A Role for Tissue Factor in Cell Adhesion and Migration Mediated by Interaction with Actin-binding Protein 280

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Abstract. Tissue factor (TF), the protease receptor initiating the coagulation system, functions in vascular development, angiogenesis, and tumor cell metastasis by poorly defined molecular mechanisms. We demonstrate that immobilized ligands for TF specifically support cell adhesion, migration, spreading, and intracellular signaling, which are not inhibited by RGD peptides. Two-hybrid screening identified actin-binding protein 280 (ABP-280) as ligand for the TF cytoplasmic domain. Extracellular ligation of TF is necessary for ABP-280 binding. ABP-280 recruitment to TF adhesion contacts is associated with reorganization of actin filaments, but cytoskeletal adaptor molecules typically found in integrin-mediated focal contacts are not associated with TF. Chimeric molecules of the TF cytoplasmic domain and an unrelated extracellular domain support cell spreading and migration, demonstrating that the extracellular domain of TF is not involved in the recruitment of accessory molecules that influence adhesive functions. Replacement of TF's cytoplasmic Ser residues with Asp to mimic phosphorylation enhances the interaction with ABP-280, whereas Ala mutations abolish coprecipitation of ABP-280 with immobilized TF cytoplasmic domain, and severely reduce cell spreading. The specific interaction of the TF cytoplasmic domain with ABP-280 provides a molecular pathway by which TF supports tumor cell metastasis and vascular remodeling.

THE coagulation protease cascade is initiated by the cell surface receptor tissue factor (TF).[1] TF is a type I transmembrane glycoprotein that is expressed either constitutively with cell-type specificity or induced in vascular cells in response to a variety of inflammatory stimuli, playing pathophysiological roles in thromboembolic disorders and the lethal coagulopathy of septicemia (Ruf and Edgington, 1994). The extracellular domain of TF has the fold of a typical type II cytokine receptor and binds the coagulation protease factor VIIa with subnanomolar affinity leading to allosteric activation of the protease to allow for efficient cleavage of protein substrates, mainly factor X (Martin et al., 1995). TF function is regulated by feedback inhibition involving a complex of the product factor Xa and a Kunitz-type inhibitor, TF pathway inhibitor (TFPI; Broze, 1995), that locks TF·VIIa in a stable and inactive quaternary complex. TFPI is found cell associated either bound to proteoglycans, such as syndecan 4 (Kojima et al., 1996), or through a mechanism involving glycosyl phosphatidylinositol (GPI) anchoring (Sevinsky et al., 1996). The latter mechanism directs the inhibited complex to caveolae, translocating TF from detergent-soluble to low density detergent-insoluble membrane domains, thus demonstrating a dynamic regulation of TF cell surface localization by specific complex formation.

TF contributes to the regulation of blood vessel development in early embryo genesis (Carmeliet et al., 1996). At these stages of development, the Tie receptor family as well as vascular endothelial cell growth factor (VEGF) and its receptors regulate the expansion and differentiation of hematopoietic precursors from blood islands into capillary plexus (Risau, 1997; Beck and D'Amore, 1997). As a consequence of increasing hypoxic stress (Maltepe et al., 1997), the capillaries mature into a vascular system that allows circulation between the embryo and the extraembryonic vasculature. The maturation of the yolk sac vasculature is critically dependent on matrix remodeling and migratory functions of cells, as demonstrated by the phenotype of the gene deletion of transforming growth factor β (Dickson et al., 1995) or its type II receptor (Oshima et

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1. *Abbreviations used in this paper*: ABP-280, actin-binding protein 280; FAK, focal adhesion kinase; GPI, glycosol phosphatidylinositol; IL-2, interleukin 2; sTF, soluble tissue factor; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TNFα, tumor necrosis factor α; VEGF, vascular endothelial cell growth factor.

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al., 1996), that possibly regulate adhesive events (Ignotz and Massagué, 1987) during remodeling of the fibronectin-rich matrix of the blood islands (George et al., 1993), as well as by the knockout of the $G\alpha_{13}$ subunit (Offermanns et al., 1997) that severely impairs cell migration in response to stimulation of G protein–coupled receptors.

The coagulation system, in particular TF, is contributing to the maturation of the yolk sack vasculature, and TF-deficient embryos have a significant reduction in mesenchymal cells (pericytes) that are normally dispersed between the TF-expressing endothelial and endodermal cells in the yolk sack (Carmeliet et al., 1996). TF may function through protease generation that activates the thrombin receptor, as suggested by the similar phenotype of deletions of both, the coagulation cofactor factor V, downstream of TF, and the thrombin receptor (Connolly et al., 1996; Cui et al., 1996). However, embryonic lethality in the case of TF is close to 100% as compared to a 50% penetrance in the other cases, indicating that the proteolytic pathway is only partially accounting for the functions of TF in vascular development.

TF has also been suggested to play a role in tumor angiogenesis, a multifactorial process of de novo vessel formation (Folkman, 1995; Folkman and D'Amore, 1996). TF expression by tumor cells has dramatic effects on the capability of the tumor to induce a supporting neovasculature (Zhang et al., 1994). The levels of TF expression appear to regulate transcription of the proangiogenic VEGF gene and the antiangiogenic thrombospondin gene. However, tumor associated endothelial cells also express TF (Contrino et al., 1996), likely a consequence of local VEGF production that is known to induce TF in endothelial cells (Clauss et al., 1990). Expression of TF in the angiogenic endothelial cells may be related to functional requirements of the sprouting and invading capillary during neovascularization of the tumor.

