Actopaxin Interacts with TESK1 to Regulate Cell Spreading on Fibronectin

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The focal adhesion protein actopaxin contributes to integrin-actin associations and is involved in cell adhesion, spreading, and motility. Herein, we identify and characterize an association between actopaxin and the serine/threonine kinase testicular protein kinase 1 (TESK1), a ubiquitously expressed protein previously reported to regulate cellular spreading and focal adhesion formation via phosphorylation of cofilin. The interaction between actopaxin and TESK1 is direct and the binding sites were mapped to the carboxyl terminus of both proteins. The association between actopaxin and TESK1 is negatively regulated by adhesion to fibronectin, and a phosphomimetic actopaxin mutant that promotes cell spreading also exhibits impaired binding to TESK1. Binding of actopaxin to TESK1 inhibits TESK1 kinase activity in vitro. Expression of the carboxyl terminus of actopaxin has previously been reported to retard cell spreading. This effect was reversed following overexpression of TESK1 and was found to be dependent on an inability of actopaxin carboxyl terminus expressing cells to promote cofilin phosphorylation upon matrix adhesion and caused by retention of TESK1 by this actopaxin mutant. Thus, the association between actopaxin and TESK1, which is likely regulated by phosphorylation of actopaxin, regulates TESK1 activity and subsequent cellular spreading on fibronectin.

Integrin-mediated adhesion to the extracellular matrix (ECM) leads to extensive actin reorganization that is regulated predominantly by the Rho family of GTPases, Cdc42, Rac, and Rho, to stimulate formation of the actin-dependent structures filopodia, lamellipodia, and stress fibers, respectively (1). Activated Rho family members interact with numerous effectors, including the p21-associated kinase (PAK), the Wiskott-Aldrich Syndrome protein (WASP), and the Rho-associated kinase (ROCK). PAK and ROCK share some common target proteins, including the LIM kinases (LIMK) (1). Closely related to the LIM family of kinases are TESK1 and TESK2 (testicular protein kinases), which were originally identified in testicular cells but have since been found to be ubiquitously expressed (2–5). Unlike LIMK, regulation of TESK1 activity by Rho GTPases has not been confirmed, although recent studies in Drosophila implicate a role in the Rac pathway associated with both eye development and spermatogenesis (6). However, this kinase has been shown to be activated upon matrix adhesion and is regulated by binding of 14-3-3β and Sprouty4 (7, 8).

TESK1, as is the case with LIMK, regulates integrin-dependent focal adhesion assembly and actin organization through phosphorylation of the amino terminus of the F-actin-severing protein coflin (3). Phosphorylation on serine 3, which is reversed by the serine phosphatases slingshot and chronophin, has been shown to decrease coflin activity by interfering with its ability to bind F-actin (9–11). Cofilin is a critical regulator of both growth factor and matrix-dependent actin reorganization, affecting lamellipodia formation, cell spreading, motility, and polarity (12–16). For instance, coflin’s F-actin severing activity potentiates Arp2/3-mediated actin assembly that is required for epidermal growth factor-induced lamellipodia formation (17).

The focal adhesion protein actopaxin is the α-isoform of the parvin family and binds actin through a pair of calponin homology (CH) domains (18–20). It interacts with the integrin-linked kinase (ILK) and the focal adhesion scaffolding proteins paxillin and Hic-5 (18, 21, 22). Affixin, the β-isoform of the parvin family, has been found to bind the putative Rac/Cdc42 guanine nucleotide exchange factor PIX suggesting the possibility of this interaction for actopaxin as well (23). The associations between actopaxin, ILK, and paxillin constitute an evolutionarily conserved integrin-actin linkage important in muscle cytoarchitecture and contribute to the regulation of cellular spreading and adhesion in mesenchymal cells (18, 22, 24–26). Recent evidence suggests that Erk-dependent phosphorylation of the actopaxin amino terminus regulates cellular spreading and motility via modulation of Rho family signaling (27).

