Binding of 14-3-3β Regulates the Kinase Activity and Subcellular Localization of Testicular Protein Kinase 1*

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Testicular protein kinase 1 (TESK1) is a serine/threonine kinase that phosphorylates cofilin and induces actin cytoskeletal reorganization. The kinase activity of TESK1 is stimulated by integrin-mediated signaling pathways, but the mechanism of regulation has remained unknown. By using the yeast two-hybrid system, we identified 14-3-3β to be the binding protein of TESK1. Specific interaction between TESK1 and 14-3-3β became evident in in vitro and in vivo co-precipitation assays. 14-3-3β interacts with TESK1 through the C-terminal region of TESK1 and in a manner dependent on the phosphorylation of Ser-439 within an RXXSXP motif. Binding of 14-3-3β inhibited the kinase activity of TESK1. During cell spreading on fibronectin, the TESK1/14-3-3β interaction significantly decreased, in a time course that inversely correlated with increase in TESK1 kinase activity. Thus, the dissociation of 14-3-3β from a TESK1/14-3-3β complex is likely to be involved in the integrin-mediated TESK1 activation. In HeLa cells, TESK1, together with 14-3-3β, accumulated at the cell periphery when cells were plated on fibronectin, whereas they were diffusely distributed in the cytoplasm in the case of non-stimulated cells. We propose that 14-3-3β plays important roles in regulating the kinase activity of TESK1 and localizing TESK1 to cell adhesion sites following integrin stimulation.

Actin cytoskeletal reorganization is essential for various cell activities, including adhesion, motility, morphological change, secretion, and cytokinesis. Cofilin and actin-depolymerizing factor (ADF) are actin-binding proteins that play a central role in regulating actin filament dynamics by depolymerizing and severing actin filaments (1, 2). The activity of cofilin/ADF is abrogated by phosphorylation on Ser-3 (1, 2). LIM motif-containing protein kinases 1 and 2 (3, 4) have the potential to phosphorylate cofilin/ADF specifically at Ser-3 in vitro and in vivo, thereby playing an important role in the actin cytoskeletal reorganization (5, 6). Testicular protein kinase 1 (TESK1) is a serine/threonine kinase, the structure of which is composed of an N-terminal protein kinase domain and a C-terminal noncatalytic region (7). The protein kinase domain of TESK1 is closely related to those of LIM kinases, but their overall domain structures differ (7). Testicular protein kinase 2 (TESK2) with a structure similar to TESK1 has also been identified (8, 9). TESK1 is expressed in various tissues and cell lines, with a particularly high expression in the testis (7, 10–12). Similar to LIM kinases, TESK1 can phosphorylate cofilin/ADF specifically at Ser-3 in vitro and in vivo (12). Formation of actin stress fibers and focal adhesions is induced after TESK1 overexpression (12). However, upstream signaling pathways regulating LIM kinases and TESK1 significantly differ; the kinase activities of LIM kinases are stimulated by Rho family small GTPases, Rho, Rac, and Cdc42, through the actions of downstream protein kinases, ROCK, Rho-associated, coiled-coil-forming protein kinase, and p21-activated kinase (5, 6, 13–18), whereas the kinase activity of TESK1 is not stimulated by either Rho-associated, coiled-coil-forming protein kinase or p21-activated protein kinase but can be stimulated by plating cells on fibronectin (12). Thus, phosphorylation of cofilin/ADF in living cells is regulated by at least two distinct types of protein kinases, LIM kinases and TESK1, of which TESK1 appears to have a role in integrin-mediated actin cytoskeletal reorganization. However, the mechanisms regulating the activity and subcellular localization of TESK1 remain to be elucidated.

14-3-3 proteins, originally named for their migration positions in two-dimensional gel electrophoresis of proteins from brain extracts, consist of several highly conserved proteins that form dimers (19–21). These proteins interact with diverse signaling proteins, such as Raf-1 kinase, protein kinase C, phosphatidylinositol 3-kinase, Cdc25 phosphatase, and an apoptosis-promoting protein BAD, and are implicated in a variety of cellular functions, including cell proliferation, cell cycle progression, differentiation, and apoptosis (19–21). As recent studies revealed that 14-3-3 proteins interact with the cytoplasmic domain of integrin β1 and a focal adhesion protein p130CAS, the potential role of 14-3-3 proteins in regulating integrin-mediated cell adhesion, spreading, migration, and related signaling events had to be considered (22, 23). 14-3-3 proteins are considered to function by binding target proteins and modulating their activity, stability, and/or subcellular localization (19–21). These proteins also function as dimeric adaptors by connecting two proteins together to produce signaling protein complexes (24). 14-3-3 proteins can interact with target proteins via serine phosphorylation-dependent and -independent mechanisms (25–28). In the former case, 14-3-3 proteins specifically recognize the phosphoserine-containing short peptide sequence motifs present in target proteins (25–27).

