protein taken as 1.0.

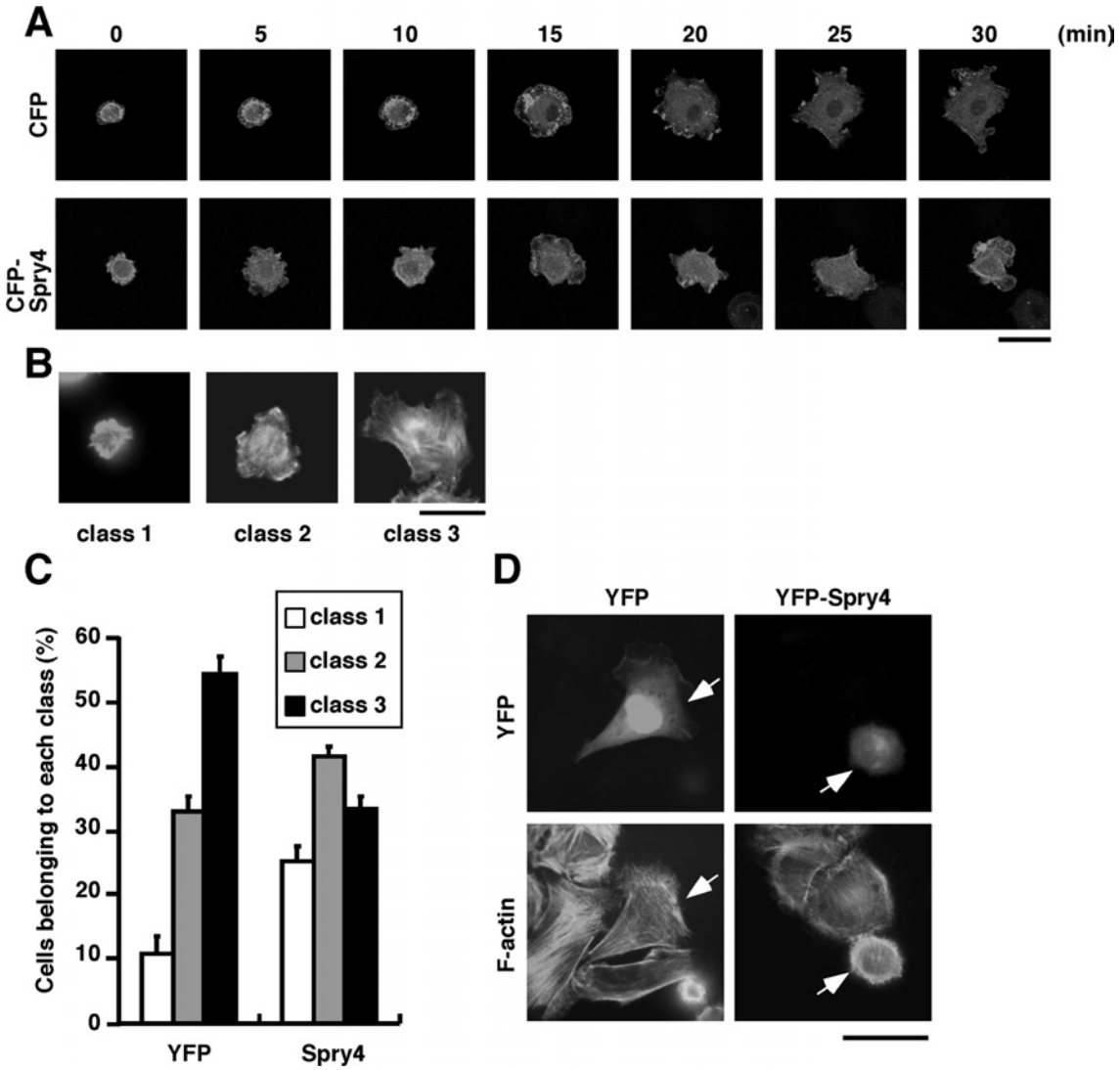

Figure 5 Spry4 suppresses cell spreading

(**A**) Time-lapse analyses of the spreading of C2C12 cells after replating on laminin. Cells transfected with CFP or CFP–Spry4 together with YFP–actin were cultured for 18 h, suspended and replated on laminin-coated coverglasses. Cells were analysed by time-lapse fluorescence microscopy, making use of YFP fluorescence. Scale bar, 40 μm. (**B**) Three categories of spreading cells. C2C12 cells transfected with YFP or YFP–Spry4 were cultured and replated on laminin-coated coverglasses. After incubation for 30 min, cells were fixed and stained with rhodamine–phalloidin for F-actin. On the basis of the area of spreading cells, cells were categorized into three classes; cell area $< 800\ \mu m^2$ (class 1), $800\ \mu m^2 <$ cell area $< 1600\ \mu m^2$ (class 2) and cell area $> 1600\ \mu m^2$ (class 3). Scale bar, 40 μm. (**C**) Quantitative analysis of the effects of Spry4 expression on the spreading of C2C12 cells. Cells transfected with YFP or YFP–Spry4 were cultured for 18 h, suspended and replated on laminin-coated coverglasses. After incubation for 30 min, cells were fixed and stained with rhodamine–phalloidin. Cells were classified into three categories and percentages of these cells in YFP-positive cells (at least 200 cells) were calculated. The results are the means $\pm$ S.E.M. for triplicate experiments. (**D**) Cell spreading morphologies of YFP- or YFP–Spry4-expressing cells. C2C12 cells transfected with YFP or YFP–Spry4 were replated on laminin and cultured for 30 min. Cells were fixed and analysed by YFP fluorescence (upper panels) and staining with rhodamine–phalloidin for F-actin (lower panels). Arrows indicate the YFP-positive cells. Scale bar, 40 μm.