Whereas it is not known whether intracellular interactions of TF are important for the above mentioned biological scenarios, the short 21 residue cytoplasmic domain of TF clearly plays essential roles in tumor cell metastasis. Deletion of the cytoplasmic domain of TF that can be Ser phosphorylated in response to phorbolester stimulation (Zioncheck et al., 1992) completely abolishes prometastatic functions of TF in an in vivo model of melanoma cell hematogenous metastasis (Bromberg et al., 1995). Although it has been suggested by this study that the prometastatic functions are independent of TF's role in triggering coagulation, our recent data indicate that extracellular protease generation, possibly by cleaving protease-activated receptors on the tumor cell surface (Fischer et al., 1995), acts in concert with crucial prometastatic interactions of the cytoplasmic domain of TF (Mueller and Ruf, 1998).

Because of the essential role of the TF cytoplasmic tail in the metastatic process, we have focused on identifying binding proteins for the TF cytoplasmic domain to elucidate the molecular pathway by which TF may influence tumor cell biology. We here demonstrate a molecular link from TF to actin-binding protein 280 (ABP-280; Gorlin et al., 1990) that plays a crucial role in stabilizing the cortical actin cytoskeleton and in cell motility (Cunningham et al., 1992). ABP-280 further regulates activation of stress-activated protein kinases in response to tumor necrosis

factor α (TNFα) and lysophosphatidic acid (Marti et al., 1997), suggesting a template function allowing assembly of intracellular signaling pathways. We demonstrate that TF supports cell adhesion and migration through recruitment of ABP-280 to TF-mediated adhesion contacts. These data define a novel pathway for TF function in the complex process of tumor cell metastasis, and possibly in vascular remodeling during angiogenesis and embryonic development.

Materials and Methods

Reagents and Antibodies

Monoclonal antibodies 6B4 and 10H10 to TF (Ruf et al., 1991), 7G7B6 to the small subunit of the IL2-receptor, and W6/32 to a monomorphic determinant of HLA A–C (both from American Tissue Culture Collection, Rockville, MD) were produced in ascites and purified on immobilized protein A followed by ion exchange chromatography on MonoQ. Fab fragments of 6B4 were prepared from papain digests followed by ion exchange and gel filtration purification. (Fab')$_2$ fragments of 7G7B6 were generated by digestion with immobilized pepsin (Pierce, Rockford, IL) in 0.1 M acetate buffer, pH 3.5, followed by purification using sequential ion exchange and gel-filtration chromatography. Recombinant VIIa, full-length TF apoprotein, soluble extracellular domain of TF (sTF), and polyclonal antibodies to TF were as described (Ruf and Edgington, 1991; Sevinsky et al., 1996). Monoclonal antibodies from commercial sources: anti-FAK and anti-paxillin (Transduction Laboratories, Lexington, KY), anti-talin, anti-vinculin, and anti–mouse IgG coupled to agarose beads (Sigma Chemical Co., St. Louis, MO), anti–ABP-280 MCA 724 and 464S (Serotec, Washington DC), horseradish peroxidase conjugated to anti-phosphotyrosine 4G10 (Upstate Biotechnology Inc., Lake Placid, NY). Secondary antibodies and reagents for chemiluminescence detection were from Amersham Corp. (Arlington Heights, IL), and FITC- and TRITC-labeled donkey anti–mouse or donkey anti–rabbit F(ab') fragment from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Poly-L-lysine (300,000 mol wt) and cytochalasin D were from Sigma Chemical Co., rhodamine phalloidin and mounting solution (Slowfade Antifade) from Molecular Probes Inc. (Eugene, OR). Fibronectin was obtained from Collaborative Biomedical Products (Bedford, MA) and GRGDSP peptide from Life Technologies Inc. (Gaithersburg, MD). Purified ABP-280 was a generous gift of J. Hartwig (Brigham and Women's Hospital, Boston, MA; Gorlin et al., 1990).

Cell Adhesion and Spreading Assays

The spontaneously transformed human umbilical vein endothelial cell line ECV304 (CRL-1998), human umbilical vein endothelial cells HUV-EC-C (CRL 1730), and the human bladder carcinoma cell line J82 (HTB-1) were obtained from the American Tissue Culture Collection. Before adhesion and migration assays, cells were serum starved overnight in serum-free DME or M199 medium. To induce TF expression, HUV-EC-C and ECV304 cells were stimulated with 10 ng/ml TNFα for five hours before the adhesion assay. Polystyrene, nontissue culture-treated, 48-well cluster plates (Costar Corp., Cambridge, MA) were coated overnight at 4°C with 10 μg/ml fibronectin, 10 μg/ml poly-L-lysine, 50 μg/ml monoclonal antibody or Fab fragment, or 50 μg/ml VIIa in Tris-buffered saline, pH 8.5, 2 mM CaCl$_2$. Before use, the wells were washed with 10 mM Tris, 130 mM NaCl, pH 7.4, (TBS), and blocked with heat-inactivated 5% BSA in TBS. Serum-starved cells were harvested and resuspended in DME supplemented with 1% BSA, 2 mM CaCl$_2$, and penicillin/streptomycin (DME-BSA), and incubated for 30 min at room temperature. After gentle mixing, 50,000 cells/well were seeded onto plates for a 1 h adherence. Nonadherent cells were removed by gentle washing in DME-BSA. Cells with a spread-out morphology were counted by phase contrast microscopy and adhesion was quantified using a colorimetric assay performed in triplicates (CellTiter 96; Promega Corp., Madison, WI).