We searched for proteins that interact with TESK1, using the yeast two-hybrid system, and we identified 14-3-3β, one of the members of a 14-3-3 protein family, as the TESK1-binding
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protein. 14-3-3β specifically interacts with TESK1 in a manner dependent on the phosphorylation of Ser-439 within the C-terminal region of TESK1. We also observed the effects of binding of 14-3-3β on the kinase activity and the subcellular localization of TESK1. Our findings suggest that 14-3-3β plays an important role in regulating the functions of TESK1 in integrin-mediated signaling pathways.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Analysis—Yeast two-hybrid screening and assays were performed according to the manufacturer’s instructions for the Prohormone Cleaving Enzyme System (Life Technologies Inc.) (12). Oligonucleotides and proteins that interact with TESK1, yeast MaV203 cells were sequentially transformed with pBluescript-TK1-1 plasmid coding for the C-terminal region of rat TESK1 (TK1-1, amino acids 288–626) fused in-frame to the DNA binding domain of GAL4, and then with a HeLa cell cDNA library constructed in pPC86 vector that codes for the activation domain of GAL4 (29). To test for transformation frequency, a small portion of the transformation mixture was plated on the medium lacking leucine and tryptophan. For screening, transformants were plated on the synthetic medium lacking leucine, tryptophan, and histidine and containing 25 mM 3-aminotriazole and then were incubated for 60 h at 30°C. After replica cleaning, plates were further incubated for 3 days, and growing colonies were selected as primary positives and further tested in three steps, using histidinol and uracil prototrophies. β-galactosidase activity, as described in the manufacturer’s instruction manual. pPC86 plasmids recovered from colonies positive in histidine prototrophy and either or both of uracil prototrophs and β-galactosidase activity were retransformed into MaV203 cells with either pBluescript-TK1-1 or pBluescript vector to assess specific protein interactions. Nucleotide sequences of the cDNA inserts in pPC86 plasmids were determined using 373A DNA sequencing (PE Biosystems, Tokyo, Japan) and analyzed using BLAST search. For binding assays, MaV203 cells were co-transformed with a pBluescript plasmid encoding the deletion mutants of TESK1 or TESK2 fused in-frame with the GAL4 DNA binding domain and a pPC86 plasmid encoding 14-3-3β protein fused in-frame with GAL4 activation domain. Transformants were streaked on the plate lacking leucine, tryptophan, and histidine and containing 25 mM 3-aminotriazole and incubated as above.

Plasmid Construction—pBluescript-TK1-1 was generated by inserting a PstI-EcoRI fragment of rat TESK1 cDNA (nucleotide (nt) residues 1994–3600) (7) into the NotI sites of pBluescript vector. pBluescript-TK1-2 was generated by inserting an EcoO109I fragment of TESK1 cDNA (nt 2147–2495) into the NotI sites of the pBluescript vector. pBluescript-TK1-3 was generated by inserting an ApaI-EcoRI fragment of TESK1 cDNA (nt 2516–3600) into SalI and StuI sites of the pBluescript vector. pBluescript-TK1-4 was generated by inserting a NotI-EcoRI fragment of TESK1 cDNA (nt 1129–2050) into NotI and Ncol sites of the pBluescript vector. pBluescript-TK1-2 was generated by inserting the PCR-amplified fragment of rat TESK2 cDNA (nt 1249–2067) (9) into the NotI site of the pBluescript vector. pBluescript-TK1-3 was generated by inserting PCR-amplified fragment of the N-terminally Myc epitope-tagged TESK1 and TESK1(D170A) mutant as described (12). Expression plasmids coding for Myc-tagged, C-terminally truncated mutants of TESK1 (Δ1, Δ2, Δ3, and Δ4) were constructed by inserting an Ncol-EcoRI fragment (nt 1129–2705), an Ncol-EcoT14I fragment (nt 1129–2386), an Ncol-NcoI fragment (nt 1129–2386), and an Ncol-EcoO109I fragment (nt 1129–2147) at the NotI site of pBluescript-TK1-1.

Purification of GST Fusion Proteins—GST fusion proteins were expressed in Escherichia coli and purified on a glutathione-Sepharose (Amersham Pharmacia Biotech), as described (35).

In Vitro Pull-down Assay—Cells were washed twice with ice-cold PBS, suspended in RIPA buffer, and incubated on ice for 30 min. After centrifugation, lysates were preclarified with glutathione-Sepharose (40 μl of 50% slurry) for 3 h at 4°C. The preclarified supernatants were incubated with anti-TESK1, anti-HA, or anti-Myc antibody, and protein A-Sepharose (20 μl of 50% slurry) overnight at 4°C. After centrifugation, immunoprecipitates were washed twice with wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) and used for immunoblot analysis or for in vitro kinase reaction.