expression of YFP–Spry4 with control CFP resulted in the inhibition of cell spreading, co-expression of YFP–Spry4 with CFP–TESK1 significantly increased the ratio of well-spreading (class 3) cells (Figure 6C). Quantitative analysis revealed that the ratio of class 3 cells was decreased and that of class 1 cells increased in cells expressing YFP–Spry4 and control CFP, but they were almost completely recovered in cells co-expressing YFP–Spry4 and CFP–TESK1, to the levels of those of control cells expressing CFP alone, when the plasmids for TESK1 and Spry4 were transfected in the ratio 9:1 (Figure 6D). Expression levels of YFP–Spry4, CFP and CFP–TESK1 proteins were analysed by immunoblotting with anti-GFP antibody (Figure 6E), which suggests that expression of TESK1 in excess of Spry4 is required to recover fully the level of cell spreading. These findings strongly suggest that Spry4 suppresses cell spreading by inhibiting the kinase activity of TESK1.

Effects of expression of Spry4 mutants and treatment with PD98059 on cell spreading

We also examined the effects of expression of Spry4 mutants on cell spreading. Similar to the case of wild-type Spry4, expression of YFP–Spry4-C or YFP–Spry4(Y75A) suppressed the spreading of C2C12 cells at 30 min after replating on laminin-coated dishes (Figure 7A); in these cells, the ratio of class 1 cells significantly increased and the ratio of class 3 cells decreased (Figure 7B). In contrast, expression of YFP–Spry4-N, which was unable to bind to and inhibit TESK1, had no significant effect on the

