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The conformational state of Tes regulates its zyxin-dependent recruitment to focal adhesions

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The function of the human Tes protein, which has extensive similarity to zyxin in both sequence and domain organization, is currently unknown. We now show that Tes is a component of focal adhesions that, when expressed, negatively regulates proliferation of T47D breast carcinoma cells. Coimmunoprecipitations demonstrate that in vivo Tes is complexed with actin, Mena, and vasodilator-stimulated phosphoprotein (VASP). Interestingly, the isolated NH_2-terminal half of Tes pulls out α-actinin and paxillin from cell extracts in addition to actin. The COOH-terminal half recruits zyxin as well as Mena and VASP from cell extracts. These differences suggest that the ability of Tes to associate with α-actinin, paxillin, and zyxin is dependent on the conformational state of the molecule. Consistent with this hypothesis, we demonstrate that the two halves of Tes interact with each other in vitro and in vivo. Using fibroblasts lacking Mena and VASP, we show that these proteins are not required to recruit Tes to focal adhesions. However, using RNAi ablation, we demonstrate that zyxin is required to recruit Tes, as well as Mena and VASP, but not vinculin or paxillin, to focal adhesions.

Introduction

The human *TES* gene is located at 7q31.1/2 and falls within the fragile chromosomal region FRA7G, a locus that shows loss of heterozygosity in many human tumors (Tatarelli et al., 2000; Tobias et al., 2001). RT-PCR analysis and DNA methylation profiles reveal that Tes is not expressed in a variety of tumor cell lines, in particular breast and ovarian cancer cell lines as well as primary tumors (Tatarelli et al., 2000; Tobias et al., 2001). However, the function of Tes is currently unknown, although it has been proposed to act as a tumor suppressor (Tatarelli et al., 2000; Tobias et al., 2001). Sequence analysis of Tes reveals that it shares a high degree of homology, particularly in the three COOH-terminal LIM domains, with a number of proteins including the focal adhesion–associated protein zyxin (Tatarelli et al., 2000; Tobias et al., 2001). The LIM domain is a conserved double zinc finger protein module that was originally identified in the transcription factors Lin-11, Isl-1, and Mec3 (Dawid et al., 1998; Bach, 2000). Numerous studies have since revealed that LIM domains are involved in interactions with many different protein domains, including other LIM domains and are found in a diverse family of proteins such as transcription factors, kinases, and cytoskeleton-associated proteins (Dawid et al., 1998; Bach, 2000). In addition to the three LIM domains, Tes also contains a PET domain in its NH_2-terminal half (Gubb et al., 1999). No function has been ascribed to the PET domain but it is found in a limited number of proteins including Prickle, which also contains three COOH-terminal LIM domains and plays a critical role in the actin-dependent establishment of planar polarity in *Drosophila* (Gubb et al., 1999; Tree et al., 2002).

Results and discussion

Given its sequence homology to a number of cytoskeletal proteins, as well as its possible role as a tumor suppressor, we set out to investigate whether Tes is a cytoskeleton-associated protein. We found that GFP-tagged human Tes was recruited to focal adhesions in HeLa cells (Fig. 1, A and B). In contrast to other focal adhesion proteins such as α-actinin, Mena, vasodilator-stimulated phosphoprotein (VASP),* and zyxin, GFP-Tes was not observed along stress fibers (Fig. S1, available at www.jcb.org/cgi/content/full/jcb.200211015/DC1). Immunofluorescence analysis with an anti-Tes anti-

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*Abbreviation used in this paper: VASP, vasodilator-stimulated phosphoprotein.

