TES is a novel focal adhesion protein with a role in cell spreading

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Summary

Previously, we identified *TES* as a novel candidate tumour suppressor gene that mapped to human chromosome 7q31.1. In this report we demonstrate that the TES protein is localised at focal adhesions, actin stress fibres and areas of cell-cell contact. TES has three C-terminal LIM domains that appear to be important for focal adhesion targeting. Additionally, the N-terminal region is important for targeting TES to actin stress fibres. Yeast two-hybrid and biochemical analyses yielded interactions with several focal adhesion and/or cytoskeletal proteins including mena, zyxin and talin. The fact that TES localises to regions of cell adhesion suggests that it functions in events related to cell motility and adhesion. In support of this, we demonstrate that fibroblasts stably overexpressing TES have an increased ability to spread on fibronectin.

Key words: TES, Focal adhesion, LIM domain, Cell spreading

Introduction

We, and others, previously identified a novel gene, *TES*, at 7q31.1, a region known to harbour a tumour suppressor gene (Tatarelli et al., 2000; Tobias et al., 2001). The promoter region of *TES* is associated with a CpG island that is frequently methylated in primary tumours and tumour-derived cell lines (Tatarelli et al., 2000; Tobias et al., 2001). In addition, lack of *TES* mRNA expression correlated with methylation of the *TES* CpG island in some tumour-derived cell lines (Tatarelli et al., 2000). We showed that forced overexpression of *TES* resulted in growth reduction of Ovcar5 (ovarian) and HeLa (cervical) carcinoma cell lines (Tobias et al., 2001), indicating that TES can be a negative regulator of cell growth.

TES is widely expressed in normal tissues and is predicted to encode a highly conserved protein of 421 amino acids containing three C-terminal LIM domains. LIM domains are approximately 55 amino-acid zinc-binding double finger motifs, with the consensus amino-acid sequence $CX_2CX_{17\text{-}19}HX_2(C/H)X_2CX_2CX_{17\text{-}19}CX_2(C/H/D)$ (Dawid et al., 1998). LIM domains were first identified in three developmentally important transcription factors Lin-11, Isl-1 and Mec-3 (Freyd et al., 1990; Karlsson, 1990; Way and Chalfie, 1988). Subsequently, many LIM-domain-containing proteins have been identified that play key roles in a diverse range of biological processes including differentiation, cytoskeletal organisation and oncogenesis (Arber et al., 1994; Brown et al., 1996; Fisch et al., 1992; Sattler et al., 2000). Several studies have demonstrated that LIM domains function as protein-protein interaction motifs that are involved in both intramolecular and intermolecular interactions (Feuerstein et al., 1994; Nagata et al., 1999). In some instances, LIM domain proteins are thought to act as scaffolds on which multiprotein complexes are formed (Arber and Caroni, 1996).

To elucidate the function of TES, we examined the subcellular localisation of the TES protein and performed yeast two-hybrid screens to identify TES-interacting proteins. Here we report that TES localises to focal adhesions and areas of cell-cell contact. In addition, we show that TES is a novel interacting partner of the known focal adhesion proteins mena, zyxin and talin. Focal adhesions are multiprotein complexes linking the cytoskeleton and signal transduction pathways with integrins at the extracellular matrix (ECM) (Burridge and Chrzanowska-Wodnicka, 1996; Critchley, 2000). Focal-adhesion-associated proteins may function to modulate cell adhesion, migration or cell signalling events (Burridge and Chrzanowska-Wodnicka, 1996; Critchley, 2000). The localisation of TES to regions of cell-cell and cell-substratum contact suggests that TES has a role in cell adherence, cell communication and, possibly, cell motility.

Materials and Methods

Materials

pEGFP-C1 (green fluorescent protein expression vector), pAS2-1 and pACT-2 (yeast expression vectors) and a human mammary gland cDNA library (HL4036AH) and mouse testis cDNA library (S1861) were purchased from Clontech. Anti-mouse HRP was purchased from New England Biolabs. Mouse anti-talin antibody (8d4), TRITC-labelled phalloidin, anti-mouse TRITC and glutathione sepharose 4B were purchased from Sigma. Protein A/G agarose and goat anti-actin antibody were purchased from Santa Cruz. Mouse anti-mena, anti-VASP and anti-paxillin antibodies were purchased from BD Transduction Laboratories. pGEX-2T was purchased from Amersham/Pharmacia. Dulbecco's modified Eagle's medium (DMEM); glutamine and fetal bovine serum (FBS) were purchased from Gibco BRL. Fibronectin purified from bovine plasma and latrunculin B were purchased from Calbiochem. Rabbit reticulocyte lysate in vitro transcription/translation kit was purchased from Promega. Mouse anti-zyxin antibodies and GFP-zyxin plasmid were generous gifts from J. Wehland and K. Rottner (Braunschweig, Germany).

Cell culture and stable transfections

Rat-1 fibroblast and HeLa cervical carcinoma cells were routinely grown at 37°C under a humidified atmosphere of 5% CO_2 in DMEM supplemented with 1% glutamine and 10% FBS. Cells were routinely passaged at 70-80% confluency using 10% trypsin.

For stable transfections, 1×10^6 cells were seeded into 100 mm dishes and transfected with either 1 μg of pEGFP-C1 vector or GFP-tagged TES constructs using Effectene reagent (Qiagen) as recommended by the manufacturer. Stable colonies were selected using G418 (600 μg/ml), and pools of stable colonies were sorted on the basis of fluorescence using a Becton Dickinson FACS vantage SE.

Plasmid construction

Plasmids used to express TES proteins were created by sub-cloning PCR-amplified human *TES* cDNA into the appropriate expression vector. Constructs were created that encoded full-length TES (amino acids 1-421 in pEGFP-C1, pGEX-2T, pAS2-1 and pACT2), LIM-less TES (amino acids 1-241 in pEGFP-C1 and 1-233 in pGEX-2T), LIM-only TES (amino acids 231-421 in pEGFP-C1), LIM 1 TES (amino acids 230-297 in pGEX-2T), LIM 1 and 2 TES (amino acids 230-360 in pGEX-2T) and LIM 3 TES (amino acids 351-421 in pGEX-2T) (see Fig. 1A). Human zyxin cDNA was obtained by restriction digest of pEGFP-N1/zxyin (gift from J. Wehland and K. Rottner, Braunschweig, Germany) with *Hin*dIII and *Bam*HI and subcloned into the *Hin*dIII and *Bam*HI sites of pcDNA3.1/HisC for in vitro transcription/translation using T7. All constructs were verified by sequencing.