In this study we performed a yeast two-hybrid screen of a human placenta library using actopaxin as bait and identified TESK1 as a direct binding partner. We have established that an association between actopaxin and TESK1 negatively regulates TESK1 kinase activity and thus phosphorylation of coflin. Furthermore, this association is negatively modulated by adhesion to fibronectin, most likely through phosphorylation of the amino terminus of actopaxin. Consequently, the association between TESK1 and actopaxin provides a mechanism for the regulation of cell spreading and potentially cell migration via modulation of coflin activity.

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§ The abbreviations used are: ECM, extracellular matrix; aa, amino acids; CH, calponin homology; Erk, extracellular signal-regulated kinase; PAK, focal adhesion kinase; ILK, integrin-linked kinase; LIMK, LIM kinase; MBP, myelin basic protein; TESK1 and TESK2, testicular protein kinases 1 and 2; XAC, Xenopus actin depolymerizing factor/cofilin; PAK, p21-associated kinase; ROCK, Rho-associated kinase; GFP, green fluorescent protein.
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**EXPERIMENTAL PROCEDURES**

**Antibodies and Materials—**Human plasma fibronectin was purchased from Sigma or BD Biosciences. Monoclonal antibody to the Xpress tag was purchased from Invitrogen. α-Actinin and MOPC monoclonal antibodies were obtained from Sigma. Focal adhesion kinase (FAK) and ILK monoclonal antibodies were purchased from BD Transduction Laboratories. The 9E10 anti-Myc monoclonal antibody developed by J. M. Bishop was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Omni-probe (directed against region of Xpress epitope tag) and GFP polyclonal antibodies were obtained from Santa Cruz Biotechnology. Polyclonal antibody to cofilin phosphorylated upon serine 3 was provided by Dr. James Bamburg (Colorado State University) (28).

**Cell Culture and Transfections—**HeLa cells were maintained in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum (Atlanta Biologica). Transfections were performed with FuGENE 6 (Roche Applied Science) according to manufacturer's protocols.

**Binding Assays—**GST binding assays were performed essentially as described previously (18). For *in vivo* binding experiments, cells were lysed in co-immunoprecipitation buffer (50 mM Tris-HCl, pH 7.6, 0.5% Nonidet P-40, 100 mM NaCl, 10% glycerol, 1 mM MgCl₂, 10 μM leupeptin, 1 mM NaN₃, and 1 mM NaF). Lysates were centrifuged to remove cellular debris, and then Xpress-actopaxin was precipitated using Omni-probe antibody (anti-epitope tag of Xpress-actopaxin) and protein A/G beads (Santa Cruz Biotechnology). Immunoprecipitated proteins were subsequently solubilized in sample buffer and analyzed by Western blotting.

**In Vitro Kinase Assays—**Kinase assays were performed essentially as described previously (3). HeLa cells expressing Myc-TESK1 were lysed in kinase immunoprecipitation buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40, 10 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 0.1 mM sodium orthovanadate, 10 μM sodium fluoride, 10 μM sodium molybdate, and 10 μM NaF), lysates were incubated at room temperature for 30 min in the presence of 5 μCi of [γ⁻³²P]ATP, 10 μg of focal adhesion kinase (MBP), and various GST-actopaxin constructs as indicated. The reaction was terminated by boiling in sample buffer and the samples resolved on a 15% SDS-PAGE gel, followed by Costammas Blue staining and autoradiography. Phosphorylation levels were quantitated with a Storm PhosphorImager (Abersham Biosciences).

**RESULTS**

**Actopaxin Interacts with TESK1 in Vitro—**To identify actopaxin-binding proteins, a yeast two-hybrid screen was performed with full-length rat actopaxin fused to LexA as a bait. Ten positive clones were isolated from the screening of 1 × 10⁶ recombinant colonies. To confirm the observed interactions, selected clones were subjected to an additional round of screening using either p53 as a nonspecific protein bait or actopaxin. Two of the plasmids that were positive for actopaxin binding and at the same time negative for p53 association encoded sequence representing the serine/threonine kinase testicular protein kinase 1 (TESK1) (data not shown).