Key words: Tes; focal adhesion; zyxin; RNAi; conformation

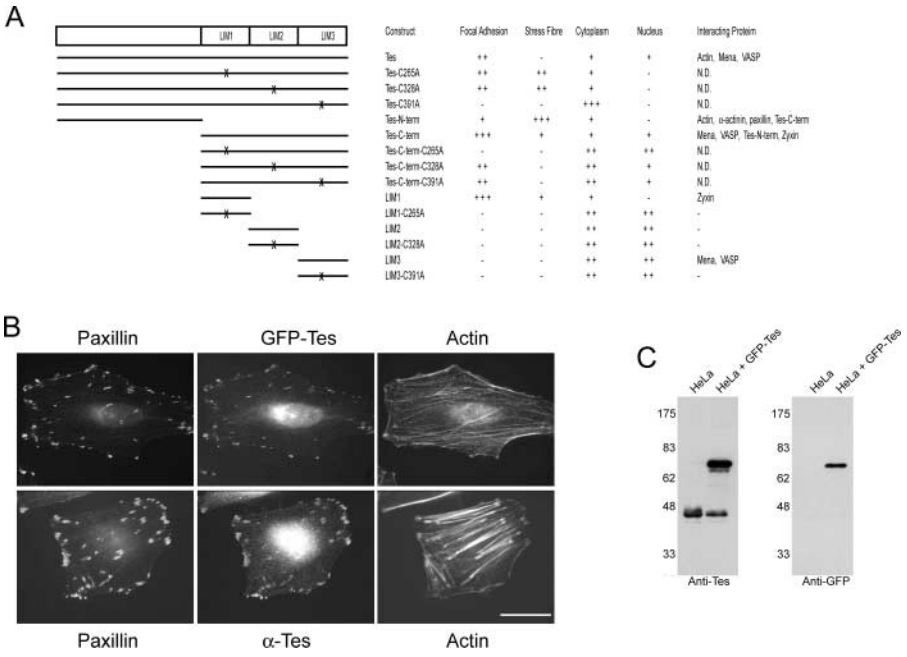


Figure 1. **Tes is recruited to focal adhesions.** (A) Schematic representation of Tes and the GFP-tagged expression clones including the location of point mutations inactivating each LIM domain used in this study. The in vivo localization of the GFP-tagged proteins and their interacting partners based on Western blot analysis of pull-down assays are indicated. Only the interactions between LIM1-zyxin and the two halves of Tes have been shown to be direct. N.D, not determined. (B) Immunofluorescence analysis of HeLa cells reveals that GFP-Tes or endogenous Tes colocalize with paxillin at focal adhesions. Bar, 20 μm. (C) Western blot analysis with Tes antibody detects a single band of the correct predicted size in HeLa cell extracts. Molecular mass markers are indicated in kDa.

body confirmed that the endogenous protein is also found at focal adhesions, whereas Western blot analysis identified a single band of the correct predicted size (Fig. 1, B and C).

To determine which region of Tes is responsible for recruitment of the protein to focal adhesions, we examined the localization of a series of GFP-tagged Tes constructs (Fig. 1 A). We found that the NH_2 terminus of the protein was recruited to stress fibers and focal adhesions (Fig. 2; and Fig. S2, available at www.jcb.org/cgi/content/full/jcb.200211015/DC1). This half of Tes, in contrast to the full-length Tes or COOH-terminal half of the protein was also recruited to actin-rich ruffles and lamellipodia as well as *Shigella* and vaccinia induced actin tails (Fig. 2; unpublished data). The COOH-terminal half of the molecule, corresponding to the three LIM domains, was strongly concentrated at focal adhesions and weakly observed along stress fibers (Fig. 2; and Fig. S2). To further delineate if a single LIM domain is required for recruitment to focal adhesions, we expressed individual GFP-tagged LIM domains in cells (Fig. 1 A). Immunofluorescence analysis of transfected cells revealed that only GFP-LIM1 is recruited to focal adhesions and also observed weakly along stress fibers (Figs. 1 A and 2; and Fig. S2). Disruption of the LIM1 domain by the introduction of a single point mutation (C265A) completely abolished this recruitment (Fig. 1 A). Likewise, only the absence of a functional LIM1 domain in the isolated COOH-terminal half of the molecule resulted in a loss of focal adhesion recruitment (Fig. 1 A). Unexpectedly, when the same point mutations were introduced into full-length Tes, we found that disruption of LIM1 or LIM2 had no effect on the targeting of Tes to focal adhesions (Figs. 1 A and 2). These mutations did, however, result in a noticeable recruitment of Tes to stress fibers (Fig. 2; and Fig. S2). In contrast, disruption of LIM3 led to a diffuse cytoplasmic localization and an absence of recruitment to focal adhesions (Fig. 2). Our observations indicate that, although the LIM3 domain is required for targeting, interaction sites in the NH_2-terminal half and LIM1 contribute to recruitment and stabilization of Tes at focal adhesions.

To examine which protein(s) might be responsible for recruiting Tes to focal adhesions, we performed pull-down assays on HeLa cell extracts using Tes produced in *E. coli*. Western blot analysis of pull-downs with a panel of antibodies against known focal adhesion proteins reveals that the Tes affinity resin retains actin, Mena, and VASP, but not α-actinin, Nck, FAK, paxillin, talin, vinculin, or zyxin, from cell extracts (Fig. 3 A). Coimmunoprecipitation experiments using anti-Tes antibody confirmed that Tes forms complexes with actin, Mena, and VASP, but not α-actinin, paxillin, or zyxin, in vivo (Fig. 3 B). Given our observations on the different in vivo localizations of the GFP-tagged Tes domains, we also performed pull-downs with the NH_2- and COOH-terminal halves of the molecule. We found that an affinity column of the NH_2-terminal half of Tes produced in *E. coli* can interact with actin, whereas the COOH-terminal half of the molecule associates with Mena and VASP (Fig. 3 A). Interestingly, the isolated halves of the protein also engage in additional interactions that are not observed with full-length Tes. The NH_2-terminal half is able to associate with α-actinin and paxillin, whereas the COOH-terminal half interacts with zyxin (Fig. 3 A). To further define which region in the COOH-terminal half of Tes is responsible for interacting with Mena, VASP, and zyxin, we performed pull-down assays using the individual LIM domains (Fig. 3 C). Western blot analysis of the pull-downs reveals that LIM1 binds zyxin, whereas LIM3 associates with Mena and VASP (Fig. 3 C). These interactions were abolished when a disrupting point mutation was introduced into the respective LIM domain (data not shown). To examine if the binding of Tes to VASP or zyxin is direct, we performed pull-down assays using protein produced in *E. coli*. We could find no evidence for a direct interaction between LIM3 and VASP (not shown). In contrast, we found that LIM1 was able to bind zyxin directly (Fig. 3 D).