Immunofluorescence

Fixation and staining of cells for immunofluorescence were performed as described previously (Gonzalez et al., 1993). Briefly, the coverslips were fixed with 3.7% formaldehyde and, where necessary, permeabilised with 0.5% (v/v) TritonX-100 and incubated with the appropriate primary antibody overnight at 4°C at the following dilutions: anti-paxillin 1/200, anti-zyxin hybridoma supernatant (164D4), phalloidin-TRITC 1/500 and anti-mena 1/50. Coverslips were incubated with the appropriate TRITC-labelled secondary antibody where necessary, mounted in vectashield (Vector Laboratories) and immunofluorescence performed using a Leica SP2 confocal microscope at 63× magnification.

Glutathione S-transferase (GST) pull-down assays and western blotting

Whole cell extracts (WCE) were obtained after incubating cells in lysis buffer (0.5% (v/v) NP40, 250 mM NaCl, 50 mM HEPES pH 7.4, 5 mM EDTA, 50 mM NaF, 200 μM sodium-orthovanadate, 50 mM β-glycerophosphate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 minutes on ice. Extracts were clarified by centrifugation at 17,500 ***g*** for 10 minutes at 4°C. pGEX-2T vector containing the cDNA sequence encoding full-length TES (Fig. 1) was expressed in *E. coli* as a GST-fusion protein. Bacterial cultures were induced using isopropyl-β-D-thiogalactopyranoside (IPTG), and lysates were incubated with glutathione sepharose 4B for 30 minutes at room temperature. 2 μg of GST-fusion protein coupled to glutathione sepharose or 2 μg of GST alone were incubated with 500 μg of WCE in cell lysis buffer overnight at 4°C. GST complexes were centrifuged at 1000 ***g*** for 1 minute, and the pellet was washed four times with cell lysis buffer before resuspending it in 20 μl of Laemmli SDS-loading buffer. GST-pull downs using in-vitro-translated zyxin were performed as above using 10 μl of rabbit reticulocyte lysate reaction.

For western blotting and immune detection, a 20 μl sample was run on a 10% SDS-PAGE gel according to the method of Laemmli (Laemmli, 1970). Blots were transferred to a PVDF membrane (Millipore) using a semi-dry transfer apparatus and membranes blocked in 5% skimmed milk TBS-T [Tris-buffered saline containing 0.2% (v/v) Tween-20] for 1 hour at room temperature. Blots were incubated overnight with mouse anti-talin (1/1000), anti-mena (1/250), anti-zyxin (1/5000) and anti-VASP (1/1000) antibodies in 5% skimmed milk TBS-T at 4°C. Blots were washed before incubation with anti-mouse HRP (1/3000 in 5% skimmed-milk TBS-T) for 1 hour at room temperature. Blots were then washed with TBS-T and protein detected using ECL reagent (Amersham) according to the manufacturer's instructions.

Yeast two-hybrid screens

Yeast two-hybrid screens and controls were performed as described in the Clontech Matchmaker II literature (BD Clontech). The full-length *TES* open reading frame cloned into the yeast two-hybrid GAL4 DNA-binding domain (DBD) vector, pAS2-1, was used as a bait to screen both a human mammary gland cDNA library and a pre-transformed mouse testis cDNA library cloned into the GAL4 activation domain (AD) vector, pACT-2. Full-length TES-expressing *S. cerevisiae* Y190 cells were either transformed with a human mammary gland cDNA library or mated with *S. cerevisiae* Y187 cells containing a pre-transformed mouse testis cDNA library and plated onto selective medium as described in the manufacturer's protocol. 1.1×10^6 transformants from the human mammary gland cDNA library and 5.6×10^7 transformants from the mouse testis cDNA library were tested for *lac*Z reporter gene expression by a colony lift filter assay using 5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside (X-gal) as the substrate. Plasmid DNA was isolated from positive colonies (Nucleon yeast DNA isolation kit, Tepnel Life Sciences, UK), amplified with pACT2-specific primers and sequenced on an ABI 3700 automated sequencer. Clone identities were determined by non-redundant and dbEST searches using the BlastN and BlastX programs at the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/).

Cell spreading

Rat-1 fibroblasts stably overexpressing pEGFP-C1 or GFP-tagged full-length TES were trypsinised, washed and pelleted. 1×10^5 cells in serum-free DMEM containing 1% (w/v) bovine serum albumin and 600 μg/ml G418 were re-seeded onto six-well plates containing fibronectin-coated coverslips (25 μg/ml) according to the manufacturer's instructions (Calbiochem). The plates were incubated at 37°C in a 5% CO_2/95% air atmosphere for the time periods specified in the results. Cells were fixed using 3.7% formaldehyde and where necessary stained with TRITC-labelled phalloidin. They were observed using a Leica SP2 confocal microscope at 63× magnification.

Results

TES is localised to focal adhesions and cell-cell contacts

To determine the subcellular localisation of TES, we produced GFP constructs produced expressing full-length TES, TES lacking the LIM domains (LIM-less TES) or the TES LIM domains (LIM-only TES) (Fig. 1A). Rat-1 fibroblasts that express endogenous *Tes* mRNA, as determined by RT-PCR (data not shown), were engineered to stably overexpress these constructs, as described in Materials and Methods. In Rat-1 fibroblasts, GFP-tagged full-length TES localised to focal adhesion structures (Fig. 1Bii,iii). Although on occasion we observed nuclear localisation of GFP-tagged full-length TES (data not shown), in the vast majority of cells we observed a clear exclusion of GFP from the nucleus (Fig. 1Bii-vi). By contrast, the vector alone shows diffuse cytoplasmic and strong