One plasmid insert contained DNA encoding aa 529–626, comprising the carboxyl terminus of TESK1, as well as 80 nucleotides from the 3'-untranslated region. The two plasmids contained aa 544–626 and 74 nucleotides from the 3'-untranslated region. It is notable that the principal difference between TESK1 and its family member TESK2 is the presence of this proline-rich carboxyl terminus extension within TESK1 (5).

An association between actopaxin and TESK1 was first confirmed using GST binding assays. These studies were restricted to the analysis of exogenous TESK1 due to the lack of an available antibody to the endogenous form. Myc-tagged TESK1 and TESK2 were expressed in HeLa cells and actopaxin binding tested using GST-actopaxin fusion proteins. TESK1 was found to specifically bind full-length GST-actopaxin (Fig. 1A). In contrast, GST-actopaxin did not precipitate TESK2, consistent with the absence of the carboxyl-terminal extension of TESK1 in this isoform (Fig. 1A). The binding of ILK served as a positive control. Additional GST pull-down assays established the carboxyl-terminal actopaxin amino acids 223–372 as the binding region for TESK1 (Fig. 1, B and C). This region of actopaxin consists of a portion of the intra-CH linker domain and the second CH domain. It also contains the binding sites for paxillin and ILK (18, 22).

The yeast two-hybrid system identified the carboxyl-terminal residues of TESK1 as the site of interaction with actopaxin. A construct was created consisting of this portion of TESK1 (529–626) fused to the carboxyl terminus of GFP. Pull-down assays confirmed the binding of GST-TESK1 529–626 to GST-actopaxin (Fig. 2A). Furthermore, a GST fusion construct of this region of TESK1 was created to verify binding with actopaxin. GST binding assays performed using HeLa cell lysates demonstrated binding of endogenous actopaxin to GST-TESK1 529–626, while the GST-paxillin LD4 motif served as a positive control for actopaxin binding, as reported previously (2B) (18). It has been suggested that ILK and actopaxin are obligate binding partners (32). However, ILK was not present in the GST-TESK1 precipitate, indicating that TESK1 can bind a pool of actopaxin that is not concurrently bound to ILK (Fig. 2B). Conversely, the presence of ILK and actopaxin in the LD4
pull-down shows that existing actopaxin/ILK associations were not disrupted in these lysates as paxillin LD4 only binds ILK indirectly through actopaxin (22, 33).

Actopaxin and TESK1 Interact in Vivo—The association between TESK1 and actopaxin was demonstrated in vivo using co-immunoprecipitation experiments. Epitope-tagged versions of these proteins were co-expressed in HeLa cells and immunoprecipitated using a polyclonal antibody (Omni-probe) to the epitope-tag of actopaxin. Myc-TESK1, but not FAK, was co-immunoprecipitated with full-length Xpress-actopaxin (Fig. 3A). Consistent with the GST pull-down assays, TESK1 co-immunoprecipitated with the carboxyl terminus of actopaxin (aa 223–372) (Fig. 3B). FAK served as a negative control, while ILK binding to the actopaxin carboxyl terminus was used as a positive control (Fig. 3B). These data demonstrate that actopaxin and TESK1 are associated in asynchronously growing cells.

The Association between TESK1 and Actopaxin Is Negatively Regulated during Adhesion to Fibronectin—Cell attachment and spreading on the ECM leads to the activation of integrins and, in turn, Rho GTPases and is a widely accepted model for lamellipodia extension associated with cell migration. To determine whether the interaction between TESK1 and actopaxin might be modulated during cell spreading, we performed co-immunoprecipitations of co-transfected proteins in HeLa cells that were growing asynchronously in culture versus cells that had been actively spreading upon 10 μg/ml fibronectin for 90 min. Actopaxin precipitated Myc-TESK1 less efficiently in spreading cells than unstimulated cells (Fig. 4A).