Cell Migration Assay

Cell migration assays were performed as described (Yebra et al., 1996). Briefly, the under surface of polycarbonate membranes in modified Boy-

den chambers, 6.5 mm diameter, 8 μm pore size (Transwell; Costar Corp.) was coated for 1 h at 37°C with 50 μg/ml antibody 6B4 or (Fab′)$_2$ fragments of 7G7B6 in RPMI, 10 mM Hepes, pH 7.4, or with Matrigel (Becton Dickinson Labware, Bedford, MA) diluted 1:100 in the same buffer. Membranes were blocked with 1% BSA in PBS and the lower compartments of the migration chamber were filled with RPMI, 10 mM Hepes, 0.5% BSA, pH 7.4., 4 × 10^4 cells per well of J82 cells or J82 cells stably transfected with the wild-type cytoplasmic domain/IL-2 receptor chimera in the same buffer were added to the upper compartment of the migration chamber. For blocking experiments, sTF was included in the upper and lower compartments. Migration chambers were incubated for 18 h at 37°C in 5% CO$_2$. After removing stationary cells from the upper side of the membrane with a cotton-tipped swab, migrated cells were fixed in 3.7% paraformaldehyde in PBS and stained with 1% crystal violet in 2% ethanol, 0.1 M borate, pH 9.0. Membranes were extensively washed, the dye eluted with 10% acetic acid for measurement of absorbance at 600 nm. Migration on BSA was subtracted from the data determined in triplicates.

Confocal Microscopy

Cells were allowed to adhere for 1 h on coverslips coated with fibronectin or VIIa as above, rinsed in ice cold TBS, and incubated for 1 h with polyclonal anti-TF antibody in TBS, 5% BSA at 4°C. The cells were then fixed and permeabilized in −20°C acetone for 2 min, followed by incubation with anti-TF and monoclonal antibodies to ABP-280 (MCA 464S), vinculin, talin, or paxillin at 4°C. After incubation with secondary antibodies for 1 h, coverslips were washed extensively and mounted in Slowfade. For dual staining of TF and F-actin, the monoclonal antibody 10H10 to TF was used in the primary staining, followed by simultaneous labeling with rhodamine phalloidin and FITC-labeled donkey anti–mouse F(ab′) fragment after fixation. Coverslips were analyzed by confocal laser scanning microscopy (MRC600; Biorad, Cambridge, MA) and fluorescence micrographs were photographed with a Focus Imagerecorder Plus (Foster City, CA) on Elite II 100 film (Eastman Kodak Co., Rochester, NY).

Analysis of FAK Phosphorylation

Cells were subjected to an adhesion assay, as described above, on 100-mm culture plates. The plates were carefully rinsed with TBS, 0.5 mM sodium vanadate, followed by lysis of the cells with 1 ml ice cold radioimmunoprecipitation buffer (RIPA: 10 mM Tris, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 1% SDS, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM PMSF, 1 mM EDTA, 20 mM NaF, 2 mM sodium vanadate, 10 mM tetrasodium pyrophosphate) at 4°C for 10 min. Cells were harvested by scraping, and the cell lysate was clarified by centrifugation at 13,000 g for 30 min. Lysates normalized for protein content were precleared with anti–mouse IgG agarose and immunoprecipitated with anti-FAK for 4 h at 4°C. Immunoprecipitates were washed five times with RIPA, separated on reducing 8–16% Tris glycine gels for Western blotting onto Immunolon P (Millipore Corp., Milford, MA). Blots blocked with 5% BSA were probed with HRP-conjugated anti-phosphotyrosine monoclonal antibody in TBS-T buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20), followed by chemiluminescent detection. After stripping with 2% SDS, 0.1 M glycine, pH 2.6, for 1 h at 65°C, the blots were reprobed with anti-FAK monoclonal antibody to assure equal loading.

Yeast Two-Hybrid Screening

For two-hybrid screening (Fields and Song, 1989), we used the Matchmaker System 2 from Clontech Laboratories (Palo Alto, CA). A bait was constructed in pAS2-1 by fusing a short segment of the transmembrane domain (for helix propensity) and the entire TF cytoplasmic domain (residue 237–263) carboxyl to the DNA-binding domain of GAL4. To allow for identification of proteins that preferentially interact with the cytoplasmic domain posttranslationally modified through phosphorylation of one or more of the Ser residues, a second copy of the cytoplasmic domain that had all three Ser mutated to Asp was fused with a short linker (Pro-Ser-Ala-Ala-Glu) to the carboxyl terminus of the bait. The bait in strain Y190 was used to screen a human HeLa or a human lymphocyte (from EBV-transformed peripheral B cells) cDNA library in pGAD GH or pACT, respectively (Clontech Laboratories). Positive clones were rescreened with bait vector fusions with single copies of the TF wild type cytoplasmic domain (residues 237–263) or specific mutants. Control baits were β_2-integrin cytoplasmic domain in pGBT9 (kindly provided by T. O'Toole, The Scripps Research Institute, La Jolla, CA), GP Ibα cytoplasmic domain

(residues 507–610; Lopez et al., 1987) in pAS2-1, or vector pAS2-1 without insert. For β-galactosidase assays, a suspension starter culture was grown in Trp, Leu selective medium and used to inoculate yeast, peptone, dextrose medium to obtain a log phase culture that was harvested at an OD$_{600}$ of 0.6–1.0. Cells were washed and resuspended in Z buffer to a density of 10 OD/ml, lysed by freeze thawing, and the supernatant was analyzed for β-galactosidase activity with Galacto-light reagents (Tropix Inc., Bedford, MA).

Affinity Precipitation of ABP-280 by His-tagged Cytoplasmic Domain of TF

A BamHI/PstI fragment encoding the wild type or mutant cytoplasmic domain of TF was recovered from pAS2-1 and subcloned in frame into BamHI/PstI cut pTrcHisC (Invitrogen, Carlsbad, CA) to generate His-tagged fusion proteins, which were expressed in *Escherichia coli* strain M15 containing pREP4 (QIAGEN Inc., Chatsworth, CA) and purified on Ni-NTA agarose after cell lysis with 8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris, pH 8. After a final wash in the same buffer at pH 6.3, beads were used for precipitation experiments. J82 cells were lysed in precipitation buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM PMSF, 20 mM NaF, 2 mM sodium vanadate, 10 mM tetra sodium pyrophosphate), followed by centrifugation at 14,000 g to clear the lysate. The Ni-NTA beads were incubated with the cell lysate for 1 h at 4°C, followed by washes in precipitation buffer and resuspension in reducing sample buffer for electrophoresis and Western blotting with antibody MCA 724 to ABP-280 and with anti-Xpress (Invitrogen) to assure equal loading of the fusion proteins.