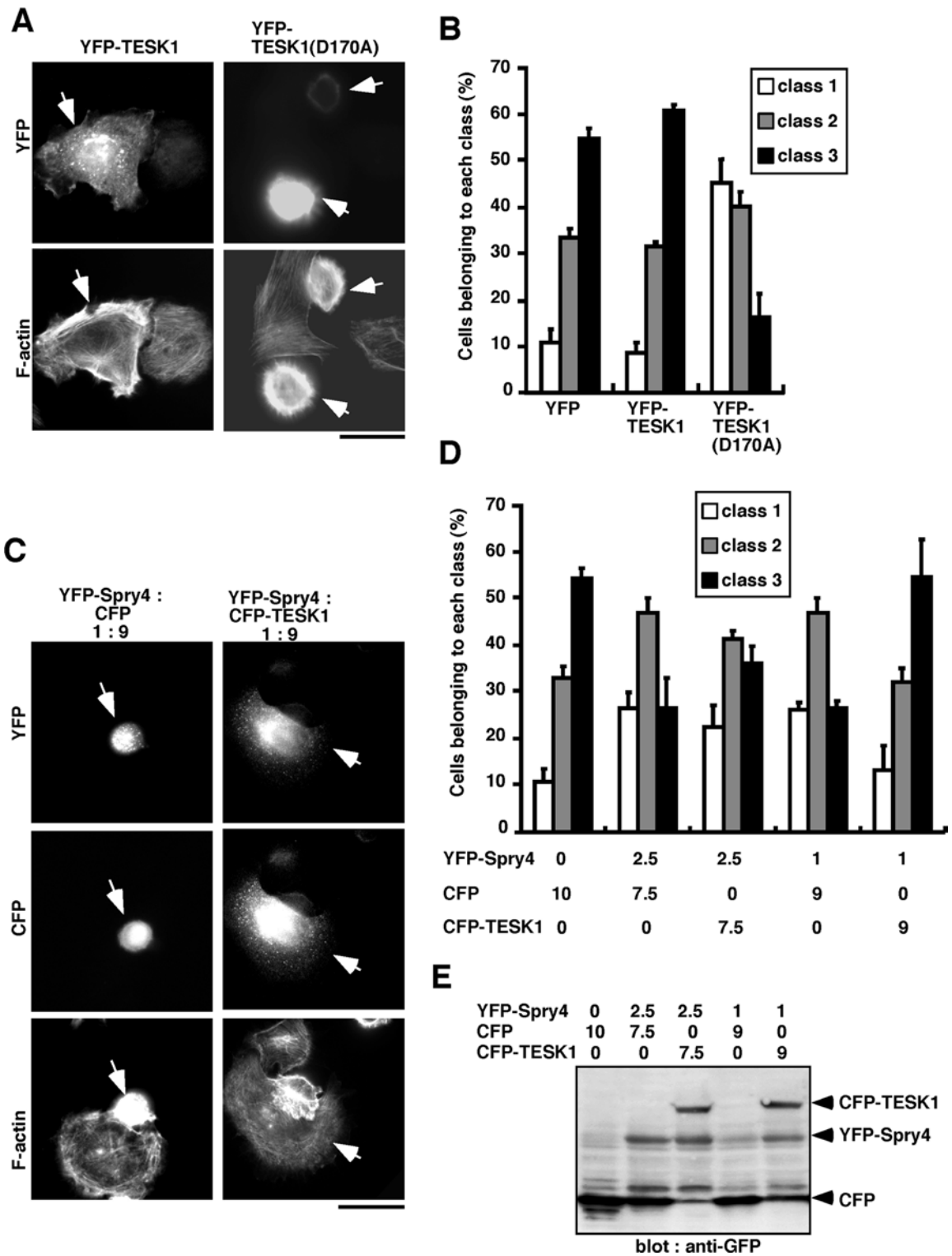

Figure 6 TESK1 rescues the inhibitory effect of Spry4 on cell spreading

(**A**) C2C12 cells were transfected with YFP–TESK1 or YFP–TESK1(D170A). Cells were cultured for 18 h, suspended and replated on laminin-coated coverglasses. After 30 min, cells were fixed and analysed by YFP fluorescence (upper panels) and rhodamine–phalloidin staining for F-actin (lower panels). Arrows indicate YFP-positive cells. Scale bar, 40 μm. (**B**) Quantitative analysis of the effects of expression of TESK1 and TESK1(D170A) on the spreading of C2C12 cells. Percentages of the cells classified into the three categories (shown in Figure 5B) in YFP-positive cells (at least 100 cells) were calculated. The results are the means $\pm$ S.E.M. for triplicate experiments. (**C**) C2C12 cells were co-transfected with YFP–Spry4 plus CFP or CFP–TESK1, as indicated. Cells were cultured and replated on laminin-coated coverglasses, as in (**A**). After 30 min, cells were fixed and analysed by YFP (top panels) and CFP fluorescence (middle panels) and rhodamine–phalloidin staining (bottom panels). Arrows indicate YFP- and CFP-positive cells. Scale bar, 40 μm. (**D**) Quantitative analysis of the effects of co-transfection with YFP–Spry4 plasmid plus CFP or CFP–TESK1 plasmid at the indicated ratio on the spreading of C2C12 cells. Percentages of the cells classified into the three categories are expressed as the means $\pm$ S.E.M. for triplicate experiments. (**E**) Immunoblot analysis of the expression of CFP, CFP–TESK1 and YFP–Spry4. Lysates of C2C12 cells transfected with CFP, CFP–TESK1 and YFP–Spry4 plasmids, as in (**D**), were analysed by immunoblotting with anti-GFP antibody.