Adhesion-dependent phosphorylation of actopaxin affects cell spreading and migration. This post-translational modification of actopaxin may exert its effects by altering its association with various binding partners. To test whether this is the case for TESK1, HeLa cells were co-transfected with Myc-TESK1 and either wild type or phosphomimetic (S4/8D) Xpress-actopaxin constructs (27). Immunoprecipitations using an antibody to the actopaxin epitope-tag showed that the phosphomimetic S4/8D Xpress-actopaxin failed to precipitate Myc-TESK1, while the wild-type Xpress-actopaxin displayed robust binding (Fig. 4B). The specificity of this result was confirmed by the observation that ILK was precipitated by both Xpress-actopaxin constructs (Fig. 4B).

As the sites of actopaxin phosphorylation are on the amino terminus of the protein, while the carboxyl terminus mediates TESK1 binding, the actopaxin phosphorylation-dependent abrogation of TESK1 binding may involve allosteric regulation between the amino and carboxyl terminus of actopaxin. Thus, we next co-transfected Myc-TESK1 with either full-length (aa 1–372) or carboxyl-terminal (aa 223–372) Xpress-actopaxin constructs. Notably, the carboxyl terminus of actopaxin contains the TESK1 binding site but lacks the phosphorylation sites (Fig. 1B). Both populations of cells were placed in suspen-
**Expression of the Carboxyl Terminus of Actopaxin Inhibits Adhesion-dependent Cofilin Phosphorylation**—Since the carboxyl terminus of actopaxin retains binding to TESK1 during adhesion and inhibits its kinase activity in *vitro*, this mutant can be used as a tool to evaluate the effects on cell function of alteration of the physiologic association between TESK1 and actopaxin. Specifically, we evaluated how introduction of this mutant influenced phosphorylation of the TESK1 substrate cofilin during cell spreading on fibronectin. HeLa cells were co-transfected with GFP-cofilin and either Xpress-actopaxin 223–372 or Xpress-β-galactosidase, as a control, and respread on 10 μg/ml fibronectin-coated culture dishes. Lysates were collected in suspension and at 30, 60, and 120 min post-re-spreading. Phospho-GFP cofilin levels were then measured by Western blotting with an antibody specific for phosphorylation on serine 3, the site phosphorylated by TESK1 that inactivates cofilin. Phosphosine 3 GFP cofilin levels are diminished in the Xpress-actopaxin 223–372-expressing cells during spreading on fibronectin (Fig. 6A). This assay was repeated following the co-transfection of Myc-TESK1 to determine whether the observed deficit of cofilin phosphorylation could be rescued. Indeed, introduction of TESK1 was found to rescue the cofilin phosphorylation defect observed in the Xpress-actopaxin 223–372 cells at the 90-min time point (Fig. 6B, lanes 4 and 5).

**TESK1 Rescues the Spreading Defect in Cells Expressing the Actopaxin Carboxyl Terminus**—The importance of the TESK1/actopaxin association in regulating cell morphology was examined by evaluating effects on cell spreading. Expression of Xpress-actopaxin 223–372 impaired spreading of HeLa cells on fibronectin at the 90-min time point, as has been reported previously (Fig. 7A) (18). Co-expression of Myc-TESK1 partially rescued this spreading defect, while TESK1 had no apparent effect on spreading when overexpressed by itself, although there was a mild increase in cortical actin as visualized by rhodamine-phalloidin staining (Fig. 7A). Equivalent expression of proteins in these experiments was confirmed by Western blotting (Fig. 7B).

We next performed quantitative analysis of spreading in these cells to confirm the TESK1-dependent rescue of the observed spreading defect caused by expression of Xpress-actopaxin 223–372. Quantitation of cell areas showed that Xpress-actopaxin 223–372 reduced the spreading of HeLa cells to 37% of that observed in GFP control cells (Fig. 7C). Co-expression of Myc-TESK1 significantly rescued the spreading of cells expressing Xpress-actopaxin 223–372 (p < 0.01) (Fig. 7C). However, this rescue was incomplete, as cells expressing Myc-TESK1 with Xpress-actopaxin 223–372 were still significantly less well spread than GFP control cells (Fig. 7C).