Surface Plasmon Resonance Analysis of ABP-280 Binding to TF

Binding of purified ABP-280 to TF was analyzed on a BIAcore 2000 instrument (Biacore, Inc., Piscataway, NJ). An antibody to the TF extracellular domain (10H10) was directly immobilized by coupling through free amino groups to a carboxylated dextran matrix activated with a mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N*′-[3-(diethylamino)propyl]carbodiimide. Full-length TF from recombinant expression in insect cells (Ruf and Edgington, 1991) or, as a control, the soluble TF extracellular domain that lacks the cytoplasmic domain of TF were captured by this antibody, followed by injection of either 500 nM VIIa or 900 nM ABP-280 in 10 mM Hepes, 150 mM NaCl, pH 7.4, 5 mM CaCl$_2$, 0.005% surfactant P20 at a 10 μl/min flow rate.

Determination of ABP-280 Concentration in J82 Cells

To determine the cellular concentration of ABP-280 and TF, serial dilutions of purified protein or J82 cells lysed in sample buffer were separated by SDS-PAGE, followed by Western blotting with anti–ABP-280 antibody MCA 724 or affinity-purified polyclonal anti-TF. The exposed films from the chemiluminescence detection were analyzed by densitometry. The dilution series of purified protein produced a linear range of intensities from which the ABP-280 and TF content of J82 cells was determined.

Extracellular Ligation and Immunoprecipitation of TF-associated ABP-280

To analyze TF extracellular ligation-dependent recruitment of ABP-280 in immunoprecipitation experiments, anti-TF monoclonal antibody 10H10 was covalently coupled to paramagnetic beads (tosylactivated Dynabeads M-450, Dynal Inc., Lake Success, NY) in 0.1 M phosphate buffer, pH 7.4. J82 cells or TNFα-stimulated ECV-304 cells were detached with trypsin/EDTA, washed once in serum containing medium and resuspended in Hepes-buffered saline (15 mM Hepes, 130 mM NaCl, pH 7.4). Cells (2–5 × 10^7/ml) were incubated with three times the number of antibody-coupled beads under gentle agitation for various times at 37°C to induce ligation-dependent clustering of TF or at 4°C to prevent cell surface receptor movement. Cells and beads were pelleted and the supernatant removed. Cells were then lysed on ice for 30 min in 20 mM Tris, pH 7.4, 5 mM EGTA, 1% Triton X-100, 50 mM NaCl, 100 μg/ml leupeptin, 2 mM PMSF, 20 mM NaF, 2 mM sodium vanadate, 50 μg/ml aprotinin, and 2 mg/ml DNAse I to depolymerize F-actin (Ohta et al., 1991). The immunoprecipitate was separated from the cell lysate by magnetic field and washed once in lysis buffer, nine times in lysis buffer without DNAse I and twice in detergent-free buffer before SDS-PAGE for Western blotting for TF and ABP-280.

Adhesion Assay with Interleukin 2 Receptor Chimeras

The small subunit of interleukin 2 (IL-2) receptor (the clone in pRSV was kindly provided by S. LaFlamme, Albany Medical College, Albany, NY) has Lys and Leu as the only cytoplasmic residues (LaFlamme et al., 1992). TF wild type or mutant cytoplasmic domain (residues 243–263) or the cytoplasmic domain of GP Ibα (residues 514–610, Lopez et al., 1987) were cloned in frame following the cytoplasmic residues of the original construct. J82 cells were transiently transfected with the chimeras using lipofectamine (Life Technologies Inc.). Forty-eight hours after transfection, cells were harvested with cell dissociation buffer (Life Technologies Inc.). Transfection efficiency was determined by FACS using 7G7B6 to the extracellular domain of the IL-2 receptor. For adhesion assays, cells were seeded onto plates coated with antibody 7G7B6 and allowed to spread for 1 h followed by a gentle wash to remove untransfected and nonadherent cells. Spreading was evaluated by phase contrast microscopy and adhesion by a colorimetric assay, as described above.

Results

TF-mediated Cell Adhesion and Spreading

J82 bladder carcinoma cells that constitutively express high levels of TF were found to adhere and spread on surfaces coated with monoclonal antibodies specific for the extracellular domain of TF. In a typical adhesion assay, serum-starved cells were detached and resuspended in serum-free medium, followed by seeding on protein-coated plastic dishes. After 1 h of incubation, unattached cells were removed by a gentle wash and plates were evaluated for adhesion and cell spreading. J82 cells did not adhere to BSA-blocked surfaces, but similar numbers of cells were found to attach to poly-L-lysine, to an antibody to a monomorphic HLA class I determinant, or to anti-TF antibody and natural ligands of TF (Fig. 1 *A*). The number of cells adhering to fibronectin was slightly higher and fibronectin adhesion was blocked by peptides containing an RGD sequence. Adhesion to VIIa, the physiological soluble ligand for TF, was not blocked by RGD peptides, but was inhibited by recombinant soluble extracellular domain (sTF) or by inhibitory monoclonal antibodies to TF (Fig. 1 *A*), both of which did not affect adhesion to fibronectin (data not shown). J82 cells adhered equally well to intact IgG and highly purified Fab fragments of anti-TF monoclonal antibody 6B4, excluding contributions of Fc-mediated effects to the adhesive event. Human umbilical vein endothelium derived ECV304 cells, stimulated with TNFα for maximum TF expression, also adhered to anti-TF monoclonal antibody 6B4 (Fig. 1 *B*). Adhesion to TF ligand, but not to fibronectin, was blocked by soluble TF extracellular domain and antibody in solution, demonstrating specificity.