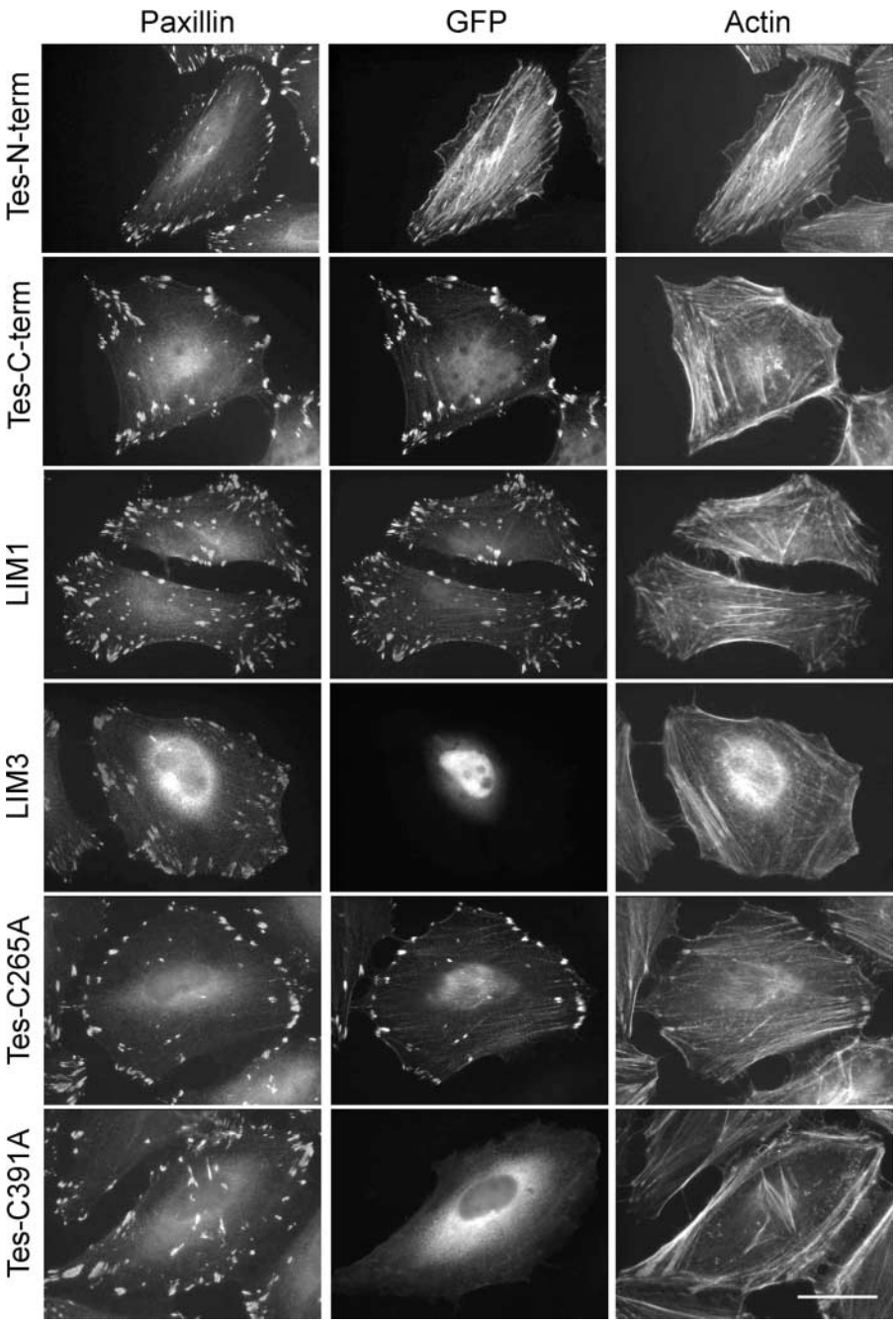


Figure 2. **Analysis of the cellular localization of GFP-tagged Tes domains.** Immunofluorescence analysis of HeLa cells expressing the indicated GFP-Tes colabeled for paxillin and phalloidin. The NH_2-terminal half of Tes (Tes-N-term) is associated with actin stress fibers, lamellipodia, and focal adhesions. The COOH-terminal half of Tes (Tes-C-term) is largely observed at focal adhesions and weakly observed along stress fibers. The LIM1, but not the LIM2 (not shown) or LIM3 domain, is strongly recruited to focal adhesions and weakly recruited along stress fibers. Disruption of LIM1 (Tes-C265A) or LIM2 (Tes-C328A) (not depicted) in full-length Tes does not affect recruitment of the protein to focal adhesions but results in increased localization along stress fibers. Disruption of the LIM3 domain (Tes-C391A) results in a diffuse cytoplasmic localization. Bar, 20 μm.