An actopaxin mutant with an altered paxillin binding site has previously been shown to be mislocalized and to also inhibit cell spreading when expressed in HeLa cells (18). We confirmed...
this finding and also determined that overexpression of TESK1 is unable to rescue this phenotype, thereby indicating the specificity of the rescue of the Xpress-actopaxin 223–372 spreading defect by TESK1 (Fig. 7C). The spreading defect of the paxillin binding site mutant caused by loss of actopaxin/paxillin association therefore operates through some alternate pathway, possibly involving mislocalization of other actopaxin binding partners.

Taken together, these results suggested that the spreading defect in Xpress-actopaxin 223–372-expressing cells is dependent upon altered cofilin signaling. Previous reports have demonstrated that phosphocofilin is localized in a region of the cell closely juxtaposed to the extending lamellipodia in a transition zone where actin filaments become stabilized (13). Thus, we examined phosphocofilin localization in cells actively spreading upon fibronectin. GFP-transfected control cells exhibited modest phosphocofilin immunostaining concentrated toward the cell periphery, while Myc-TESK1 expression increased this signal (Fig. 4B).

FIG. 4. The association between TESK1 and actopaxin is decreased during cell spreading. A, HeLa cells were co-transfected with Xpress-actopaxin and Myc-TESK1. Omni-probe immunoprecipitations for Xpress-actopaxin were then performed either from cells growing in culture or from cells that had been spread on 10 μg/ml fibronectin for 90 min. Actopaxin more readily precipitates TESK1 from asynchronously growing cells than from those spreading on fibronectin. α-Actinin does not bind actopaxin in either condition. B, HeLa cells were co-transfected with Myc-TESK1 and either phosphomimetic S4/8D or wild-type Xpress-actopaxin constructs. Xpress-actopaxin constructs were immunoprecipitated with Omni-probe polyclonal antibody, resolved using SDS-PAGE, and transferred to nitrocellulose. Myc-TESK1 co-immunoprecipitated with the wild-type but not the phosphomimetic S4/8D Xpress-actopaxin construct. Equivalent amounts of ILK bound to each actopaxin construct. C, HeLa cells were co-transfected with Myc-TESK1 and either Xpress-actopaxin 1–372 (full-length) or 223–372 (carboxyl terminus). These cells were then spread on 10 μg/ml fibronectin for 90 min followed by Omni-probe immunoprecipitations. The association between full-length actopaxin and TESK1 is diminished during spreading. However, the carboxyl terminus of actopaxin retains its association with TESK1. Conversely, both Xpress-actopaxin constructs still precipitate ILK.
The cDNA were standardized with Xpress and Xpress actopaxin 223–372. HeLa cells were transfected with: Xpress-actopaxin 223–372. Cells expressing Xpress-actopaxin 223–372 were able to rescue the defect in cofilin phosphorylation displayed in the cells expressing Xpress-actopaxin 223–372. HeLa cells were transfected and respread as in Fig. 9. The combination of Myc-TESK1 and Xpress-actopaxin 223–372 significantly increases spreading as compared with cells expressing Xpress-actopaxin 223–372 (p < 0.01). An Xpress-actopaxin construct containing a mutated paxillin binding site (PBS) also displays impaired spreading. However, this defect is not reversed by TESK1, indicating the specificity of the rescue of the Xpress-actopaxin 223–372 construct.