Immunoblot Analysis—For immunoblot analysis, cell lysates or immunoprecipitated proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes. The membrane was blocked overnight with 5% nonfat dry milk in PBS containing 0.05% Tween 20, and incubated for 2 h at room temperature with the primary antibody diluted in PBS containing 1% nonfat dry milk and 0.05% Tween 20. After washing in PBS containing 0.05% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG (Amersham Pharmacia Biotech). The autoradiographic bands were visualized by using an ECL chemiluminescence reagent (Amersham Pharmacia Biotech).

Phosphotase Treatment—Lysates of COS-7 cells expressing Myc-TESK1 were immunoprecipitated with anti-Myc antibody. Immunoprecipitates were washed twice with phosphate reaction buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM MgCl2) and incubated for 30 min at 30°C in 50 μl of phosphate reaction buffer, with or without 30 units of calf intestinal alkaline phosphatase (Takara Shuzo, Tokyo, Japan). After washing, the protein complexes were eluted with 0.1% SDS for 5 min at 95°C, diluted 1:10 with RIPA buffer, and subjected to in vitro pull-down assay.

Purification of GST Fusion Proteins—GST fusion proteins were expressed in Escherichia coli and purified on a glutathione-Sepharose (Amersham Pharmacia Biotech), as described (35).

In Vitro Pull-down Assay—Lysates of HeLa or COS-7 cells expressing TESK1, Myc-TESK1, or Myc-TESK1(S439A) were immunoprecipitated with anti-TESK1 or 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 MnCl2, 5 mM MgCl2) and incubated for 30 min at 30°C in the kinase reaction buffer containing 0.1–100 μM purified GST-14-3-3β protein and 10 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech), and 50 μg His6-tagged cofilin. The reaction mixture was solubilized in Laemmli’s sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1 mM dithiothreitol, 1% SDS, 0.002% bromphenol blue) for 5 min at 95°C and subjected to SDS-PAGE. Proteins were transferred onto membranes and 32P incorporation into cofilin was visualized by autoradiography.
**RESULTS**

**Identification of 14-3-3β as the TESK1-interacting Protein**—To identify proteins that interact with TESK1, we used the yeast two-hybrid system. A HeLa cell cDNA library constructed in the GAL4 transactivation domain vector (pPC86) was screened using the yeast two-hybrid system. As shown in Fig. 1A, 14-3-3β interacted with TK1-2 (amino acid residues 339–455) but not with TK1-3 (residues 462–626) or TK1-4 (residues 1–306). These results suggest that 14-3-3β interacts with TESK1 via the region of amino acid residues 339–455. In this sequence, we noted three regions that were highly conserved between the sequences of TESK1 and TESK2 (termed conserved regions 1–3 (CR1, CR2, and CR3), Fig. 1A) (9). The specific interaction of 14-3-3β with the C-terminal region of rat TESK2 (TK2-1, residues 299–570) was also detected using the two-hybrid assay (Fig. 1B). These results suggest that 14-3-3β interacts with the C-terminal regions of TESK1 and TESK2, and their conserved regions, CR1 to CR3, are presumably involved in the interaction.

**Fig. 1. Interaction of 14-3-3β with the C-terminal region of TESK1 and TESK2 in the yeast two-hybrid system.** A, schematic diagrams of TESK1, TESK2, and their deletion constructs used in this assay and results of two-hybrid analysis. Amino acid residue numbers at boundaries of deletion constructs are indicated above the schematics. Black and gray boxes indicate the protein kinase (PK) domain and the highly conserved regions (CR1, CR2, and CR3) between the C-terminal regions of TESK1 and TESK2, respectively. Results of two-hybrid analysis are shown on the right. B, two-hybrid analysis. Yeast cells were co-transformed with a pDBLeu-based bait vector fused to the cDNA for TESK1 or TESK2 fragments and a pPC86-based prey vector fused to the cDNA for 14-3-3β. + indicates vector alone. Transformed cells were streaked on the plate lacking leucine, tryptophan, and histidine and containing 20 μM 3-aminotriazole. Yeast cells co-transformed with pDBLeu-TK1-1 and pPC86–14-3-3β were grown in selection medium, but not so for the cells co-transformed with pDBLeu-TK1-1 and pPC86 vector or the cells with pDBLeu vector and pPC86–14-3-3β (Fig. 1B), thus indicating the specific interaction between the C-terminal region of TESK1 and 14-3-3β.
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**Fig. 2. Interaction of 14-3-3β with TESK1 in cultured cells.** A, co-precipitation of 14-3-3β with TESK1. Full-length TESK1 cDNA and/or HA-tagged 14-3-3β cDNA were transfected into COS-7 cells, as indicated. Cell lysates were immunoprecipitated with anti-TESK1 antibody and immunoblotted with anti-HA antibody (top panel) or anti-TESK1 antibody (middle panel). Expression of HA-tagged 14-3-3β was analyzed by immunoprecipitation and immunoblotting with anti-HA antibody (bottom panel). B, co-precipitation of TESK1 and TESK1(D170A) with 14-3-3β. COS-7 cells were transfected with HA-tagged 14-3-3β cDNA and/or TESK1 or TESK1(D170A) cDNA. Cell lysates were immunoprecipitated with anti-HA antibody (top panel) or anti-HA antibody (middle panel). Expression of TESK1 and TESK1(D170A) was analyzed by immunoprecipitation and immunoblotting with anti-TESK1 antibody (bottom panel). IP, immunoprecipitation; blot, immunoblotting.