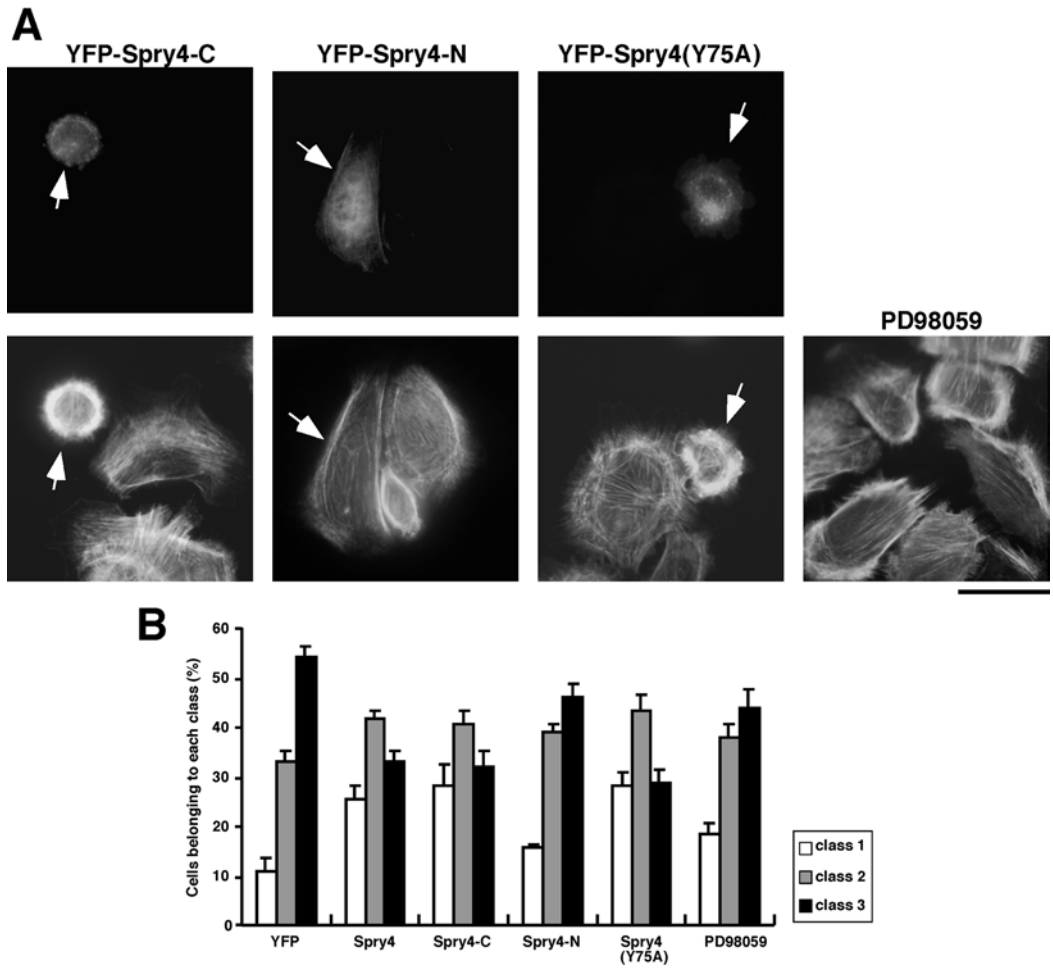

Figure 7 Effects of expression of Spry4 mutants and treatment with PD98058 on cell spreading