The differences in the ability of Tes and its individual halves to associate with α-actinin, paxillin, and zyxin from cell extracts (Figs. 1 A and 3 A) suggest that the interaction sites for these proteins are not accessible in the full-length molecule. One possible way to achieve this is via an intramolecular interaction between the halves of the molecule, as is observed for vinculin (Johnson and Craig, 1994). To test this hypothesis, we examined whether the NH_2- and COOH-terminal halves of Tes could interact with each other. We found that the NH_2-terminal half of Tes was able to interact with the COOH-terminal half of the molecule in vitro, but showed negligible binding to full-length Tes or to itself (Fig. 3 E). This suggests that Tes produced in *E. coli* is in a "closed" conformation. To test whether our in vitro observations reflect the situation in vivo, we examined whether GFP-Tes-N-term would copurify with His-Tes-C-term from HeLa extracts. Western blot analysis of nickel resin pull-downs revealed that GFP-Tes-N-term does copurify with the COOH-terminal half of the molecule, demonstrating that the two halves of Tes interact in vivo (Fig. 3 F).

Our localization studies suggest that in the context of the full molecule, the LIM3 domain is required for recruitment of the protein to focal adhesions. This could imply that Ena/VASP proteins are involved in recruiting Tes to focal adhesions. However, immunofluorescence analysis of MV^{D7} cells deficient in Mena and VASP (Bear et al., 2000) revealed that Tes is still present at focal adhesions (Fig. 4 A). That Tes recruitment to focal adhesions is independent of Ena/VASP proteins is consistent with the observation that mutation of the LIM3 domain in the isolated COOH-terminal half of

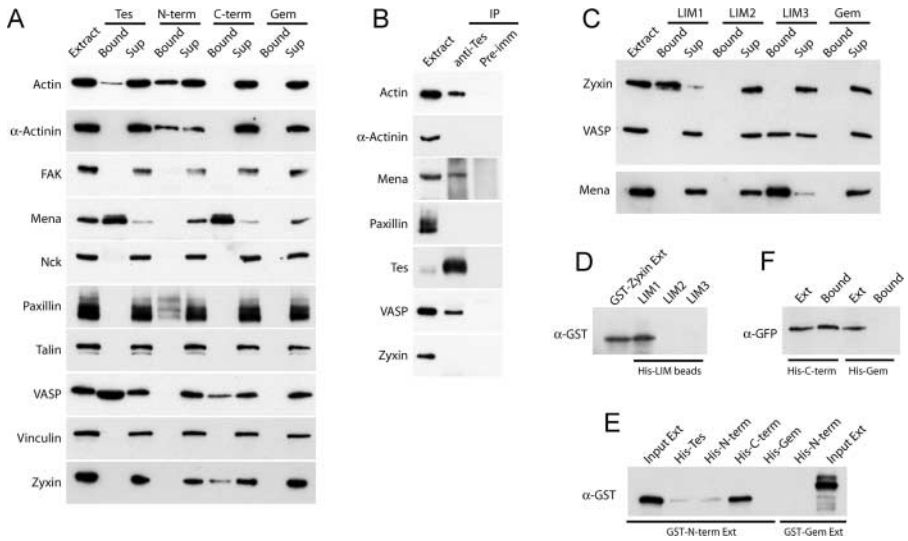
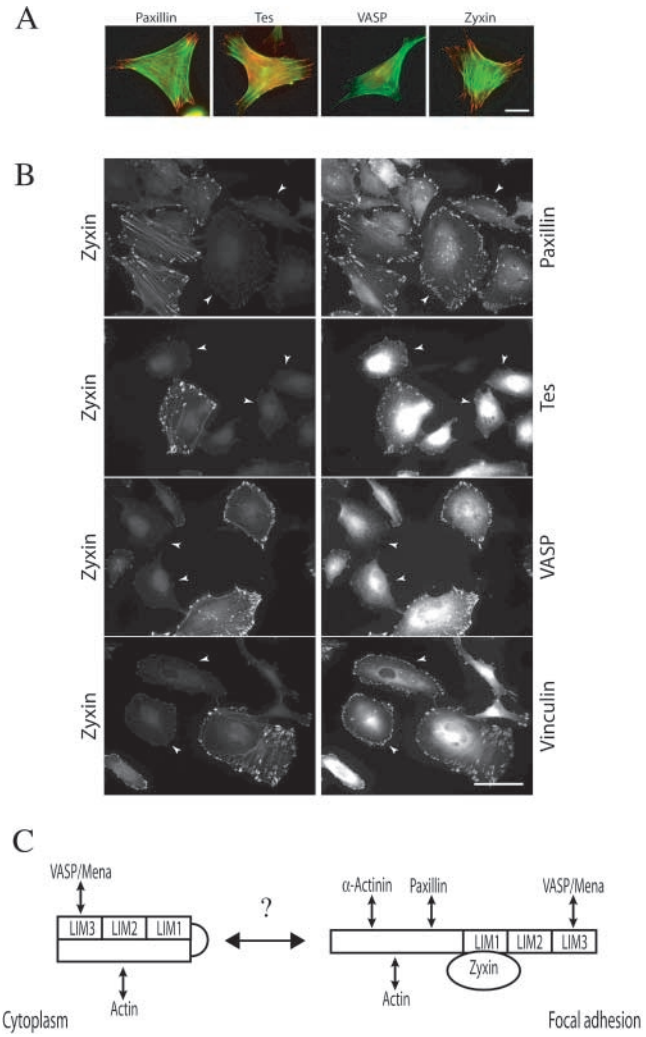