As previously shown for other cell types, adhesion to the positively charged surface generated by coating with poly-L-lysine did not result in spreading of J82 cells, whereas J82 cells spread on fibronectin (Fig. 2 *A*). Spreading was also observed on plates coated with intact IgG and Fab fragments of anti-TF monoclonal antibody 6B4 (not shown) and the immobilized natural ligand for TF, VIIa. A RGD peptide known to block several matrix-binding integrins did not abolish spreading on VIIa (Fig. 2 *A*). It is notable that J82 cells spreading on TF ligand have a different morphology compared to cells adherent to fibronectin through integrins (Fig. 2 *A* and higher power views in Fig. 2 *B*). This suggests qualitative differences in the two adhesive events.

Spreading on all TF ligands was divalent cation dependent, as demonstrated by inhibition with 1 mM EDTA. Adhesion to VIIa was inhibited by sTF (Fig. 2 *A*) and by monoclonal antibody 6B4 to the VIIa binding site of TF (not shown; Ruf et al., 1991), demonstrating specificity. Monoclonal antibodies to other epitopes on TF also supported adhesion and spreading. However, immobilized anti-HLA antibody did not induce spreading (Fig. 2 *A*), indicating that receptor ligation per se is insufficient to induce actin-filament reorganization in J82 cells. The morphology of cells attached to this antibody was similar to cells adherent on VIIa in the presence of cytochalasin D, which prevents actin-filament assembly. These data indicate that actin polymerization occurs subsequently to TF-mediated cell attachment and emphasize that TF is capable of inducing cytoskeleton reorganization.

Spreading on TF ligand was not limited to J82 bladder carcinoma cells. The spontaneously transformed endothelial cell line ECV304 or human HUV-EC-C endothelial cells both adhered and spread on TF ligand, when stimulated with TNFα to induce TF. The morphology of endothelial cells spreading on TF ligand resembled the typical TF-mediated adhesion of J82 cells (Fig. 2 *B*). Endothelial cells thus specifically adhere to and spread on TF ligands.
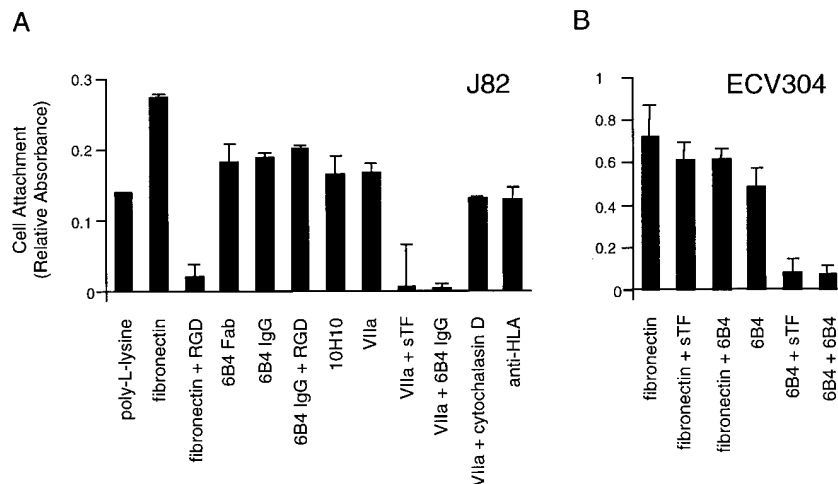
A

B

Figure 1. (*A*) Adhesion of J82 bladder carcinoma cells to the indicated immobilized ligands. For competition experiments, GRGDSP (+ *RGD*) at 1 mg/ml, soluble TF extracellular domain (+ *sTF*) at 100 μg/ml, 6B4 IgG to the VIIa-binding site on TF (+ *6B4 IgG*) at 50 μg/ml, or cytochalasin D (1 μM) were included during the adhesion assay. (*B*) Adhesion of TNFα-stimulated ECV304 endothelial cells to immobilized anti-TF antibody 6B4 or fibronectin in the absence of competitors or the presence of 60 μg/ml 6B4 IgG (+ *6B4*) or 50 μg/ml soluble TF extracellular domain (+ *sTF*).
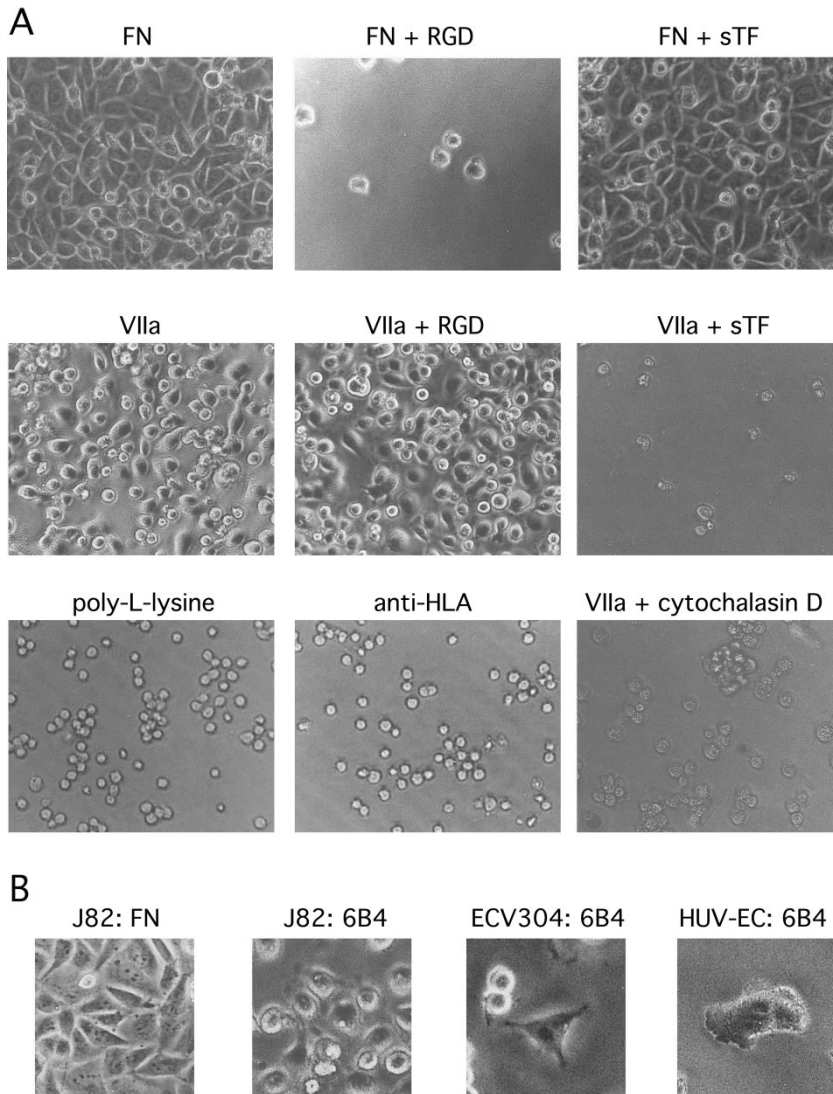
FN   FN + RGD   FN + sTF