(**A**) C2C12 cells were transfected with YFP–Spry4 mutants or pretreated with 20 μM PD98059 for 20 min and then replated on laminin-coated dishes. After 30 min, cells were fixed and stained with rhodamine–phalloidin for F-actin. Arrows indicate cells expressing YFP-fusion proteins. Scale bar, 40 μm. (**B**) Quantitative analysis of the effects of expression of Spry4 mutants or treatment with PD98059 on the spreading of C2C12 cells. Percentages of the cells classified into the three categories (shown in Figure 5B) in YFP-positive cells (at least 100 cells) were calculated. The results are the means $\pm$ S.E.M. for triplicate experiments.

level of cell spreading (Figures 7A and 7B). These results suggest that Spry4 inhibits cell spreading by inactivating TESK1 through the C-terminal cysteine-rich region and that Tyr-75 phosphorylation is not involved in the inhibitory activity of Spry4 on cell spreading.

To determine further if the inhibitory activity of Spry4 on cell spreading is related to its inhibitory activity on MAP kinase signalling, we tested the effect of PD98059, a specific inhibitor of MEK (MAP kinase kinase/ERK kinase) that is an upstream protein kinase for ERK activation, on cell spreading. Treatment of C2C12 cells with 20 μM PD98059 had no significant effect on the spreading of C2C12 cells plated on laminin (Figures 7A and 7B). These findings suggest that MAP kinase activation is not essential for C2C12 cell spreading on laminin and the inhibition of cell spreading by Spry4 is caused by a mechanism independent of its inhibitory activity on the ERK activation pathway.

Spry4 inhibits the increase in the P-cofilin level during cell spreading

We reported previously that the kinase activity of TESK1 and the level of P-cofilin increased during cell spreading after plating on fibronectin [10]. Ectopic expression of TESK1 enhanced the increase in level of P-cofilin during cell spreading, whereas expression of a kinase-negative TESK1(D170A) mutant suppressed it [10]. These findings suggest that TESK1 plays a critical role in integrin-mediated cofilin phosphorylation during cell spreading. If Spry4 inhibits TESK1 activity during cell spreading, expression of Spry4 is expected to suppress the increase in P-cofilin level during cell spreading. To address this issue, we examined changes in the level of P-cofilin in Spry4-expressing cells after plating on laminin. C2C12 cells transfected with HA–Spry4 or control vector were plated on laminin for 30–60 min and then analysed for the level of P-cofilin by immunoblotting with an anti-P-cofilin antibody. In control cells mock-transfected with vector plasmid, the level of P-cofilin slightly increased at 30–60 min after plating on laminin (Figure 8A). In contrast, the level of P-cofilin in HA–Spry4-expressing cells remained unchanged after plating cells on laminin (Figure 8A). When C2C12 cells were transfected with Myc–TESK1, the basal level of P-cofilin increased at zero time and the level increased further at 30–60 min after plating on laminin (Figure 8B). In cells co-expressing HA–Spry4 with Myc–TESK1, the level of P-cofilin reverted to the basal level at zero time and did not change even after plating the cells on laminin