Figure 3. **Tes interacts directly with zyxin; the two halves of Tes interact with each other.** (A) Western blot analysis with the indicated antibodies (left) of pull-down assays performed on HeLa cell extracts using the Ni affinity resins indicated (top). The His-Gem affinity resin (Gem) represents a negative control. The input extract (Extract) as well as the bound fraction (Bound) and supernatant (Sup) for each resin are indicated. (B) Western blot analysis with the indicated antibodies (left) of Tes immunoprecipitation assays. The input extract (Extract), anti-Tes antiserum (anti-Tes) or the pre-immune serum (Pre-imm) are indicated. (C) Western blot analysis of pull-down assays performed on HeLa cell extracts using the His-LIM resins indicated (top) shows that the LIM1 domain interacts with zyxin, whereas LIM3 binds Mena and VASP. (D) Western blot analysis with anti-GST antibody of pull-down assays performed on *E. coli* soluble fraction containing GST-zyxin with the His-LIM resins shows that only the LIM1 domain is able to interact directly with zyxin. (E) Western blot analysis with anti-GST antibody of pull-down assays performed on *E. coli* soluble fraction containing the GST fusion protein (bottom) with purified His protein resins (top) demonstrates that the NH_2-terminal half of Tes can interact directly with the COOH-terminal half of the molecule in vitro. (F) Western blot analysis of Ni resin pull-downs from extracts of HeLa cells cotransfected with His-Tes-C-term and GFP-Tes-N-term. The anti-GFP blot demonstrates that GFP-Tes-N-term copurifies with His-Tes-C-term but not His-Gem, indicating the halves of the molecule interact with each other in vivo.



the molecule does not inhibit its recruitment to focal adhesions (Fig. 1 A). Furthermore, LIM3 alone is not recruited to focal adhesions (Figs. 1 A and 2). We suggest that disruption of the LIM3 domain abolishes recruitment of Tes to focal adhesions because it is involved in regulating the conformational state of the full-length protein and thus its ability to be recruited to focal adhesions. One can imagine that upon transition to an "open" conformational state, Tes is recruited and/or stabilized at focal adhesions through a direct interaction of its LIM1 domain with zyxin (Fig. 4 C). The recruitment of Tes to focal adhesions by zyxin would also be stabilized by the ability of the NH_2-terminal domain to associate with α-actinin and paxillin (Fig. 3 A). Consistent with this hypothesis, disruption of LIM1 but not LIM2 or LIM3

Figure 4. **Recruitment of Tes and VASP to focal adhesions is dependent on zyxin.** (A) Immunofluorescence analysis demonstrates that endogenous paxillin, zyxin, and Tes (red) are still recruited to focal adhesions in the absence of Ena/VASP proteins in MV^{D7} cells. The actin cytoskeleton is visualized with phalloidin (green). Bar, 20 μm. (B) Immunofluorescence analysis of mixed populations of HeLa cells transfected with zyxin and control siRNA oligos. The left column corresponds to the zyxin signal, whereas the right column shows the indicated protein. In cells lacking zyxin (white arrowheads), there is a corresponding absence of Tes, VASP, and Mena (not depicted) but not paxillin or vinculin at focal adhesions. Bar, 20 μm. (C) Schematic representation of the conformation changes in Tes and the proteins with which it associates. Double-headed arrows indicate associations based on pull-downs or immunoprecipitations, which may not represent direct interactions. In the cytoplasm, the molecule is in a "closed" conformation but can still associate with actin and VASP/Mena. Upon "activation" by an unknown mechanism, the molecule adopts a more "open" conformation and is recruited to and/or stabilized at focal adhesions. In this "open" conformation, Tes is able to associate with α-actinin, actin, paxillin, Mena, VASP, and bind directly to zyxin.

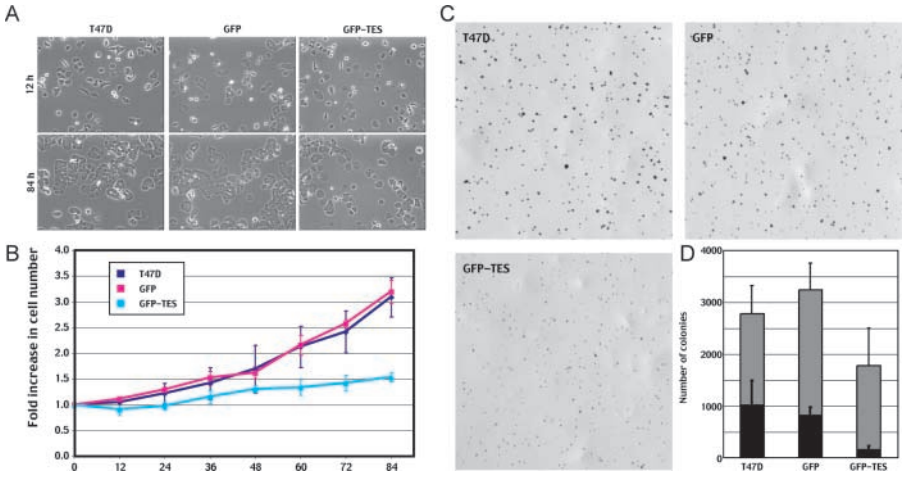


Figure 5. **Tes negatively regulates growth of T47D cells.** (A) Phase–contrast images of T47D cells, wild-type or expressing GFP or GFP-Tes, taken from movie sequences at the times indicated. Video available at www.jcb.org/cgi/content/full/jcb.200211015/DC1. (B) Graph showing average fold increase in cell number over times indicated. Error bars represent standard deviation of three independent experiments. (C) T47D colonies in soft-agar stained with nitroblue tetrazolium after 14-d growth. (D) Graph showing number of colonies (gray) formed by T47D cells (wild-type or expressing GFP or GFP-Tes) and the number of colonies with diameter greater than 100 μm (black). Data represent mean ± standard deviation between three independent experiments. Expression of GFP-Tes in T47D cells reduces the number and size of colonies formed when compared with cells expressing GFP.

in the isolated COOH-terminal half of the molecule results in the elimination of its recruitment to focal adhesions (Fig. 1 A).