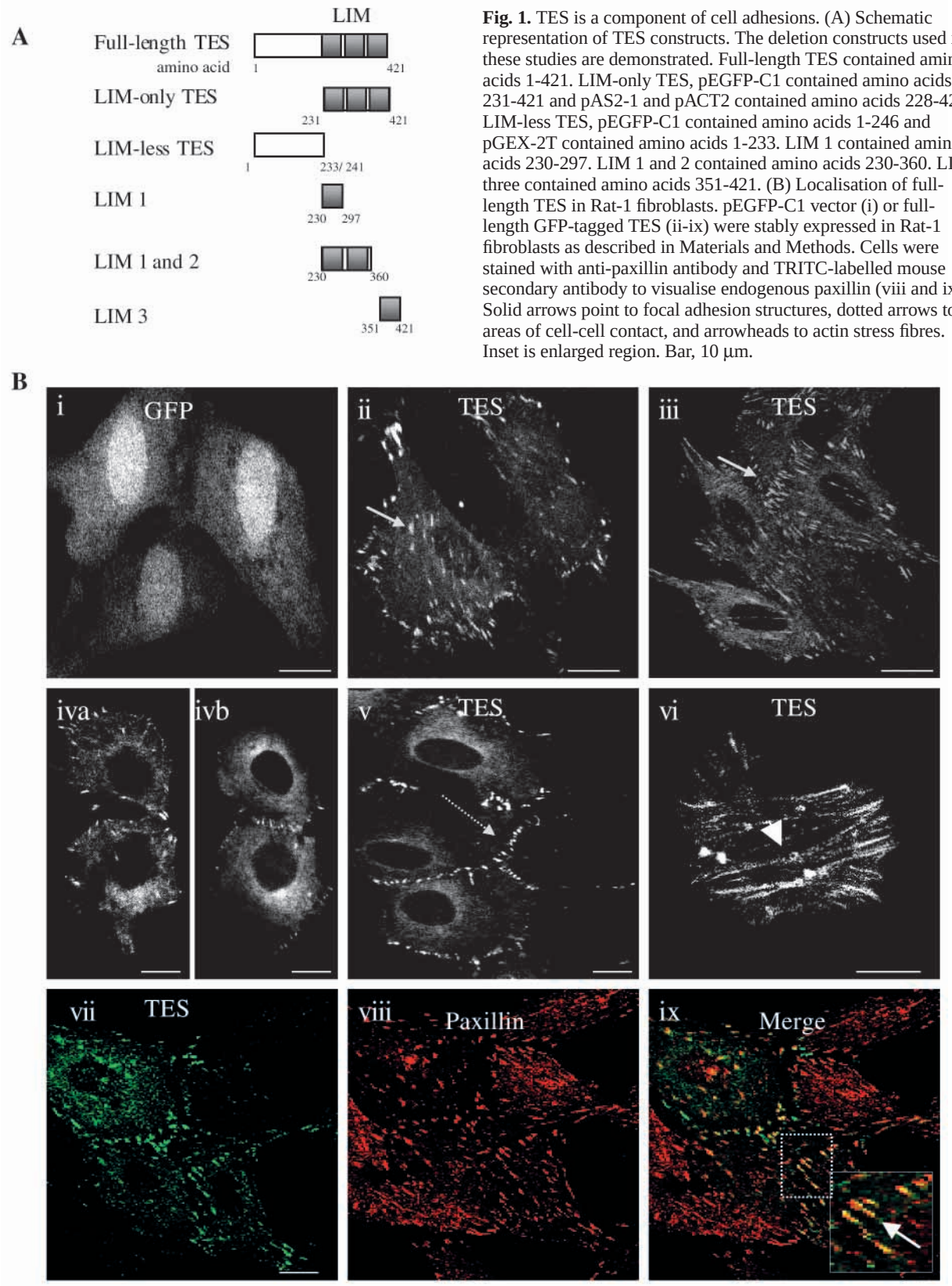

Fig. 1. TES is a component of cell adhesions. (A) Schematic representation of TES constructs. The deletion constructs used in these studies are demonstrated. Full-length TES contained amino acids 1-421. LIM-only TES, pEGFP-C1 contained amino acids 231-421 and pAS2-1 and pACT2 contained amino acids 228-421. LIM-less TES, pEGFP-C1 contained amino acids 1-246 and pGEX-2T contained amino acids 1-233. LIM 1 contained amino acids 230-297. LIM 1 and 2 contained amino acids 230-360. LIM three contained amino acids 351-421. (B) Localisation of full-length TES in Rat-1 fibroblasts. pEGFP-C1 vector (i) or full-length GFP-tagged TES (ii-ix) were stably expressed in Rat-1 fibroblasts as described in Materials and Methods. Cells were stained with anti-paxillin antibody and TRITC-labelled mouse secondary antibody to visualise endogenous paxillin (viii and ix). Solid arrows point to focal adhesion structures, dotted arrows to areas of cell-cell contact, and arrowheads to actin stress fibres. Inset is enlarged region. Bar, 10 μm.

nuclear GFP localisation (Fig. 1Bi). In addition, full-length TES localised to areas of cell-cell contact (Fig. 1Bivb,v). Note that focal adhesion localisation of full-length TES occurred at the basal surface of the cell (Fig. 1Biva), and adjusting the plane of focus revealed that full-length TES at areas of cell-cell contact was on the apical surface (Fig. 1Bivb). In some cells, full-length TES could also be observed along actin stress fibres (Fig. 1Bvi). Identical results were seen with both

transient and stably overexpressing His-tagged full-length TES in Rat-1 cells and in chick embryo fibroblasts (CEF) stably overexpressing myc-tagged full-length TES (data not shown). In addition, similar subcellular localisation of GFP-tagged TES was seen in several other cell lines stably overexpressing TES, including HT1080, HeLa and SUSM-1 (data not shown).

To confirm that TES was in fact localised to focal adhesion structures, cells stably overexpressing GFP-tagged full-length TES were stained with an antibody to the well characterised focal adhesion protein paxillin. As expected, endogenous paxillin strongly localised to distinct focal adhesions (Fig. 1Bviii), and full-length TES often colocalised with paxillin at focal adhesions (Fig. 1Bix), confirming that TES is a novel component of focal adhesions.

Additionally, the intracellular localisation of LIM-only TES (Fig. 1A) was determined. We observed that the LIM-only region of TES stably overexpressed in Rat-1 cells strongly localised to focal adhesions (Fig. 2A,D) where it colocalised with paxillin (Fig. 2F). The LIM domains alone localised to areas of cell-cell contact (Fig. 2B,C); however, we did not observe localisation along actin stress fibres (Fig. 2A-D). By contrast, removing the LIM-domains from TES (Fig. 1) resulted in the loss of the majority of focal adhesion localisation (Fig. 2G-I) with little colocalisation with paxillin at focal adhesions (Fig. 2M-O). The majority of LIM-less TES localised throughout the cell along actin stress fibres (Fig. 2G,H,J,M) where it colocalised with actin (Fig. 2J-L). There was also some localisation of LIM-less TES at areas of cell-cell contact (Fig. 2I). Similar results were seen with Rat-1 cells transiently and stably expressing His-tagged LIM-less TES (data not shown).

Together, these data demonstrate that the LIM-domains of TES contain information important for targeting full-length TES to focal adhesions, and both the LIM domains and the LIM-less region of TES can be targeted to regions of cell-cell contact. Note that there were no gross qualitative differences in focal adhesion appearance in any of the TES stable cell lines that we have seen. In addition, our data suggest that the LIM-less region of TES contains sequences that may be involved in targeting TES to actin stress fibres. Co-staining Rat-1 cells stably overexpressing GFP-tagged full-length TES with phalloidin demonstrated that TES localised predominantly to the tips of actin stress fibres found at both focal adhesions and sites of cell-cell contact (Fig. 3A). Treatment of Rat-1 cells with the actin-disrupting agent latrunculin B prevents the formation of stress fibres and prevents localisation of TES to focal adhesions and regions of cell-cell contact (Fig. 3B), which suggests that the localisation of TES is actin dependent. Similar results were seen with cytochalasin B treatment (data not shown).