**Interaction between 14-3-3β and TESK1 in Mammalian Cells**—To determine whether 14-3-3β interacts with TESK1 in mammalian cells, we co-expressed HA epitope-tagged, full-length 14-3-3β (HA-14-3-3β) and full-length TESK1 in COS-7 cells. When lysates of COS-7 cells co-expressing HA-14-3-3β and TESK1 were immunoprecipitated with anti-TESK1 antibody, HA-14-3-3β was detected in the TESK1 immune complex (Fig. 2A, lane 3). Similarly, when lysates of COS-7 cells expressing HA-14-3-3β alone were immunoprecipitated with anti-TESK1 antibody, a faint band of HA-14-3-3β was detected in the TESK1 immune complex (Fig. 2A, lane 2); this band seems to be HA-14-3-3β which co-precipitated with endogenous TESK1. Conversely, when lysates of COS-7 cells co-expressing HA-14-3-3β with TESK1 were immunoprecipitated with anti-HA antibody, TESK1 was detected in the HA-14-3-3β immune complex (Fig. 2B, lane 3). In addition, a kinase-inactive mutant, TESK1(D170A), in which the presumptive catalytic residue Asp-170 is replaced by alanine, was also co-precipitated with HA-14-3-3β (Fig. 2B, lane 4), indicating that the kinase activity of TESK1 is not required for the interaction with 14-3-3β. In some experiments (Fig. 2B, lane 3), TESK1 protein was detected as a doublet. Based on the elution position on SDS-PAGE, the upper band may be a phosphorylated form of TESK1.

**In Vitro Binding of TESK1 to 14-3-3β**—We next examined whether TESK1 would directly bind to 14-3-3β, using in vitro pull-down assays. Expression plasmids coding for Myc-tagged full-length TESK1 and a series of C-terminally truncated TESK1 mutants, termed Δ1 to Δ4, were constructed and transfected into COS-7 cells (Fig. 3A). Immunoblot analysis of COS-7 cell lysates with anti-Myc antibody confirmed the expression of Myc-TESK1 and its truncated mutants with their expected molecular masses (Fig. 3B, left panel). Cell lysates were incubated with GST-14-3-3β fusion protein bound to glutathione-Sepharose beads, and the bound proteins were analyzed by immunoblots with anti-Myc antibody. The pull-down assay revealed that full-length TESK1 and Δ1 co-precipitated with GST-14-3-3β but Δ2, Δ3, and Δ4 mutants did not do so (Fig. 3B, right panel). No binding was detected between Myc-tagged TESK1 and control GST (data not shown). These results suggest that TESK1 interacts with 14-3-3β through the region of amino acid residues 421–526. Combined with the data on the yeast two-hybrid assay (Fig. 1), the region of amino acid residues 421–455 (including a CR3 region) of TESK1 is likely to be involved in the association with 14-3-3β.

**Interaction between TESK1 and 14-3-3β Depends on the Phosphorylation of Ser-439 in TESK1**—Previous studies (25) revealed that 14-3-3 proteins bind to several proteins via the specific sequence motif, RXX(S/K)X, in a manner dependent on the phosphorylation of the underlined serine residue. We found that TESK1 and TESK2 contain the corresponding sequences within the conserved region CR3; RCRSLP (residues 436–441) in TESK1 and RWRLSP (residues 453–458) in TESK2 (Fig. 4A). To determine whether the phosphorylation of Ser-439 in the CR3 region of TESK1 is necessary for binding to 14-3-3β, we constructed a Myc-tagged TESK1(S439A) mutant, in which Ser-439 is replaced by alanine (Fig. 4B). In vitro pull-down assay revealed that both wild-type TESK1 and a kinase-inactive D170A mutant bind to GST-14-3-3β but not so an S439A mutant (Fig. 4C), thus indicating that Ser-439 (probably phosphorylation of Ser-439) is essential for the interaction of TESK1 with 14-3-3β. To determine whether the interaction of TESK1 with 14-3-3β depends on the phosphorylation, TESK1 was treated with or without calf intestinal alkaline phosphatase and subjected to the in vitro pull-down assay with GST-14-3-3β. As shown in Fig. 4D, phosphatase treatment significantly decreased the amount of TESK1 bound to GST-14-3-3β, compared with the phosphatase-untreated control. To examine further the importance of Ser-439 phosphorylation, in vitro pull-down assay with GST-14-3-3β was done in the presence of excess amounts of the Ser-439-phosphorylated peptide (pS439-peptide, VRRCR(pS)LPSSPEL) corresponding to residues 434–446 of TESK1. As shown in Fig. 4E, pS439-peptide inhibited the binding of TESK1 with 14-3-3β, but the control non-phosphorylated peptide (S439-peptide, NRRCRSLPSSPEL) did not. Taken together these results suggest that TESK1 interacts with 14-3-3β in a manner dependent on the phosphorylation of Ser-439.