**A TESK1 Construct That Does Not Bind Actopaxin Increases Cell Spreading on Fibronectin**—To further examine the importance of the actopaxin/TESK1 interaction in the regulation of cell spreading, we created a GFP-TESK1 1–528 construct, which lacks the actopaxin binding site but contains the kinase domain and the autophosphorylation and previously characterized regulatory sites. GST binding assays confirmed the lack of binding of actopaxin to this construct (Fig. 10A). We then expressed this construct in HeLa cells and respread them on 10 μg/ml fibronectin for 90 min. The cells were then processed for immunofluorescence microscopy. In contrast to full-length phosphocofilin staining (Fig. 8). Consistent with our biochemical evaluation (Fig. 6), cells expressing Xpress-actopaxin 223–372 had diminished phosphocofilin staining, which was notably absent from the periphery when compared with an adjacent non-transfected cell. Importantly, phosphocofilin staining was restored by co-expression of Myc-TESK1 with Xpress-actopaxin 223–372 (Fig. 9). No apparent difference in total cofilin cell staining was observed (data not shown). These data support a role for actopaxin in regulating TESK1 signaling to cofilin.

We further tested a role for cofilin by evaluating the ability of phosphomimetic (S3E) cofilin constructs to rescue the spreading on fibronectin in these cells. Significantly, co-expression of a non-active S3E phosphomimetic cofilin construct with Xpress-actopaxin 223–372 was able to partially rescue spreading, similar to that seen with Myc-TESK1 (Fig. 9). Conversely, expression of an S3A construct, which mimics a non-phosphorylated, active cofilin, decreased spreading to levels comparable with those seen in HeLa cells expressing Xpress-actopaxin 223–372 (Fig. 9). Co-expression of the S3A cofilin construct with Xpress-actopaxin 223–372 did not result in further inhibition of spreading (Fig. 9).

These data demonstrate that expression of the carboxyl terminus of actopaxin severely inhibits cell spreading on fibronectin in part through alteration of cofilin phosphorylation, most likely mediated through the TESK1 kinase. The ability of TESK1 to only partially rescue this phenotype indicates the possible perturbation of other actopaxin interactions, for instance the association with actin.

**Fig. 6.** Expression of the carboxyl terminus of actopaxin inhibits adhesion-dependent cofilin phosphorylation. A, HeLa cells were co-transfected with GFP-cofilin and either β-galactosidase (β-Gal), as control, or the actopaxin carboxyl terminus (Xpress-actopaxin 223–372). Cells were then plated in suspension for 1 h and either lysed or spread onto plates coated with 10 μg/ml fibronectin. Samples were then collected at 30, 60, and 120 min and analyzed by SDS-PAGE for levels of phosphoserine 3 GFP-cofilin and total GFP-cofilin. Cells expressing the carboxyl terminus of actopaxin displayed reduced phosphocofilin levels upon cell attachment and spreading on fibronectin. B, to determine whether TESK1 could rescue the observed cofilin phosphorylation defect, HeLa cells were transfected and respread as in A, with the exception that samples were collected solely at 90 min post-respreading. HeLa cells were transfected with: Xpress-actopaxin 223–372 (lane 1), GFP-cofilin (lane 2), GFP-cofilin and Myc-TESK1 (lane 3), GFP-cofilin and Xpress-actopaxin 223–372 (lane 4), or GFP-cofilin, Myc-TESK1, and Xpress-actopaxin 223–372 (lane 5). Total amounts of transfected cDNA were standardized with Xpress β-galactosidase. Myc-TESK1 is able to rescue the defect in cofilin phosphorylation displayed in the cells expressing Xpress-actopaxin 223–372.