VIIa   VIIa + RGD   VIIa + sTF

poly-L-lysine   anti-HLA   VIIa + cytochalasin D

B

J82: FN   J82: 6B4   ECV304: 6B4   HUV-EC: 6B4

Figure 2. (*A*) Representative overviews of J82 cells attached to the indicated immobilized ligand. For competition experiments, GRGDSP (+ *RGD*) at 1 mg/ml and soluble TF extracellular domain (+ *sTF*) at 100 µg/ml, or cytochalasin D (1 µM) were included during the adhesion assay. (*B*) High power views of the morphology of J82 cells on fibronectin or anti-TF antibody 6B4, and of TNFα-stimulated human umbilical vein endothelial cell derived ECV304 and HUV-EC adherent to 6B4.

TF-dependent Phosphorylation of Focal Adhesion Kinase (FAK)

Because the TF cytoplasmic domain does not contain a protein-kinase domain, we explored whether TF-dependent adhesion could induce the phosphorylation of FAK, a nonreceptor tyrosine kinase involved in cell adhesion. FAK was immunoprecipitated from cells adhering to different ligands, and tyrosine phosphorylation was determined by Western blotting with anti-phosphotyrosine antibody. When serum-starved J82 cells were kept in suspension or attached without spreading to poly-L-lysine or anti-HLA, FAK showed little tyrosine phosphorylation. Adhesion to monoclonal antibodies or Fab fragments specific for the TF extracellular domain induced phosphorylation of FAK to levels comparable to cells adherent to fibronectin (Fig. 3 *A*). The immobilized physiological ligand of TF, recombinant VIIa, similarly supported phosphorylation of FAK (Fig. 3 *B*). The phosphorylation of FAK was time dependent, reaching a maximum at 60–120 min (Fig. 3 *C*). Cells typically had a well spread morphology 2 h after seeding onto TF ligands. Actin polymerization was required for

FAK phosphorylation, as shown by the reduction in FAK phosphorylation in the presence of cytochalasin D to levels observed with anti-HLA that supported adhesion, but not spreading of J82 cells (Fig. 3 *D*). Consistently, ligation of TF by antibody on cells in suspension did not induce FAK phosphorylation (not shown). We conclude that phosphorylation of FAK follows the TF-dependent actin–filament organization, indicating that FAK activation is not an early event in TF-mediated adhesion.

TF-mediated Migration of J82 Cells

FAK plays a central role in migratory functions of cells (Ilic et al., 1995). We therefore tested whether the extracellular ligation of TF is sufficient for migration of J82 cells in a haptotactic migration assay with immobilized ligand. The lower part of a Boyden chamber was coated with reconstituted basement membrane (Matrigel) to support integrin-dependent migration or with monoclonal antibody 6B4 as a ligand for TF. J82 cells migrated to Matrigel leading to accumulation of cells in the lower compartment of the migration chamber after 16 h (Fig. 4). Cells also mi-
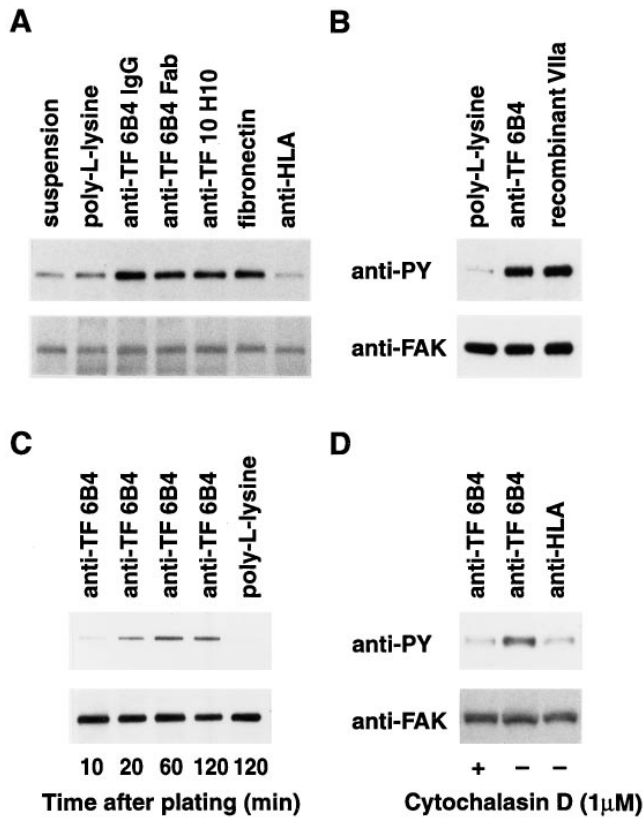
Figure 3. Phosphorylation of FAK following adhesion of J82 cells to various immobilized ligands. (*A*) Lack of FAK phosphorylation in cells on ligand that failed to support cell spreading (*poly-L-lysine*; *anti-HLA*). FAK is phosphorylated in cells spread on fibronectin or antibodies (IgG and Fab fragments) to TF. (*B*) Phosphorylation of FAK in cells spreading on immobilized VIIa, the physiological ligand for TF. (*C*) Time course of FAK phosphorylation on immobilized anti-TF. (*D*) Inhibition of FAK phosphorylation by cytochalasin D (1 μM) treatment of cells adherent on immobilized anti-TF.