**14-3-3β Inhibits the Kinase Activity of TESK1**—Previous studies (19–21) indicated that the binding of 14-3-3 proteins alters the enzymatic activities of various target proteins. We therefore examined the effects of 14-3-3β binding on the kinase activity of TESK1. Lysates of COS-7 cells transfected with Myc-TESK1 cDNA were immunoprecipitated with anti-Myc antibody or control IgG and subjected to in vitro kinase assay, using recombinant coflin as a substrate. As shown in Fig. 5A, immunoprecipitates with anti-Myc antibody exhibited the kinase activity to phosphorylate coflin, but those with control IgG did not, which indicates the specificity of the kinase activity in anti-Myc immunoprecipitates. The kinase activity of...
Myc-TESK1 was significantly inhibited in the presence of excess amounts of GST-14-3-3β (Fig. 5B, left panel). In contrast, a faint decrease was observed in the kinase activity of a S439A mutant of TESK1 in the presence of GST-14-3-3β (Fig. 5B, right panel). The kinase activity of TESK1 was not affected in the presence of the similar doses of GST control protein (data not shown). These results suggest that 14-3-3β inhibits the kinase activity of TESK1 by binding to TESK1 via the sequence surrounding the phosphorylated Ser-439. The partial inhibition of the kinase activity of TESK1 is probably due to the existence of the Ser-439 non-phosphorylated form of TESK1 (that is unable to bind to 14-3-3β) in COS-7 cell lysates. It is likely that TESK1 is not sufficiently phosphorylated on Ser-439 because of its overexpression in COS-7 cells. Thus, we also examined the effects of 14-3-3β on the kinase activity of TESK1 from HeLa/TESK1 cells, in which TESK1 is stably expressed and the level of TESK1 expression is lower than that in COS-7 cells. As expected, a further decrease (about 60% inhibition) in the kinase activity was observed for TESK1 from HeLa/TESK1 cells (Fig. 5C).

Interaction between TESK1 and 14-3-3β Is Decreased after Integrin-mediated Cell Adhesion—We reported (12) that the kinase activity of TESK1 is enhanced by plating cells on fibronectin-coated dishes. We thus asked if cell adhesion onto fibronectin would affect interactions between TESK1 and 14-3-3β. HeLa/TESK1 cells stably expressing TESK1 were suspended, replated onto fibronectin- or poly-L-lysine-coated dishes, and cultured under serum-free conditions. At the indicated times, cells were lysed, and lysates were subjected to the in vitro pull-down assay with GST-14-3-3β. As shown in Fig. 6A, the interaction between TESK1 and 14-3-3β was significantly reduced 15–30 min after plating cells onto fibronectin and then partially reverted at 60 min. In contrast, the interaction was not affected when the cells were plated on poly-L-lysine-coated dishes (Fig. 6B). We also examined changes of the TESK1/14-3-3β interaction after cell adhesion on fibronectin by co-immunoprecipitation assays. Lysates of HeLa/TESK1 cells expressing HA-14-3-3β were analyzed by immunoprecipitation with anti-TESK1 antibody, followed by immunoblotting with anti-HA antibody. The amount of 14-3-3β co-precipitated with TESK1 was reduced after cell adhesion to fibronectin (Fig. 6C). These results suggest that the TESK1/14-3-3β interaction is negatively regulated by plating cells on fibronectin. Under the above conditions, the kinase activity of TESK1 was slightly increased after cell adhesion on fibronectin (Fig. 6D), which is consistent with the results we reported (12). Thus, the kinase activity of TESK1 inversely correlated with the level of interaction between TESK1 and 14-3-3β during cell adhesion and spreading on fibronectin. Together with the finding that binding of 14-3-3β inhibits the kinase activity of TESK1 (Fig. 5), these results suggest that the dissociation of TESK1 from 14-3-3β may be involved in the increase in the kinase activity of TESK1 after cell adhesion on fibronectin.