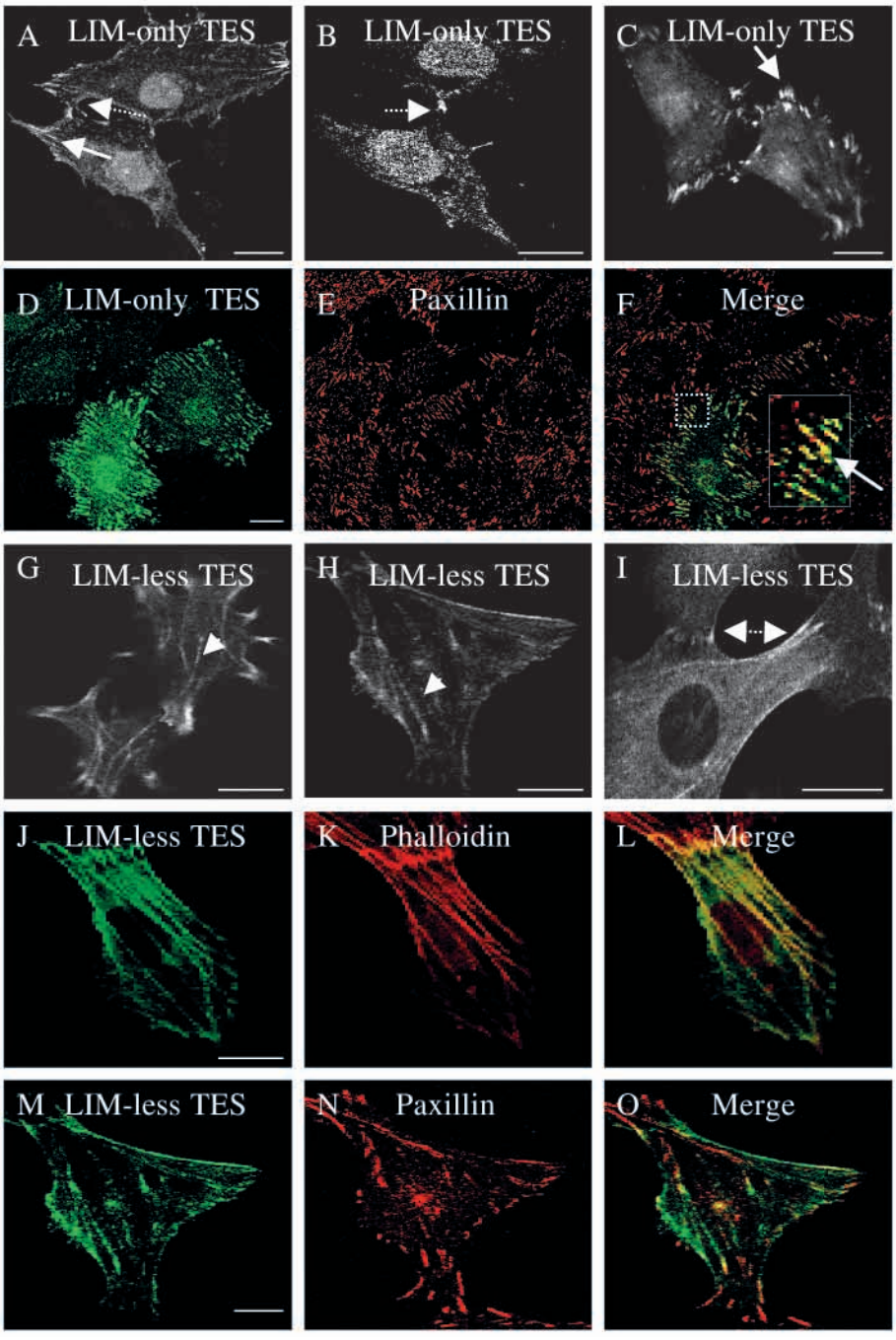

Fig. 2. Localisation of LIM-less and LIM-only regions of TES. Rat-1 fibroblasts stably expressing GFP-tagged LIM-only TES (A-F) or GFP-tagged LIM-less TES (G-I). Cells were stained with anti-paxillin antibody and TRITC-labelled mouse secondary antibody to visualise endogenous paxillin (E,F,N,O) or TRITC-labelled phalloidin to visualise actin stress fibres (K,L). Solid arrows point to focal adhesions, dotted arrows to areas of cell-cell contact and arrowheads to actin stress fibres. Bar, 10 μm.

Furthermore, GST-tagged TES can precipitate endogenous actin from HeLa whole cell extracts, suggesting that TES may interact with actin (Fig. 4H).

Yeast two-hybrid screens to identify potential TES interacting proteins

In order to identify TES-interacting proteins, yeast two-hybrid screens were performed using full-length human TES as bait to screen both a human mammary gland and a mouse testis cDNA library. Table 1 summarises some of the proteins identified after screening approximately 7×10^6 transformants from the two cDNA libraries as described in the Materials and Methods. Most of the proteins identified in the screens were focal-adhesion- and/or cytoskeleton-related proteins, supporting our data on the localisation of TES. Interestingly, two of the TES-interacting clones from the human library encoded TES from amino acid 25 onwards (Table 1A) and one of the TES-interacting clones from the mouse library encoded mouse Tes from amino acid 189 onwards (Table 1B). The mouse *Tes* gene has previously been cloned (Divecha and Charleston, 1995), but no function has thus far been described. The interaction between full-length TES and TES in yeast suggests that it may interact with itself, and data we have obtained from yeast mating experiments and in vitro pull-down assays suggest that the LIM domains of TES are able to interact with the LIM-less region of TES (data not shown).

TES interacts with the adhesion proteins mena, zyxin and talin

TES interacted with several well characterised focal adhesion proteins in yeast two-hybrid assays (Table 1), which supports our findings that TES is localised to focal adhesions. One of the TES-interacting clones encodes zyxin from amino acid 381 onwards (Table 1A). Zyxin is a 572 amino acid, low abundance focal adhesion protein that, like TES, contains three C-terminal LIM domains (Macalma et al., 1996). GFP-tagged TES colocalised with endogenous zyxin at focal adhesions and areas of cell-cell contact in Rat-1 cells (Fig. 4A). In addition, strong colocalisation was seen with endogenous zyxin in primary CEF cells stably expressing myc-tagged full-length TES (data not shown). Despite a positive interaction in yeast and colocalisation of TES and zyxin in vivo, an interaction of the zyxin with full-length TES in either immunoprecipitation or GST pull-down assays was not consistently observed despite exhaustive efforts. Interestingly, we found that endogenous zyxin from HeLa cell extracts interacted most strongly with the isolated GST-tagged LIM domain 1 of TES (Fig. 4C). This interaction appears to be direct as in-vitro-translated zyxin interacts with GST-tagged LIM 1 (Fig. 4D). Given the fact that the LIM domains of TES can interact with the N-terminal LIM-less region (Table 1; data not shown), it is likely that either an intramolecular interaction or conformational change in TES accounts for the difficulties that we have observed in identifying an interaction between full-length TES and zyxin.