Collectively, our data suggest that zyxin is the major determinant in the recruitment of Tes to focal adhesions. To address whether this is the case, we examined the effects of RNAi-induced loss of zyxin expression on the localization of Tes. Western blot analysis revealed that the zyxin expression was severely reduced in siRNA-treated cells (Fig. S3, available at www.jcb.org/cgi/content/full/jcb.200211015/DC1). In contrast, the expression levels of Grb2, paxillin, VASP, and Tes were unaffected (Fig. S3; unpublished data). Immunofluorescence analysis revealed that when zyxin expression was ablated, there was a corresponding loss of Tes at focal adhesions (Fig. 4 B). Interestingly, although the loss of zyxin did not appreciably affect the recruitment of vinculin or paxillin, it did result in the loss of VASP and Mena from focal adhesions (Fig. 4 B; unpublished data). Thus, although Tes is able to associate with several different focal adhesion components, it is the conformation-dependent interaction with zyxin that is the main determinant in the recruitment of the protein to focal adhesions (Fig. 4 C).

What is the cellular role of Tes? Previous observations have demonstrated that forced expression of Tes inhibited stable colony formation during antibiotic selection (Tobias et al., 2001). These experiments, however, do not rule out the possibility that reduced colony formation was due to cell death rather than suppression of cell growth. Notwithstanding this possibility, the expression profiles of Tes are consistent with a possible role as a tumor suppressor (Tatarelli et al., 2000; Tobias et al., 2001). To investigate whether Tes is able to suppress cell growth, we performed long-term video analysis of T47D cells and those stably expressing GFP or GFP-Tes (Fig. 5 A). T47D cells are human invasive ductal breast carcinoma cells, which do not express Tes based on Northern blot and RT-PCR analysis (Tatarelli et al., 2000), as well as immunofluorescence and Western blot analysis with anti-Tes antibody (not shown). GFP-Tes is detected at focal adhesions when stably expressed in T47D cells (not shown). We found that although expression of Tes had no appreciable effect on the motility of T47D cells, it severely reduced their overall growth rate (Fig. 5, A and B; and Videos 1–3, available at www.jcb.org/cgi/content/full/jcb.200211015/DC1). We also found that expression of GFP-Tes had an inhibitory effect on anchorage-independent growth of T47D cells in soft agarose (Fig. 5, C and D). Fewer colonies were formed by GFP-Tes expressing cells compared with those with GFP. Furthermore, those colonies that did form were significantly smaller (Fig. 5, C and D). The role of focal adhesions in mediating integrin-dependent modulation of cell growth through the action of MAP kinases is well established (Schwartz and Assoian, 2001). Given its numerous interactions, it is likely that Tes is also involved in this complex signaling cascade. Our future studies will aim to understand the regulation of the conformational state of Tes and how this influences cell growth.

Materials and methods

Construction of mammalian Tes expression vectors

Tes was amplified by PCR from a human fetal Marathon-Ready cDNA library (BD Biosciences; CLONTECH Laboratories, Inc.) using the primers NotTesFor and TesHindRev and was cloned into the mammalian expression vector CB6-N-GFP (Rietdorf et al., 2001) to generate GFP-Tes. DNA corresponding to the Tes NH_2 terminus (residues 1–234), Tes COOH terminus (residues 230–421), LIM1 (residues 230–295), LIM2 (residues 296–356), and LIM3 (residues 357–421) was amplified by PCR and cloned into CB6-N-GFP. All LIM domain–inactivating point mutants (C265A, C328A, and C391A), based on the observations of Taira et al. (1994), were generated using the QuikChange site-directed mutagenesis kit (Stratagene). The fidelity of all Tes expression clones was confirmed by sequencing.

E. coli protein expression, purification and affinity resin production

The required regions of Tes were subcloned from CB6-N-GFP into pMW172-HIS or pMW172-GST to generate His- or GST-tagged Tes *E. coli* T7 expression clones. Human Gem was amplified by PCR from pMT2TGem (Piddini et al., 2001) and cloned into pMW172-HIS. All pMW172 expression clones were transformed into *E. coli* strain BL21 (DE3) and protein was produced via leaky expression by growth overnight at 30°C. All proteins were purified from the *E. coli* soluble fraction except His-Tes-C-term, which was renatured from inclusion bodies using standard methods. The soluble fraction or urea-solublized inclusion bodies were

clarified by centrifugation and loaded on Ni-NTA Agarose (QIAGEN) in the presence of 50 mM Tris, pH 8.0, 25 mM imidazole, 500 mM NaCl, and 0.1% Triton X-100 ± 6 M urea (resuspension buffer). The resin was washed with resuspension buffer and stored in 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM β-mercaptoethanol, 20 μM $ZnCl_2$ (storage buffer) at 4°C or protein eluted with resuspension buffer containing 250 mM imidazole, pH 8.0. His-Tes-C-term was eluted with 500 mM imidazole in resuspension buffer containing 6 M urea and dialyzed overnight at 4°C against storage buffer containing 1 mM $MgCl_2$. The dialyzed soluble protein was rebound to Ni-NTA Agarose to use as an affinity resin for pull-downs.