Subcellular Localization of TESK1 and 14-3-3β in Cells Plated on Fibronectin—To examine the subcellular localization of TESK1 and 14-3-3β, HeLa cells were co-transfected with the plasmids for Myc-TESK1 and HA-14-3-3β and analyzed by immunofluorescence staining with anti-Myc and anti-HA antibodies. In HeLa cells not stimulated with fibronectin, TESK1 and 14-3-3β were diffusely distributed in the cytoplasm (Fig. 7A). Dense staining of TESK1 was observed at the perinuclear region, as reported (12). It was reported (22, 23) that 14-3-3
proteins accumulate at the cell periphery during cell attachment to the extracellular matrix. Thus, we determined if TESK1 would co-localize with 14-3-3 in HeLa cells plated on fibronectin. When HeLa cells expressing HA-14-3-3β alone were suspended and replated on fibronectin-coated dishes, HA-14-3-3β accumulated at the periphery of cells during adhesion and spreading (Fig. 7B). When HeLa cells co-expressing Myc-TESK1 and HA-14-3-3β were plated on fibronectin, TESK1 co-localized with 14-3-3β at the periphery of cells (Fig. 7C, left panels). A kinase-inactive Myc-TESK1(D170A) also co-localized with HA-14-3-3β at the cell periphery, when these cells were cultured on fibronectin (Fig. 7C, middle panels). In contrast, when HeLa cells co-expressing Myc-TESK1(S439A) and HA-14-3-3β were plated on fibronectin, TESK1(S439A) localized diffusely in the cytoplasm, whereas 14-3-3β was concentrated at the cell periphery (Fig. 7C, right panels). Myc-TESK1 or TESK1(D170A), when expressed alone in HeLa cells, was distributed diffusely in the cytoplasm, and accumulation at the cell periphery was not significant even after plating cells on fibronectin, as reported (12). These results suggest that 14-3-3β plays an important role to localize TESK1 to the cell periphery when cells are stimulated by fibronectin, and the localization of TESK1 to the cell periphery depends on the Ser-439 phosphorylation of TESK1 and the binding to 14-3-3β.

**DISCUSSION**

By using the yeast two-hybrid system, we identified 14-3-3β to be a TESK1-binding protein. 14-3-3 proteins interact with diverse proteins related to cell signaling and regulation (19–21). These proteins associate with target proteins through two known mechanisms as follows: one dependent on the phosphorylation of the serine residue in target proteins (25–27), and the other independent of the phosphorylation (28). By using several deleted mutants of TESK1, we mapped the 14-3-3β-binding site in TESK1 to the C-terminal short sequence (amino acids 421–455) that contains the CR3 consensus region. The CR3 region of TESK1 contains the sequence CRCSLP (amino acids 436–441), which is consistent with the RXXYSP motif, one of the consensus sequence motifs for the phosphoserine-dependent 14-3-3-binding, where the phosphorylation of an underlined serine residue is critical (25). Accordingly, TESK1 appears to interact with 14-3-3β via the CR3 region by the serine phosphorylation-dependent mechanism. Indeed, an
Fig. 5. Binding of 14-3-3β inhibits the kinase activity of TESK1. A, specificity of the kinase activity of Myc-TESK1 in anti-Myc immunoprecipitates (IP). Lysates of COS-7 cells transfected with Myc-TESK1 cDNA were immunoprecipitated with anti-Myc antibody or control IgG and subjected to in vitro kinase reaction, using His6-cofilin as a substrate. Reaction mixtures were separated on SDS-PAGE and analyzed, using autoradiography (32P) and Amido Black staining for cofilin and immunoblotting with anti-Myc antibody for Myc-TESK1. B, effects of GST-14-3-3β on the kinase activity of Myc-TESK1 expressed in COS-7 cells. Myc-tagged wild-type (WT) TESK1 and TESK1(S439A) expressed in COS-7 cells were immunoprecipitated with anti-Myc antibody, incubated with excess amounts (50 and 100 µg/ml, which corresponds to about 50- and 100-fold the concentration of Myc-TESK1) of GST-14-3-3β, and then subjected to in vitro kinase reaction as in A. GST-14-3-3β in reaction mixtures was also analyzed by Amido Black staining. Relative kinase activity of TESK1 and TESK1(S439A) is shown in the bottom panels as means ± S.E. of triplicate experiments, with the activity of TESK1 and TESK1(S439A) in the absence of GST-14-3-3β taken as 1.0. C, effects of GST-14-3-3β on the kinase activity of TESK1 from HeLa/TESK1 cells. Lysates of HeLa/TESK1 cells were immunoprecipitated with anti-TESK1 antibody, incubated with excess amounts of GST-14-3-3β, and then subjected to in vitro kinase reaction as in B. Relative kinase activity of TESK1 is shown in the bottom panel as means ± S.E. of triplicate experiments, with the activity of TESK1 in the absence of GST-14-3-3β taken as 1.0.

S439A mutant of TESK1 in which Ser-439 is replaced by a non-phosphorylatable alanine failed to bind to 14-3-3β, and treatment of wild-type TESK1 with alkaline phosphatase significantly reduced the amount of TESK1 that bound to 14-3-3β. Furthermore, the interaction was competed by the peptide containing a phosphorylated Ser-439 but not by the control non-phosphorylated peptide. We therefore propose that TESK1 interacts with 14-3-3β via the CR3 region in a manner dependent on the phosphorylation of Ser-439. The interaction of 14-3-3β with the C-terminal region of TESK2 was also detected in the yeast two-hybrid analysis. As the CR3 region of TESK2 contains the sequence RWRSILP (amino acids 453–458) (9) that also matches an RXXXP motif, TESK2 probably interacts with 14-3-3β via the CR3 region, an event that depends on the phosphorylation of Ser-456 of TESK2.