Several TES-interacting clones were identified that encoded amino acid 2 onwards of mena (Table 1B). Mena [mammalian Enabled (Ena)] belongs to the Ena/VASP (vasodilator-stimulated phosphoprotein) protein family (Gertler et al., 1996) and localises to focal adhesions and adherens junctions (Gertler et al., 1996; Vasioukhin et al., 2000). Immunofluorescence studies demonstrated that mena colocalised with GFP-tagged full-length TES in Rat-1 fibroblasts at focal adhesions and at areas of cell-cell contact (Fig. 4B). GST-tagged full-length TES precipitated endogenous mena and VASP from HeLa cell extracts (Fig. 4E,F). The fact that mena interacts

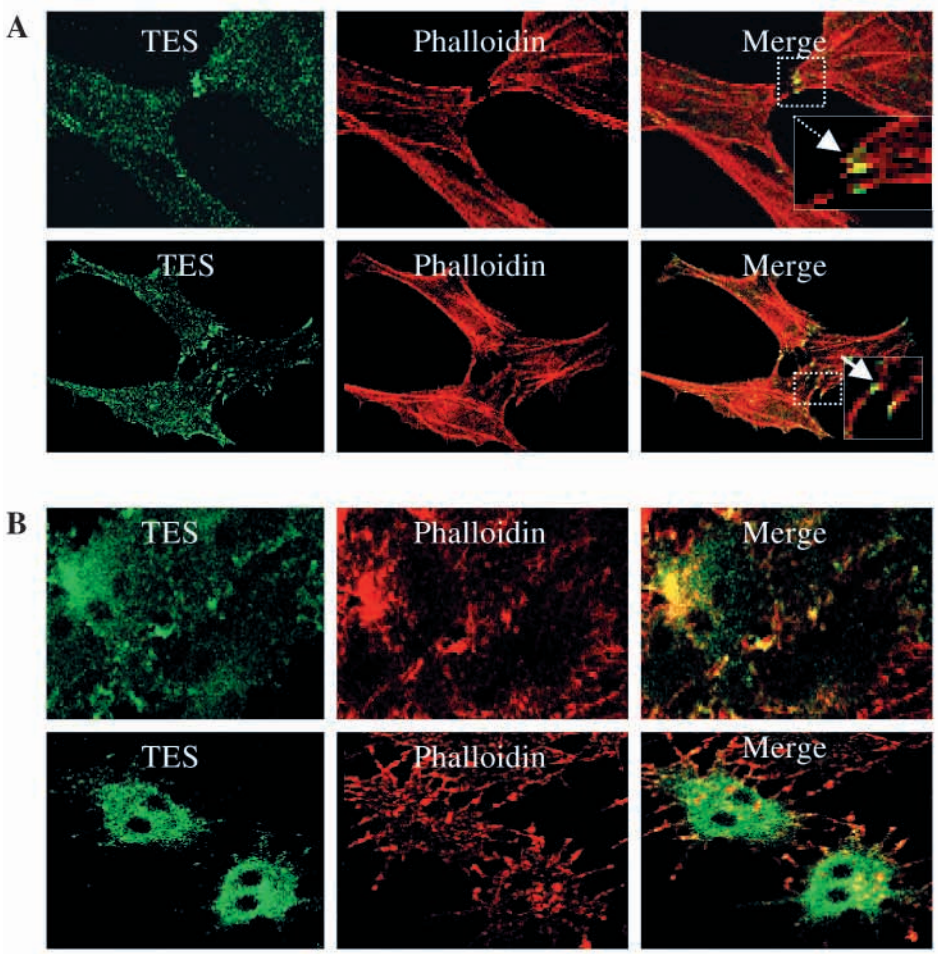

Fig. 3. TES localises to the tips of actin filaments. (A) Rat-1 fibroblasts stably expressing GFP-tagged full-length TES were stained with TRITC-labelled phalloidin to visualise actin stress fibres. Solid arrows indicate focal adhesions; dotted arrows indicate areas of cell-cell contact. Insets are enlarged regions as denoted. (B) Actin stress fibres were disrupted by treating Rat-1 fibroblasts stably expressing GFP-tagged full-length TES with 2 μM (top panel) or 10 μM (lower panel) latrunculin B for 24 hours. Cells were then fixed and stained with TRITC-labelled phalloidin.