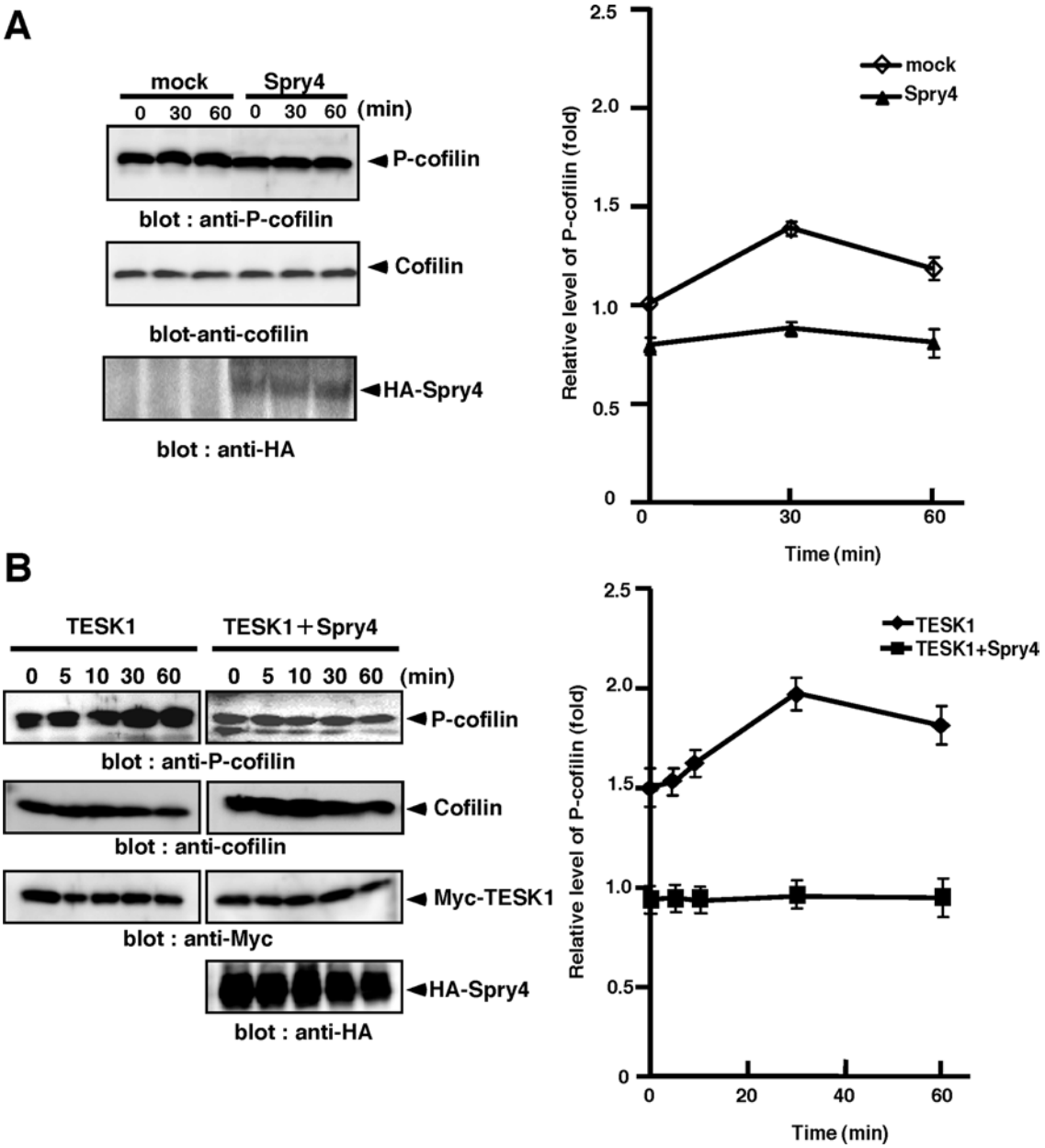

Figure 8 Spry4 inhibits integrin- and TESK1-mediated cofilin phosphorylation

(**A**) C2C12 cells transfected with HA–Spry4 or control vector were suspended and replated on laminin-coated dishes. At the indicated times, cells were lysed and the lysates were analysed by immunoblotting with anti-P-cofilin and anti-cofilin antibodies. Expression of HA–Spry4 was analysed by immunoblotting with anti-HA antibody. In the right panel, the relative amounts of P-cofilin in C2C12 cells are plotted against the time after plating cells on laminin. The results are the means ± S.E.M. for triplicate experiments, with the amount of P-cofilin in mock-transfected cells at zero time of plating taken as 1.0. (**B**) C2C12 cells transfected with Myc–TESK1 with or without HA–Spry4 were suspended and replated on laminin-coated dishes. At the indicated times, cells were lysed and the lysates were analysed as in (**A**). Expression of Myc–TESK1 was analysed by immunoblotting with anti-Myc antibody. In the right panel, the relative amounts of P-cofilin in C2C12 cells are plotted against the time after plating cells on laminin, as in (**A**).