Figure 4. Migration of J82 cells on immobilized anti-TF 6B4 (IgG) and Matrigel. Specificity of TF-dependent migration is demonstrated by the inclusion of soluble TF extracellular domain (sTF) at the indicated concentration in the upper and lower migration chamber.

grated to 6B4 and this migration was inhibited by 4 or 20 μM of recombinant sTF. We excluded nonspecific effects of sTF on cell migration, based on the unaltered migration to Matrigel. These data demonstrate that adhesive interactions of TF can support migratory functions of cells.

Identification of ABP-280 as a Ligand for the Cytoplasmic Domain of TF

TF has a short 21-residue cytoplasmic domain with no apparent homology to known sequence motifs. We used the yeast two-hybrid system to identify intracellular ligands for TF. From the screening of cDNA libraries established from HeLa cells and EBV-transformed B cells, we obtained two clones of different length that encoded for the carboxyl terminus of the 2647 residue containing ABP-280 (nonmuscle filamin), which was originally cloned from endothelial cells (Gorlin et al., 1990). The shorter clone encoded for the carboxyl-terminal half of the 24th β sheet structured repeat of ABP-280 (residues 2608–2647), whereas the longer clone initiated in repeat 22 (residues 2366–2647). The shorter clone of ABP-280 interacted specifically with
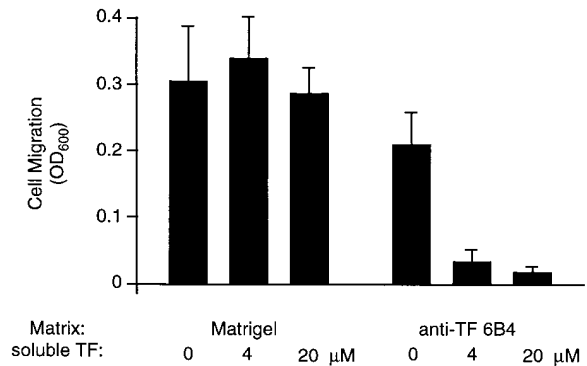
TF cytoplasmic domain constructs that mimicked phosphorylation by Asp for Ser substitution, but the longer clone that encoded repeats 22–24 demonstrated ~50-fold higher activity in the β-galactosidase assay. These data are consistent with a role for the 24th repeat in binding to the TF cytoplasmic domain, but the 23rd repeat and possibly dimer formation of the carboxyl terminus of ABP-280 (Gorlin et al., 1990) may contribute to affinity of the interaction. The clone that encoded for repeats 22–24 lacked nonspecific interaction in cells that harbored the DNA-binding domain vector or constructs that encoded the cytoplasmic domains of the β[2] integrin subunit or glycoprotein Ibα, the latter of which interacts with repeat 17–19 of ABP-280 (Meyer et al., 1997; Fig. 5 *A*). β-galactosidase activity with the cytoplasmic domain that incorporated Asp substitutions for each of the Ser residues was about threefold higher than with unmodified TF wild-type cytoplasmic domain. A mutant TF in which Ser[253] and Ser[258] were replaced by Ala showed even further decreased activity, consistent with the importance of these two residues and Ser phosphorylation of the cytoplasmic domain for the high affinity interaction with ABP-280.

Precipitation of Full-length ABP-280 by Immobilized Cytoplasmic Domain of TF

To test whether full-length ABP-280 can interact with the TF cytoplasmic domain, we expressed His-tagged fusion proteins of wild type and mutant cytoplasmic domains of TF in *E. coli* for affinity precipitation of ABP-280 from J82 cell lysates (Fig. 5 *B*). ABP-280 did not bind to the His-tagged peptide encoded by the vector polylinker sequence, but ABP-280 was detected in precipitates with wild type cytoplasmic domain of TF. Precipitation appeared to be enhanced with constructs that encoded Asp substitutions for each of the cytoplasmic Ser residues to mimic phosphorylation. Ala replacement for Ser[253] and Ser[258] essentially abolished binding of ABP-280 in the precipitation experiment, consistent with the suggested diminished affinity for the mutant in the two-hybrid assay. Taken together, these data indicate that Ser phosphorylation of the TF cytoplasmic domain can modulate affinity for ABP-280.
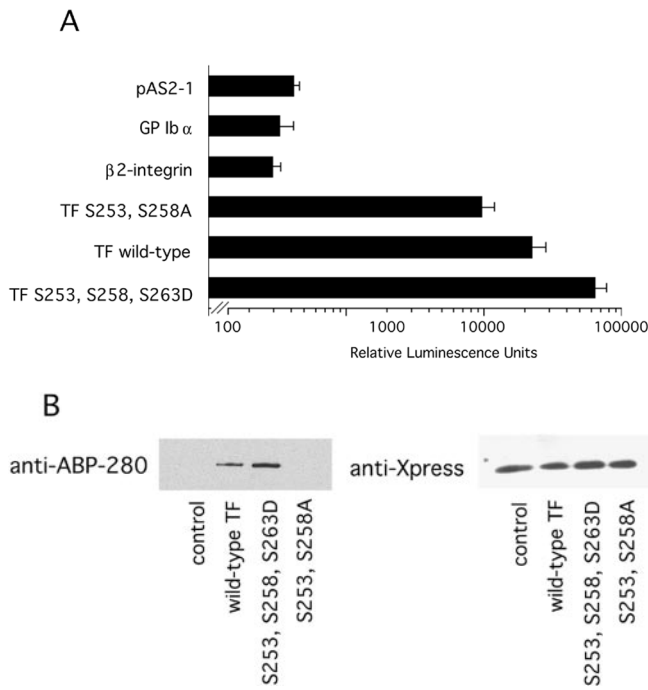
A