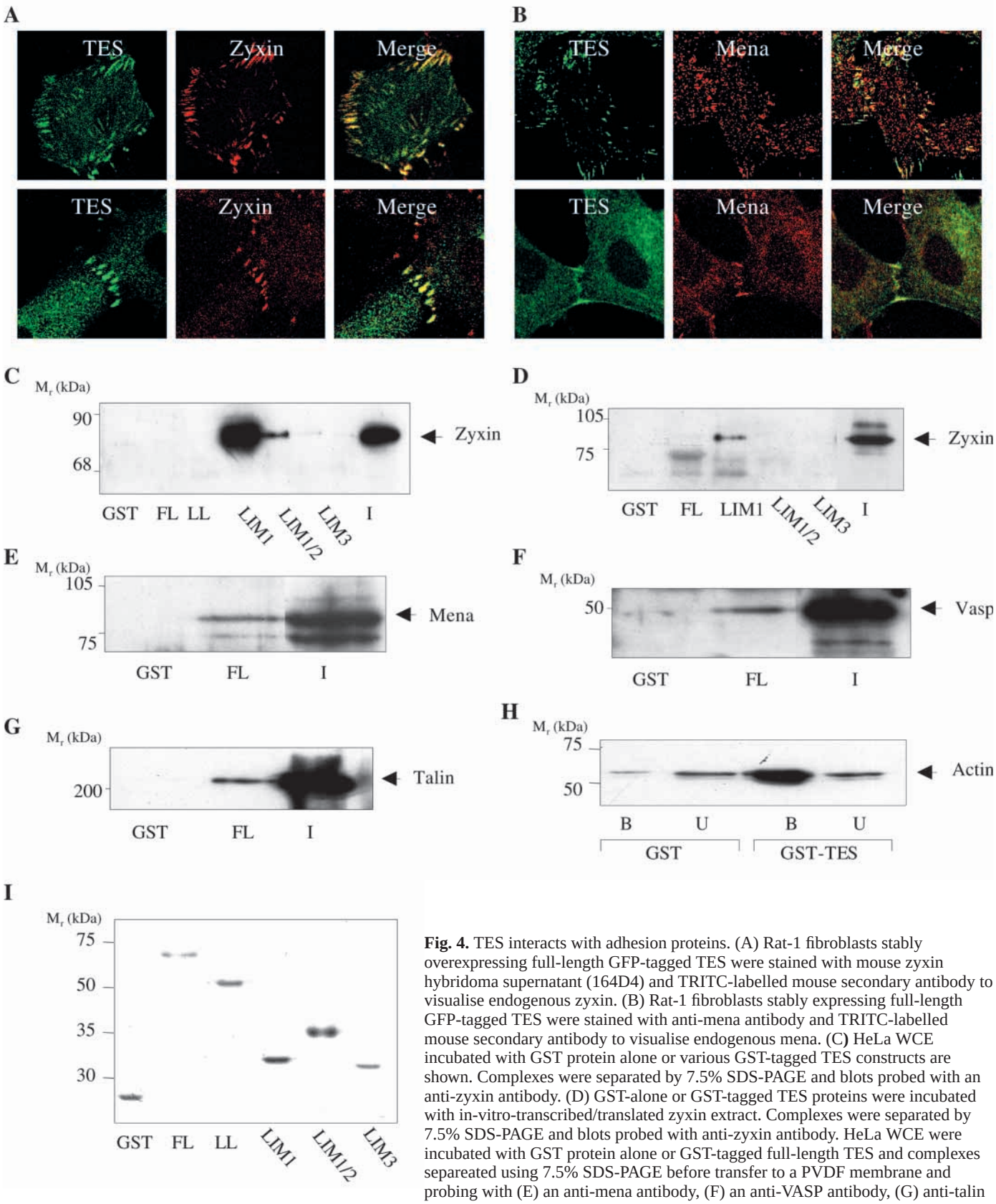

Fig. 4. TES interacts with adhesion proteins. (A) Rat-1 fibroblasts stably overexpressing full-length GFP-tagged TES were stained with mouse zyxin hybridoma supernatant (164D4) and TRITC-labelled mouse secondary antibody to visualise endogenous zyxin. (B) Rat-1 fibroblasts stably expressing full-length GFP-tagged TES were stained with anti-mena antibody and TRITC-labelled mouse secondary antibody to visualise endogenous mena. (C**)** HeLa WCE incubated with GST protein alone or various GST-tagged TES constructs are shown. Complexes were separated by 7.5% SDS-PAGE and blots probed with an anti-zyxin antibody. (D) GST-alone or GST-tagged TES proteins were incubated with in-vitro-transcribed/translated zyxin extract. Complexes were separated by 7.5% SDS-PAGE and blots probed with anti-zyxin antibody. HeLa WCE were incubated with GST protein alone or GST-tagged full-length TES and complexes separeated using 7.5% SDS-PAGE before transfer to a PVDF membrane and probing with (E) an anti-mena antibody, (F) an anti-VASP antibody, (G) anti-talin antibody or **(**H**)** an anti-actin antibody. (I) GST and GST-tagged proteins used in the pull-down assays were run on 10% SDS-PAGE and stained with Coomassie brilliant blue to visualise protein bands. Positions of molecular weight markers (Amersham) are shown. GST, GST control; FL, GST-tagged full-length TES; LL, GST-tagged LIM-less TES; LIM1, GST-tagged 1st LIM domain; LIM1/2, GST-tagged 1st and 2nd LIM domains; LIM3, GST-tagged 3rd LIM domain; I, input (10% of input WCE was run alongside as a comparison). B, bound protein; U, unbound.

Table 1. TES interacting proteins identified in yeast

Protein	Region (starting amino acid)	Number of clones	Description
A*			
TES	25	2	LIM-domain protein
Zyxin	381 (start of 1st LIM domain)	1	LIM domain focal adhesion protein, in cytoskeleton which is thought to function and cell-signalling events
B†			
Talin	1541	3	Focal adhesion protein
Mena‡	2	9	Focal adhesion protein
Actin-like 7A§	1	200	Actin-related protein, function unknown
GRIP1	28	7	Plasma membrane-associated protein.
Tes¶	189 (just before the 1st LIM domain)	1	LIM domain protein, which function unknown

*Screen performed using a human mammary cDNA library. †Screen performed using a mouse testis cDNA library. ‡Insert contained approximately 60 amino acids before amino acid 2 of Mena, which may represent an alternative first exon. §Name refers to the human homologue. ¶We have recently submitted a corrected mouse *Tes* sequence to the GenBank/EMBL database (accession number AJ344065).

directly with zyxin (Drees et al., 2000; Gertler et al., 1996) suggests that TES may form part of a complex containing both zyxin and mena.

Furthermore, a number of TES-interacting clones from the mouse testis library encoded talin, a 225 kDa phosphoprotein found in adhesion complexes (Critchley et al., 1999). Talin was present in GST-TES complexes obtained after incubating TES fusion proteins with HeLa cell extracts (Fig. 4G). Currently, we are delineating the regions of TES that are important for these interactions as well as investigating whether these interactions are direct.

TES affects cell spreading

Rat-1 fibroblasts stably overexpressing GFP-tagged TES constructs (Fig. 1A) were generated to identify the cellular function of TES. Although cells stably expressing TES were obtained, we found that initially fewer full-length TES-expressing clones grow in comparison with vector controls, suggesting that overexpression of TES has a detrimental effect on the cell. Indeed, previously we showed that TES can have a growth inhibitory effect, as measured by colony forming assays (Tobias et al., 2001). Despite this, once stably overexpressing cells were obtained there appeared to be no obvious differences in growth parameters as measured by doubling times and cell cycle analysis (data not shown) nor any obvious phenotypic differences or differences in the ability of transfected cells to invade matrigel (data not shown).

Since TES localises to areas of actin-based structures we were interested in examining its effects on cell adhesion as measured initially by cell spreading on fibronectin. Fig. 5 demonstrates that in the early stages of spreading on fibronectin, Rat-1 fibroblasts stably expressing TES have a distinctly altered morphology. Full-length TES-overexpressing cells spread with an enhanced rate, appear larger and contain increased numbers and lengths of cell protrusions, an effect that persists for several hours after re-seeding (Fig. 5A). To ensure that the effects we observed were not due to an inability to visualise the entire GFP-vector-expressing cells (due to the differential localisation of GFP and GFP-TES), similar experiments were performed and the cells were stained with TRITC-labelled phalloidin to enable complete visualisation of both the GFP and full-length TES-expressing cells. Again we observed that TES overexpressing fibroblasts appeared larger and more spread on fibronectin compared with the vector-transfected control cells and contained increased numbers of actin-based protrusions (Fig. 5B). TES overexpression appears not to have an effect on the percentage of cells spread (data not shown); instead it appears to affect the degree of cell spreading. The proportion of enlarged TES-expressing Rat-1 fibroblasts was significantly increased compared with GFP-expressing cells (Fig. 5C). Interestingly, LIM-less and LIM-only TES-expressing cells were not as enlarged and protrusive as the full-length TES-expressing cells (data not shown). Similar results were also obtained with CEF cells stably overexpressing myc-tagged TES (data not shown). The effects of TES on cell spreading appear to be transient, as the cells growing normally do not appear to be different in size, have no gross observable differences in the level or distribution of F-actin and do not appear to contain altered focal adhesions (data not shown). Therefore, increased levels of TES appear to increase initial cell spreading on a fibronectin matrix and, together with the localisation of TES, would suggest a role for TES in actin dynamics and/or cell adhesion, a hypothesis that is currently being tested.