(Figure 8B). These observations suggest that Spry4 has the potential to suppress the integrin-mediated and TESK1-stimulated increase in the P-cofilin level during cell spreading.

DISCUSSION

Previous studies have shown that Sprys are negative regulators for growth factor-induced RTK signalling by inhibiting signalling pathways leading to Ras and MAP kinase activation [18–30]. In the present study, we provided evidence for a novel cellular function of Spry4 that negatively regulates TESK1 activity and integrin-mediated cell spreading. Overexpression of Spry4, similar to that of a kinase-inactive TESK1 mutant [10,12], significantly suppressed integrin-mediated cell spreading, and TESK1 reverted Spry4-induced inhibition of cell spreading. We also showed that Spry4 inhibits integrin-mediated and TESK1-stimulated cofilin phosphorylation during cell spreading on laminin. Together with the finding that Spry4 binds to TESK1 and inhibits its kinase activity *in vitro*, these observations suggest that Spry4 inhibits cell spreading by directly repressing TESK1 activity in cultured cells.

Phosphorylation of the conserved tyrosine residue in the N-terminal region of Spry is essential for its inhibitory activity on MAP kinase signalling [26,29]. A non-phosphorylatable mutant, Spry4(Y75A), failed to inhibit, or rather enhanced, FGF-induced ERK activation, but it did inhibit the kinase activity of TESK1 *in vitro* and integrin-mediated cell spreading on laminin to a similar extent as wild-type Spry4, which suggests that tyrosine phosphorylation is not required for the inhibitory activity of Spry4 on cell spreading. Furthermore, treatment of cells with the MEK inhibitor PD98059 had no apparent effect on cell spreading. These findings strongly suggest that Spry4 inhibits cell spreading through a mechanism distinct from that for the inhibition of the MAP kinase pathway. Thus Spry seems to be an inhibitor of at least two distinct cell-signalling pathways, an integrin-mediated actin reorganization pathway for inhibiting TESK1 and an RTK-mediated MAP kinase activation pathway.

Spry was originally identified to be an inhibitor of FGF-induced branching and sprouting morphogenesis in the *Drosophila* tracheal system [17]. Subsequent studies on vertebrate Sprys revealed that they similarly inhibit branching morphogenesis in lung and blood vessels (angiogenesis) during development [23,31], which indicates that Sprys have the conserved function to regulate negatively tubular branching morphogenesis. At the cellular level, Sprys have the potential to inhibit growth factor-induced cell proliferation and migration [23–25], both of which are essential for tubular formation. Whereas the inhibitory action of Spry on cell proliferation is supposed to be based on its inhibitory activity on a Ras/MAP kinase signalling pathway, the mechanism of anti-migratory action of Spry is not clear. Yigzaw et al. [32] reported that the increase in the amount of soluble PTP-1B (protein tyrosine phosphatase-1B) contributes to the anti-migratory, but not anti-mitogenic action of mouse Spry2, although the mechanism by which PTP-1B is rendered soluble by Spry2 and how the increase in soluble PTP-1B suppresses cell migration are still not known [32]. In the present study, we provided evidence that Spry4 inhibits cell spreading by suppressing the kinase activity of TESK1. Since TESK1 phosphorylates cofilin and inhibits actin-depolymerizing and -severing activities of cofilin [10], Spry4 probably regulates the actin cytoskeletal reorganization by modulating the level of cofilin activity through TESK1 inactivation. We previously showed that cofilin phosphorylation by LIM kinase is required for chemokine-induced T-cell migration [33]. Cofilin phosphorylation seems to contribute to cell spreading and migration by establishing and stabilizing actin filaments at the leading edge of spreading and migrating cells. Thus we propose that suppression of cofilin phosphorylation through TESK1 inactivation is at least one of the important mechanisms for the anti-migratory action of Spry.

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