**Fig. 7.** TESK1 rescues the spreading defect in cells expressing the actopaxin carboxyl terminus. A, HeLa cells transfected with the indicated constructs were spread on 10 μg/ml fibronectin-coated coverslips for 90 min and then processed for immunofluorescence. Transfected cells were determined by co-transfection with GFP and are indicated by asterisks. Cells expressing the actopaxin carboxyl terminus (Xpress-actopaxin 223–372) displayed impaired spreading upon fibronectin that was rescued by co-expression of Myc-TESK1. Bar, 10 μm. B, lysates from experiments in A were blotted to demonstrate equivalent expression of proteins under each condition. C, cells were respread as in A, followed by area measurement and analysis as detailed under “ Experimental Procedures.” Error bars represent standard deviation. A minimum of 40 cells was quantified per condition per trial (n was a minimum of three per condition). * indicates a condition significantly different from GFP (p < 0.01). The combination of Myc-TESK1 and Xpress-actopaxin 223–372 significantly increases spreading as compared with cells expressing Xpress-actopaxin 223–372 (p < 0.01). An Xpress-actopaxin construct containing a mutated paxillin binding site (PBS) also displays impaired spreading. However, this defect is not reversed by TESK1, indicating the specificity of the rescue of the Xpress-actopaxin 223–372 construct. **Cell Spreading on Fibronectin**—To further examine the importance of the actopaxin/TESK1 interaction in the regulation of cell spreading, we created a GFP-TESK1 1–528 construct, which lacks the actopaxin binding site but contains the kinase domain and the autophosphorylation and previously characterized regulatory sites. GST binding assays confirmed the lack of binding of actopaxin to this construct (Fig. 10A). We then expressed this construct in HeLa cells and respread them on 10 μg/ml fibronectin for 90 min. The cells were then processed for immunofluorescence microscopy. In contrast to full-length...
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FIG. 8. Cells expressing the actopaxin carboxyl terminus display aberrant phosphocofilin localization during spreading. HeLa cells were transfected and spread on fibronectin as in figure 7. Cells were then fixed after 90 min and stained for phosphocofilin. GFP control cells exhibited an enrichment of phosphocofilin toward the cell periphery. Myc-TESK1-expressing cells showed slightly elevated phosphocofilin levels. In contrast, cells expressing the actopaxin carboxyl terminus (Xpress-actopaxin 223–372) displayed substantially reduced levels of phosphocofilin as compared with adjacent non-transfected cells. Localized phosphocofilin staining and cell spreading are restored when Myc-TESK1 is co-transfected with the Xpress-actopaxin 223–372 construct. Transfected cells are indicated by GFP co-transfection. Bar, 10 μm.

TESK1, which was found not to have an effect on spreading by itself, the GEF-TESK 1–528 increased spreading above that seen in GFP control cells (Figs. 10B and 7C). An increase in cortical actin structures was observed, consistent with elevated TESK1 activity (Fig. 10B). We confirmed this effect by quantifying areas of respread cells. GFP-TESK1 1–528 significantly increased spreading to 1.34 times that seen in control cells. Furthermore, this result was blocked by co-expression of cofilin S3A, indicating the effect is dependent on cofilin phosphorylation. Finally, expression of TESK1 1–528 lacking the actopaxin binding site completely rescued spreading in HeLa cells expressing Xpress actopaxin 223–372, confirming a role for TESK1 downstream of actopaxin in cell spreading (Fig. 10C).

DISCUSSION

Actopaxin performs a critical, evolutionarily conserved, role in stabilizing integrin-actin interactions at sites of cell adhesion to the extracellular matrix in muscle and non-muscle cells (18, 26). Herein, we detail a functional interaction between actopaxin and the serine/threonine kinase TESK1, which is an important modulator of integrin-mediated actin dynamics due to its ability to phosphorylate and thereby regulate the activity of the F-actin-severing protein cofilin (3). Using a combination of yeast two-hybrid analysis, GST pull-down, and co-immunoprecipitation assays we have localized the sites of interaction to the carboxyl terminus but likely involves long distance allosteric changes. This model is supported by the loss of adhesion-dependent regulation of the TESK1 association exhibited by the carboxyl terminus of actopaxin.