Discussion

Evidence for the location of a multi-tissue tumour suppressor gene at human chromosome 7q31 was provided by the frequent loss of heterozygosity (LOH) in a variety of tumour types. In addition, functional studies mapped a senescence gene to this region. We carried out analyses of human chromosome 7q31.1 in order to identify the putative senescence and/or tumour suppressor gene(s) believed to map to this region (Black, 1999) and have so far reported the detailed characterisation of three candidate tisssue suppressor genes mapping to this region (Hughes et al., 2001; Hurlstone et al., 1999; Tobias et al., 2001). We observed promoter methylation of the *TES* gene in the vast majority of human ovarian tumours and tumour-derived cell lines of diverse origin (Tobias et al., 2001). Further, expression of the TES protein in tumour-derived cell lines resulted in significant growth suppression (Tobias et al., 2001). We therefore began to functionally characterise TES, and, as a first step, we report here the subcellular localisation of TES and the identification of TES-interacting proteins.

We have presented data demonstrating that TES is a novel focal adhesion protein. In addition, several well characterised focal adhesion proteins interact with and colocalise with TES. One of the TES-interacting proteins we identified in yeast is zyxin, which, like TES, contains three C-terminal LIM domains. Zyxin is a low-abundance phosphoprotein that has been localised not only to focal adhesions but also to adherens junctions, actin stress fibres and the nucleus (Beckerle, 1986; Nix and Beckerle, 1997; Vasioukhin et al., 2000). Zyxin may play a role in actin assembly (Golsteyn et al., 1997), cell motility (Drees et al., 1999) and intracellular communication (Nix and Beckerle, 1997). Several zyxin-binding partners have been previously identified including mena (Drees et al., 2000; Gertler et al., 1996). Mena is a member of the mena/VASP protein family; members of this family localise to regions of dynamic actin formation within cells and are present at focal adhesions, at the tips of filopodia and the leading edge of lamellipodia, and at adherens junctions (Gertler et al., 1996; Reinhard et al., 1992). Mena/VASP proteins interact with actin as well as the actin-monomer-binding protein profilin and play a role in actin assembly (Bachmann et al., 1999; Bear et al., 2000; Drees et al., 2000; Gertler et al., 1996). Studies have also demonstrated an important role for mena/VASP proteins in cell motility. Rottner et al. demonstrated that GFP-tagged VASP accumulates at the lamellipodial front in melanoma cells and this closely correlates with the protrusion rate (Rottner et al., 1999). In addition, fibroblasts lacking endogenous mena/VASP proteins migrate more slowly than cells re-expressing mena (Bear et al., 2000). More recently, it has been shown that mena/VASP promotes actin filament elongation within lamellipodia to control cell motility (Bear et al., 2002).

The interaction of zyxin with mena/VASP family members also suggests a role for zyxin in actin dynamics. In fibroblasts, membrane targeting of zyxin resulted in increased actin-rich