The requirement of the serine phosphorylation for interaction of TESK1 with 14-3-3β indicates that protein kinase(s) and phosphatase(s), which specifically phosphorylate and dephosphorylate Ser-439 of TESK1, play critical roles in the regulation of the TESK1/14-3-3β interaction. Similar to wild-type TESK1, a kinase-inactive D170A mutant of TESK1 has the potential to interact with 14-3-3β and co-localizes with 14-3-3β in fibronectin-stimulated cells. Thus, the autophosphorylation activity of TESK1 is not involved in the TESK1/14-3-3β interaction. Several protein kinases have been identified that can phosphorylate the serine residue participating in the 14-3-3 binding in target proteins as follows: Chk1 and Cds1 for phosphorylation of Cdc25 (34), protein kinase A for NFAT (a nuclear factor of activated T cells) (35), and Akt for BAD (an apoptosis-promoting protein) and FKHRL1 (a forkhead-type transcription factor) (36, 37). It remains to be determined if any of these protein kinases or uncharacterized kinases are involved in the Ser-439 phosphorylation in TESK1. As the binding of 14-3-3β seems to play a key role in the kinase activity and subcellular localization of TESK1, identification of the protein kinase(s) and phosphatase(s) responsible for the phosphorylation and dephosphorylation of Ser-439 of TESK1 will be important to understand the mechanisms regulating cellular functions of TESK1.

Previous studies revealed that 14-3-3 proteins associate with various signaling proteins, such as Raf-1 kinase, protein kinase C, and phosphatidylinositol 3-kinase, and stimulate or inhibit their enzyme activities, although contradictory data have been reported (19–21, 38–40) as to the positive or negative regulation of the enzyme activity. We found that incubation with 14-3-3β significantly reduced the kinase activity of TESK1 but not the activity of TESK1(S439A), which indicates that 14-3-3β negatively regulates the kinase activity of TESK1 by binding to the site of phosphorylated Ser-439. The partial inhibition even in the presence of excess amounts of 14-3-3β in experiments in
Fig. 5 is probably due to the existence of the Ser-439 non-phosphorylated form of TESK1 in cell lysates. 14-3-3/H9252 may suppress the kinase activity of TESK1 by inducing and/or stabilizing the inactive conformation of TESK1. Alternatively, 14-3-3/H9252 may inhibit TESK1 by masking the catalytic site or substrate-binding site present in the N-terminal half of TESK1. The non-catalytic region of protein kinases often serves as the domain that negatively regulates the kinase activity, and release from its suppressive effect results in activation of the kinase (33, 41). As TESK1 mutants lacking the C-terminal non-catalytic region have about a 2–4-fold higher kinase activity, compared with wild-type TESK1, the C-terminal region of TESK1 seems to negatively regulate the kinase activity of TESK1. As in the cases for many protein kinases, activation of TESK1 may occur by binding of activator proteins to the C-terminal regulatory domain of TESK1. If so, binding of 14-3-3β may have a role to sequester TESK1 as an inactive complex to prevent accession of activator proteins. Further studies are required to elucidate the mechanisms by which the

Fig. 6. Interaction between TESK1 and 14-3-3β is decreased after integrin-mediated cell adhesion. A and B, changes in the TESK1/14-3-3β interaction after plating cells on fibronectin (A) or poly-l-lysine (B). HeLa/TESK1 cells were suspended and replated on dishes coated with fibronectin (A) or poly-l-lysine (B). At the indicated times, cell lysates were prepared and subjected to in vitro pull-down binding assay using GST-14-3-3β-coupled glutathione-Sepharose. Bound proteins were separated on SDS-PAGE and immunoblotted with anti-TESK1 antibody (top panel) or anti-14-3-3β antibody (2nd panel). Initial lysates were also separated on SDS-PAGE and immunoblotted with anti-TESK1 antibody (3rd panel). These immunoblots are representative of three separate experiments. Relative levels of TESK1 bound to GST-14-3-3β are shown as means ± S.E. of triplicate experiments, with the level of TESK1 bound to GST-14-3-3β at zero time of plating taken as 1.0 (bottom panel). C and D, changes in the amount of 14-3-3β co-precipitated with TESK1 (C) and the kinase activity of TESK1 (D) after plating cells on fibronectin. HeLa/TESK1 cells expressing HA-14-3-3β were suspended and replated on fibronectin-coated dishes. C, at the indicated times, cell lysates were immunoprecipitated (IP) with anti-TESK1 antibody and analyzed by immunoblotting with anti-HA (upper panel) or anti-TESK1 antibody (middle panel). Initial lysates were also separated on SDS-PAGE and immunoblotted with anti-HA antibody (bottom panel). These immunoblots are representative of three separate experiments. D, immunoprecipitates prepared as in C were subjected to in vitro kinase reaction, using His₆-cofilin as a substrate. Relative kinase activities of TESK1 are shown as means ± S.E. of triplicate experiments, with the activity of TESK1 at zero time of plating taken as 1.0.