Pull-down assays and immunoprecipitation

Washed HeLa cells were resuspended in an equal volume of 50 mM Tris.HCl, pH 7.5, 2% Triton X-100, 1% Nonidet P-40, 200 mM NaCl containing 20 μg/ml pepstatin, leupeptin, aprotinin, antipain, and chymostatin, 500 μg/ml Pefabloc SC (Roche), and 2 mM sodium vanadate. Detergent extracts were prepared after incubation for 1.5 h at 4°C by centrifugation at 20,000 g for 15 min. Imidazole was added to a final concentration of 50 mM, and the extract incubated with the desired Ni affinity resin for 4 h at 4°C. All resins were washed with 25 mM Tris.HCl, pH 7.5, 1% Triton X-100, 0.5% Nonidet P-40, 100 mM NaCl and 50 mM imidazole before addition of Laemmli sample buffer. For direct interaction assays, bacterial soluble fractions containing GST-Tes-N-term, GST-Gem, or GST-zyxin were incubated with the Ni affinity resins in storage buffer containing 25 mM imidazole for 1 h at 4°C. Resins were washed three times with this buffer before gel sample preparation and Western blot analysis. For immunoprecipitations, the detergent extract was incubated with polyclonal anti-Tes serum bound to Protein A–Sepharose beads (Amersham Pharmacia Biotech) for 4 h at 4°C and extensively washed with 25 mM Tris.HCl, pH 7.5, 1% Triton X-100, 0.5% NP-40, 100 mM NaCl. Washed beads were boiled in gel sample buffer and processed for Western blotting. Where the Tes antibody heavy chain would interfere with the visualization of the desired protein by Western blot, it was first cross-linked to the Protein A–Sepharose with dimethyl pimelimidate (Harlow and Lane, 1999), and bound proteins were eluted with 100 mM glycine, pH 2.5, and precipitated with trichloroacetic acid.

Antibodies, cell culture, transfections, immunofluorescence, and Western blot analysis

Anti–β-actin (AC-74), anti–α-actinin (BM75.2), anti-talin (8d4), and anti-vinculin (hVIN1) were obtained from Sigma-Aldrich. The anti-p125FAK and anti-Nck antibody were from Upstate Biotechnology. The anti-paxillin and anti-VASP antibodies were from Transduction Labs; the anti-GFP (FL) antibody was from Santa Cruz. Anti-Mena (2197) (Gertler et al., 1996), antizyxin B71 (Hoffman et al., 2003), and zyxin monoclonal antibodies (184A3 and 164D4) (Rottner et al., 2001) were gifts from Drs. F. Gertler (Massachusetts Institute of Technology, Boston, MA), M. Berkerle (University of Utah, Salt Lake City, UT), and M. Krause and J. Wehland (Gesallschaft für Biotechnologische Forschung, Braunschweig, Germany), respectively. Anti-Tes antibodies were produced in rabbits by immunization with His-Tes or His-Tes-N-term produced in *E. coli*. Rabbit serum was used directly or blot affinity purified against His-Tes (Harlow and Lane, 1999).

MV^{D7} cells were grown as previously described (Bear et al., 2000). HeLa or T47D cells were transfected with CB6 expression constructs using standard methods. For the stable cell lines, T47D cells were FACS sorted 24 h after transfection, and the GFP-positive cells were allowed to recover for a further 24 h before selection by the addition of 400 μg/ml G418. Subsequently, G418-resistant T47D cells were FACS sorted to recover populations expressing GFP-Tes or GFP. All experiments were performed with these cells in the presence of G418. Immunofluorescence and Western blot analysis were performed as described previously (Moreau et al., 2000).

Colony assays

A single-cell suspension of 5×10^3 viable T47D cells/ml (wild-type or stably expressing GFP-Tes or GFP) in 0.3% agarose solution was plated onto a 2-ml base layer of hardened 0.3% agarose with same media composition. Cultures were grown for 14 d, and colonies visualized using nitroblue tetrazolium. Colony-forming assays were scanned, and the number and size of colonies was determined using the Metamorph "integrated morphometry analysis" function (Universal Imaging Corp.), to count objects with pixel areas between 5 and 150. Data represent three independent experiments, with five replica dishes per experiment.

Videomicroscopy

10^5 viable T47D cells/ml (wild-type or stably expressing GFP-Tes or GFP) were plated onto fibronectin (10 μg/ml) coated MatTek dishes. Cells were allowed to settle overnight before acquistion of single images every 5 min in humidified chambers supplied with 10% CO_2 and maintained at 37°C. Cell numbers were counted in Metamorph using the "manually count objects" function and scaled to give fold increase from the first frame. Data represent a mean ± standard deviation of three independent experiments.