Although both LIMK, which is activated downstream of Rho-ROCK or Cdc42/Rac-PAK pathways, and TESK can phosphorylate cofilin (1), TESK1 has been suggested to be the primary regulator of adhesion-dependent cofilin phosphorylation, as supported by a significant loss of this signaling event in cells expressing kinase-dead TESK1 (3). This translates into an inability of these cells to spread efficiently on fibronectin (8). Interestingly, overexpression of the carboxyl terminus of actopaxin, which, as opposed to full-length actopaxin, maintains binding to TESK1 during adhesion, inhibits both cell spreading and cofilin phosphorylation. While it has previously been suggested that this spreading defect is due to perturbation of the F-actin binding site on actopaxin and thus disruption of the integrin-actin linkage (18), our current results, showing that the spreading defect can be partially rescued following overexpression of TESK1, provide evidence that cell spreading can also be controlled through actopaxin-mediated regulation of TESK signaling to cofilin. The introduction of an S3E phosphomimetic cofilin construct also reverts the spreading defect. As this mutant does not bind actin, its rescue is likely mediated through competition for binding partners with endogenous cofilin (9). This may potentially act through binding and seques-
from GFP (comparably with GFP control cells. * indicates significantly different
combination of GFP-TESK1 1–528 and Xpress-actopaxin 223–372 spreads
with GFP control cells. This effect is blocked by S3A cofilin. The com-
of GFP-TESK1 1–528 significantly increases spreading as compared
quantified as described under “Experimental Procedures.” Expression
of GFP-TESK1 1–528 was co-expressed with cofilin S3A or Xpress-acto-
and respread on 10
1–528 followed by GST pull-down binding assays. GST-acto-
paxin 223–372, consistent with the ability of this mutant to
cofilin staining was reduced in cells expressing Xpress-acto-
trast, a pool of phosphorylated cofilin is enriched a short dis-
tance away from the edge of the lamellipodia, where it func-
tions to stabilize actin structures necessary to support further
membrane protrusions (13). Consistent with a role for phos-
phocofilin in lamellipodia extension during cell spreading, we
have found phosphocofilin to be enriched toward the cell pe-
rimery following integrin ligation. Importantly, the phospho-
cofilin staining was reduced in cells expressing Xpress-acto-
paxin 223–372, consistent with the ability of this mutant to
interfere with TESK1 activity and thus phosphorylation of
cofilin.

Incorporating our data into the model of TESK1 regulation of
cofilin function, we propose (Fig. 11) that actopaxin and TESK1
associate with one another in the cytosol of asynchronously
growing adherent cells or cells held in suspension, and this
serves to inhibit TESK1 kinase activity. Upon integrin-medi-
atted attachment to the ECM, as occurs during cell spreading or
sites immediately proximal to the leading edge of an extend-
lamellipodium, actopaxin is recruited to the nascent focal
complexes and becomes phosphorylated within its amino ter-
minus. This likely promotes an intramolecular rearrangement
between the amino and carboxyl terminus of actopaxin that
reduces TESK1 binding, thereby relieving an inhibition of
TESK1 kinase activity. TESK1 then phosphorylates the local
pool of cofilin and thereby stabilizes actin filaments adjacent to
focal complexes. Interestingly, nascent focal complexes have
been shown to form at the transition zone between the extend-
lamellipodium, which is rich in cofilin and Arp2/3 activity (36–39).

The phosphoserine binding adaptor protein 14-3-3 has previ-
ously been shown to regulate adhesion-dependent activation of
TESK1 via a similar mechanism (8). Interestingly, we found that
expression of GFP-TESK1 1–528, which lacks actopaxin binding
but retains serine 439, the site of 14-3-3 binding, promoted aberrant
cell spreading and cortical actin structure formation, while wild-type TESK1 overexpression exerted minimal effects.
This likely suggests that 14-3-3β and actopaxin contribute to the
regulation of TESK1 function via overlapping as well as distinct
mechanisms. For instance, it will be important to determine
whether actopaxin binding influences TESK1 phosphorylation
on serine 439 and thus 14-3-3β binding.

Finally, it has previously been suggested that a stable asso-
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Changes in the balance of these multiple interactions may contribute to focal adhesion turnover and cell migration (40, 41). It has also been shown that actopaxin reported herein, it will be important to determine whether β-parvin binds TESK1 and if so how this may affect its activity. These possibilities are made more intriguing by the ability of TESK1 to facilitate cell spreading on fibronectin, while ILK has been shown to negatively affect spreading on fibronectin (6). These interactions exert opposing effects on ILK kinase activity (5), which, in turn, could contribute to focal adhesion turnover and cell migration (40, 41).

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