$^a$ J. Y. Toshima, J. Toshima, T. Watanabe, and K. Mizuno, unpublished data.
kinase activity of TESK1 is regulated.

Integrins are a family of cell-surface heterodimer proteins, each composed of an α-subunit and a β-subunit, that bind to extracellular matrix proteins such as fibronectin and transduce signals to control cell survival, proliferation, differentiation, and migration (42, 43). On binding to extracellular matrix proteins, integrins become clustered and form large protein complexes known as focal adhesion complexes, through which integrins link to the actin cytoskeleton (42, 43). We reported that the kinase activity of TESK1 and the level of cofilin phosphorylation are increased by integrin-mediated cell stimulation, and we suggested that TESK1 plays an important role in integrin-mediated cofilin phosphorylation and actin reorganization (12). However, the mechanism by which TESK1 is activated downstream of integrin remained to be determined. In the present study, we found that 14-3-3β inhibits the kinase activity of TESK1 by interacting with it, and this interaction is decreased when plating cells on fibronectin. These results suggest that the dissociation of 14-3-3β from a TESK1/14-3-3β complex after integrin stimulation is at least one of the causes for the integrin-induced activation of TESK1.

When cells are stimulated by fibronectin, 14-3-3β accumulates to cell attachment sites at the cell periphery (22, 23). Han et al. (22) identified the specific interaction between 14-3-3β and the cytoplasmic domain of integrin β1 and suggested the involvement of 14-3-3β in the integrin-mediated cell spreading and migration. In our study, we found that TESK1 co-localizes with 14-3-3β and accumulates to cell adhesion sites when cells are plated on fibronectin, whereas they are diffusely distributed in the cytoplasm in non-stimulated cells. As an S439A

![Fig. 7](image-url)
after plating cells on fibronectin, the association of TESK1 with 14-3-3 revealed that TESK1 co-localizes with 14-3-3 comes activated there (Fig. 9). Because cell staining studies of the kinase activity and localization of TESK1 after integrin stimulation, TESK1 is translocated to adhesion sites by the help of 14-3-3. Once TESK1 is localized at the adhesion sites, it is dissociated from 14-3-3, becomes activated, and induces actin reorganization by phosphorylating cofillin. See text for details.

mutant of TESK1 did not accumulate at the cell periphery even after plating cells on fibronectin, the association of TESK1 with 14-3-3 through phosphorylated Ser-439 seems to be essential for the integrin-mediated peripheral localization of TESK1. 14-3-3β may function as the dimeric adaptor to connect TESK1 with integrins (Fig. 9). On the other hand, the interaction between TESK1 and 14-3-3β is decreased, and the kinase activity of TESK1 is inversely increased, after cell adhesion on fibronectin. Thus, we postulate that once TESK1 is localized at the cell adhesion sites it is dissociated from 14-3-3β and becomes activated there (Fig. 9). Because cell staining studies revealed that TESK1 co-localizes with 14-3-3β at the cell periphery during cell spreading, it is likely that TESK1 is retained at cell adhesion sites even after dissociation from 14-3-3β. TESK1 activated at the cell periphery is probably involved in cell adhesion and spreading by regulating actin filament dynamics through cofillin/ADF phosphorylation. Dissociation of TESK1 from a TESK1-14-3-3β complex may be induced by competitive binding of 14-3-3 with other proteins present at cell adhesion sites. Because p130CSK interacts with 14-3-3 proteins and the interaction is increased after plating cells onto fibronectin (23), it may play a role in the dissociation of TESK1 from a TESK1-14-3-3β complex. In addition, the dephosphorylation of Ser-439 seems to be involved in the dissociation of TESK1 from the complex (Fig. 9).

In conclusion, we identified the interaction between TESK1 and 14-3-3β and found that 14-3-3β negatively regulates the kinase activity of TESK1. When cells are plated on fibronectin, TESK1 and 14-3-3β are localized at cell adhesion sites, and TESK1 is dissociated from 14-3-3β and becomes activated. Expression of a kinase-dead TESK1 significantly suppresses cell spreading on fibronectin, which strongly suggests the role of TESK1 in integrin-mediated cell spreading. Together with recent studies showing that 14-3-3β directly binds to the cytoplasmic domain of integrin β1 and modulates cell spreading and migration (22), our findings will provide new insight into the integrin-mediated signaling pathways that induce actin cytoskeletal remodeling and focal adhesion formation.

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