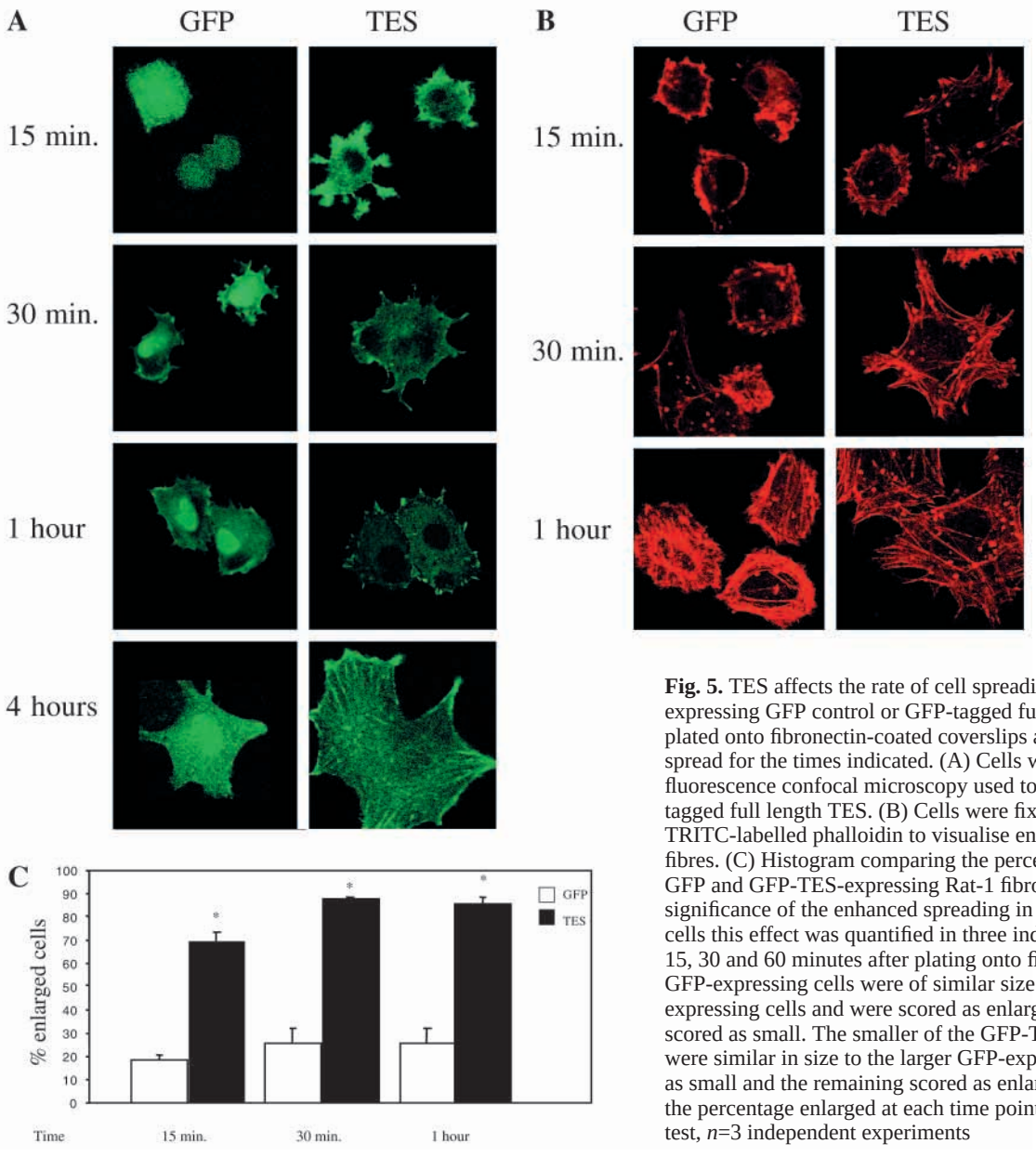

Fig. 5. TES affects the rate of cell spreading. Rat-1 fibroblasts stably expressing GFP control or GFP-tagged full-length TES or were re-plated onto fibronectin-coated coverslips and allowed to adhere and spread for the times indicated. (A) Cells were fixed and direct fluorescence confocal microscopy used to visualise GFP or GFP-tagged full length TES. (B) Cells were fixed and stained with TRITC-labelled phalloidin to visualise endogenous actin stress fibres. (C) Histogram comparing the percentage of enlarged cells in GFP and GFP-TES-expressing Rat-1 fibroblasts. To determine the significance of the enhanced spreading in the GFP-TES-expressing cells this effect was quantified in three independent experiments at 15, 30 and 60 minutes after plating onto fibronectin. The largest GFP-expressing cells were of similar size to smaller GFP-TES-expressing cells and were scored as enlarged and the remaining scored as small. The smaller of the GFP-TES-expressing cells, which were similar in size to the larger GFP-expressing cells, were scored as small and the remaining scored as enlarged. The results represent the percentage enlarged at each time point. *P<0.002, Student's t-test, n=3 independent experiments

surface structures. This effect was believed to be due to the ability of zyxin to bind mena/VASP proteins, as cells containing a zyxin mutant that was unable to interact with mena/VASP had a reduced number of actin-rich surface projections (Drees et al., 2000). More recently, Fradelizi et al. have demonstrated that zyxin is capable of inducing actin polymerisation, which is dependent on VASP, but independent of the Arp2/3, actin polymerisation complex (Fradelizi et al., 2001). Interestingly, Drees et al. have demonstrated that perturbations of the zyxin-mena/VASP interaction that result in the mislocalisation of mena/VASP reduce the degree of fibroblast spreading on fibronectin (Drees et al., 2000).

Whether or not TES has a function in actin dynamics remains to be elucidated, but the fact that fibroblasts overexpressing TES can spread to an enhanced degree and contain increases in actin-based protrusions suggests that TES, similar to its binding partners, functions in actin dynamics. Intriguingly, we have also identified an actin-related protein, actin-like 7A (ACTL7A) (Table 1), as the most frequent TES-interacting protein from our yeast two-hybrid screens. ACTL7A, identified by Chadwick et al., is predicted to encode a 48.6 kDa protein with more than 40% amino-acid identity to members of the actin gene family (Chadwick et al., 1999), which suggests that, similar to other actin-related proteins, it may play a role in actin dynamics.

Talin is thought to play a role in focal adhesion formation and in linking integrins to the actin cytoskeleton. Several binding partners for talin have been described, including integrins, focal adhesion kinase, vinculin (which can interact with VASP) and F-actin (Buck and Horwitz, 1987; Burridge and Mangeat, 1984; Muguruma et al., 1990). Studies have demonstrated that downregulation of talin expression in HeLa cells result in a decreased rate of cell spreading (Albiges-Rizo et al., 1995). Similarly, in undifferentiated mouse embryonic stem cells, disruption of both copies of the talin gene resulted in cells that were unable to assemble focal adhesions and had defects in cell spreading (Priddle et al., 1998).

Binding of both mena and zyxin to TES suggests that TES may be part of a complex containing both of these proteins. In addition, the fact that talin interacts with VASP further links talin to a common complex. Interestingly, an interaction of zyxin with glutamate-receptor-interacting protein 1 (GRIP1) in yeast two-hybrid analysis was recently reported; the function of this interaction remains to be elucidated (Li and Trueb, 2001). We also identified GRIP1 as a potential TES-interacting protein in yeast (Table 1) and verified this interaction in biochemical analyses (data not shown), which further link TES and zyxin to a complex that may contain one or more common binding partners.

We have demonstrated that GFP-tagged TES localises to focal adhesions and that TES interacts with the well-described focal adhesion proteins, talin, zyxin and mena, to provide compelling evidence that TES is a novel component of focal adhesions. Focal adhesions are specialised structures that connect the actin cytoskeleton to the extracellular matrix (ECM) via transmembrane integrins. Integrin receptor engagement triggers multiple signal transduction pathways that are thought to affect cell motility and spreading as well as cell proliferation and apoptosis (Gumbiner, 1996; Shen and Guan, 2001). Cell adhesion and spreading are complex processes involving dynamic rearrangements of the actin cytoskeleton. How overexpression of TES affects these processes is not known, but studies are currently ongoing to identify a potential role for TES in actin dynamics.

Our data also provide evidence that TES, in common with its binding partners, is a constituent of cell-cell adhesion sites. The cadherin-catenin adhesion complex plays a role in tissue and organ morphogenesis as well as cell migration (Gumbiner, 1996; Vasioukhin and Fuchs, 2001). Vasioukhin et al. have demonstrated that in epithelial cells cadherin-mediated intercellular adhesion generates a two-rowed zipper of puncta and that mena, VASP and zyxin are recruited to these adhesion zippers and this is dependent on α-catenin (Vasioukhin et al., 2000). Adherence zippers are a precursor to adherens junctions (Vasioukhin et al., 2000) and these structures look quite similar to the puncta we see TES, zyxin and mena localised to in fibroblasts. This suggests that similar structures may occur in fibroblasts and that TES is a component of these structures. Indeed Ko et al. demonstrated that adherens junction structures are present in fibroblasts (Ko et al., 2000). Transient expression of TES in epithelial cells does not result in localisation of TES to adherens junctions (data not shown), which suggests either that the localisation of TES may be cell-type specific or other proteins not expressed in the cell lines we examined are involved in the subcellular localisation of TES.

Initially, we identified TES as a candidate tumour suppressor gene at 7q31.1, a region where LOH is frequently observed in a variety of cancers. Evidence also suggests that 7q31 harbours a potential tumour metastasis inhibitor (Ichikawa et al., 2000), and in prostate cancer increases in LOH at 7q31.1 were seen in metastatic lesions (Saric et al., 1999). In cancerous cells, the breakdown of adhesion structures is a necessary prerequisite for the invasive phenotype. It is therefore plausible given the subcellular localisation and protein interactions identified in this report to suggest that TES is involved in events related to cell motility and adhesion, and currently we are investigating this possibility.

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