RNAi silencing

HeLa cells at 80–90% confluency in 24 well plates were transfected with 20 pmol of synthetic double-stranded siRNA that corresponds to position 1597 in the open reading frame of human zyxin mRNA (AAGTGTTACAAGTGTGAGGAC) or control GFP-22 siRNA (QIAGEN) using Lipofectamine 2000 (Invitrogen). 48 h after transfection, the two populations of cells were trypsinized and mixed at a ratio of 3:1 (zyxin/GFP-22 siRNA) before plating onto coverslips. 24 h later, the mixed cells were processed for immunofluorescence analysis.

Online supplemental material

Supplementary material is available at www.jcb.org/cgi/content/full/jcb.200211015/DC1 and includes additional figures showing localization of endogenous Tes, GFP-Tes, GFP-Tes-N-term, GFP-Tes-C-Term, GFP-LIM, and GFP-Tes-C265A with respect to endogenous α-actinin, VASP, or zyxin (Figs. S1 and S2). Western blot analysis is also provided showing that the cellular levels of Grb2 and Tes are not affected in the absence of zyxin (Fig. S3). Movies of the effect of GFP-Tes expression on growth and motility of T47D cells are also available (Videos 1–3).

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References

Bach, I. 2000. The LIM domain: regulation by association. *Mech. Dev.* 91:5–17.

Bear, J.E., J.J. Loureiro, I. Libova, R. Fassler, J. Wehland, and F.B. Gertler. 2000. Negative regulation of fibroblast motility by Ena/VASP proteins. *Cell.* 101: 717–728.

Dawid, I.B., J.J. Breen, and R. Toyama. 1998. LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet.* 14: 156–162.

Gertler, F.B., K. Niebuhr, M. Reinhard, J. Wehland, and P. Soriano. 1996. Mena, a relative of VASP and *Drosophila* Enabled, is implicated in the control of microfilament dynamics. *Cell.* 87:227–239.

Gubb, D., C. Green, D. Huen, D. Coulson, G. Johnson, D. Tree, S. Collier, and J. Roote. 1999. The balance between isoforms of the prickle LIM domain protein is critical for planar polarity in *Drosophila* imaginal discs. *Genes Dev.* 13:2315–2327.

Harlow, E., and D. Lane. 1999. Using Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 495 pp.

Hoffman, L.M., D.A. Nix, B. Benson, R. Boot-Hanford, E. Gustafsson, C. Jamora, A.S. Menzies, K.L. Goh, C.C. Jensen, F.B. Gertler, et al. 2003. Targeted disruption of the murine zyxin gene. *Mol. Cell. Biol.* 23:70–79.

Johnson, R.P., and S.W. Craig. 1994. An intramolecular association between the head and tail domains of vinculin modulates talin binding. *J. Biol. Chem.* 269:12611–12619.

Moreau, V., F. Frischknecht, I. Reckmann, R. Vincentelli, G. Rabut, D. Stewart, and M. Way. 2000. A complex of N-WASP and WIP integrates signalling cascades that lead to actin polymerization. *Nat. Cell Biol.* 2:441–448.

Piddini, E., J.A. Schmid, R. de Martin, and C.G. Dotti. 2001. The Ras-like GTPase Gem is involved in cell shape remodelling and interacts with the novel kinesin-like protein KIF9. *EMBO J.* 20:4076–4087.

Rietdorf, J., A. Ploubidou, I. Reckmann, A. Holmström, F. Frischknecht, M. Zettl, T. Zimmermann, and M. Way. 2001. Kinesin dependent movement on microtubules precedes actin based motility of vaccinia virus. *Nat. Cell Biol.* 3:992–1000.

Rottner, K., M. Krause, M. Gimona, J.V. Small, and J. Wehland. 2001. Zyxin is not colocalized with vasodilator-stimulated phosphoprotein (VASP) at

lamellipodial tips and exhibits different dynamics to vinculin, paxillin, and VASP in focal adhesions. *Mol. Biol. Cell.* 12:3103–3113.

Schwartz, M.A., and R.K. Assoian. 2001. Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. *J. Cell Sci.* 114:2553–2560.

Taira, M., H. Otani, J.P. Saint-Jeannet, and I.B. Dawid. 1994. Role of the LIM class homeodomain protein Xlim-1 in neural and muscle induction by the Spemann organizer in Xenopus. *Nature.* 372:677–679.

Tatarelli, C., A. Linnenbach, K. Mimori, and C.M. Croce. 2000. Characterization of the human TESTIN gene localized in the FRA7G region at 7q31.2. *Genomics.* 68:1–12.

Tobias, E.S., A.F. Hurlstone, E. MacKenzie, R. McFarlane, and D.M. Black. 2001. The TES gene at 7q31.1 is methylated in tumours and encodes a novel growth-suppressing LIM domain protein. *Oncogene.* 20:2844–2853.

Tree, D.R., J.M. Shulman, R. Rousset, M.P. Scott, D. Gubb, and J.D. Axelrod. 2002. Prickle mediates feedback amplification to generate asymmetric planar cell polarity signaling. *Cell.* 109:371–381.