B

anti-ABP-280   anti-Xpress

A

B

Figure 5. (*A*) Interaction of the TF cytoplasmic domain with the carboxyl terminus of ABP-280. Yeast harboring the coding sequence for residues 2366–2647 of ABP-280 in pACT and the indicated cytoplasmic domain as carboxyl-terminal fusions of the GAL4 DNA-binding domain were tested for β-galactosidase activity. (*B*) Affinity precipitation of ABP-280 from J82 cell lysate using His-tagged fusion proteins of the TF cytoplasmic domain bound to Ni-NTA agarose. Associated ABP-280 was detected by Western blotting. Phosphorylation of the cytoplasmic Ser residues in TF was mimicked by Asp substitutions (*TF S253, 258, 263D*). Ala substitutions were introduced to evaluate the importance of Ser[253] and Ser[258] (*TF S253, 258A*). As control, the fusion protein created by the vector polylinker was used. Equal loading of the fusion proteins onto the Ni-NTA beads was confirmed by Western blotting with anti-Xpress antibody to an epitope in the fusion protein. The sizes of the TF cytoplasmic domain fusion proteins (61 residues) and of the control fusion protein (55 residues) were comparable.

Binding of Purified ABP-280 to Purified TF

The interaction of ABP-280 with TF was further characterized by surface plasmon resonance analysis using purified ABP-280, soluble TF extracellular domain (TF_{1-218}) and full-length, recombinant TF (Fig. 6 *A*). In these experiments, a noninhibitory monoclonal antibody to TF was immobilized on the sensorchip and used to capture either full-length TF or soluble TF extracellular domain that lacks the presumed ABP-280 interactive cytoplasmic domain. Both molecules bound VIIa, the extracellular ligand of TF (Fig. 6 *B*). ABP-280 at 900 nM bound only to full-length TF, but not the TF extracellular domain (Fig. 6 *B*). The response on the sensorgrams with ABP-280 was significantly lower than the response that was seen with the extracellular ligand VIIa, possibly indicating low affinity of the interaction under the experimental condition. It is conceivable that the cytoplasmic domain of recombinant TF lacks specific posttranslational modifications for high affinity binding or that only a fraction of the immobilized
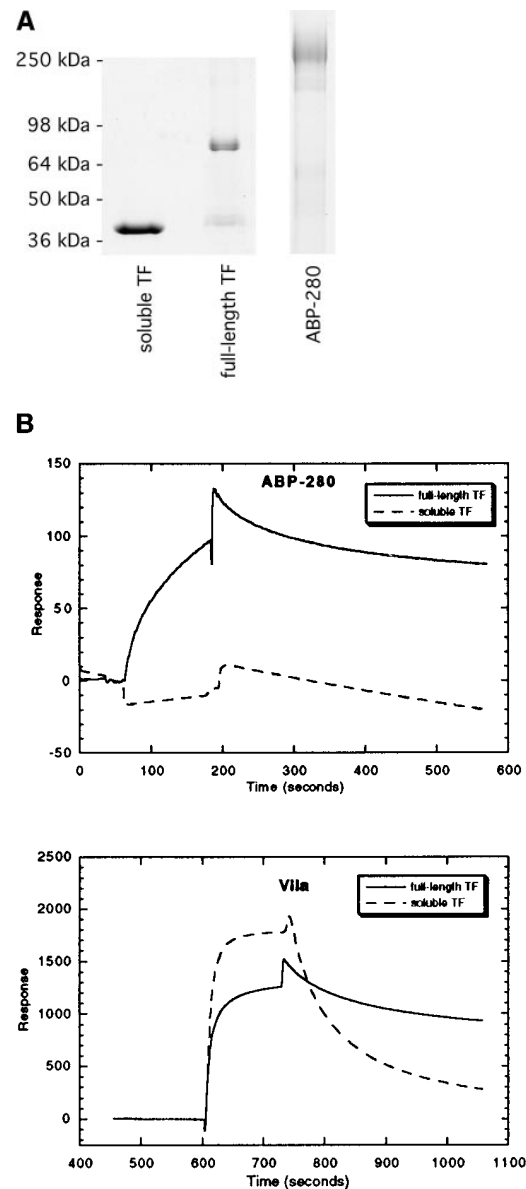
Figure 6. (*A*) Protein preparations used for the surface plasmon resonance experiment. 5 µg of protein was separated by SDS-PAGE under nonreducing condition and the gels were stained with Coomassie brilliant blue. Full-length TF is predominantly dimeric due to disulfide bond formation of the unpaired cytoplasmic cysteine residue. (*B*) Full-length TF or soluble TF extracellular domain (*soluble TF*) were captured by noninhibitory anti-TF monoclonal antibody (10H10) that was covalently bound to the sensorchip. Sensorgrams of injections of 500 nM VIIa or 900 nM ABP-280 onto the sensorchip are shown.

TF molecules is oriented properly for binding of ABP-280. We were limited by increasing nonspecific binding at higher concentrations of ABP-280 to more conclusively address the binding kinetics of ABP-280 and full-length TF. The cytoplasmic domain of TF contains one unpaired cysteine residue that frequently forms a disulfide bond with a second TF molecule during purification from overexpressing insect cells, resulting in the formation of dimers that are detectable as the predominant species in the pro-
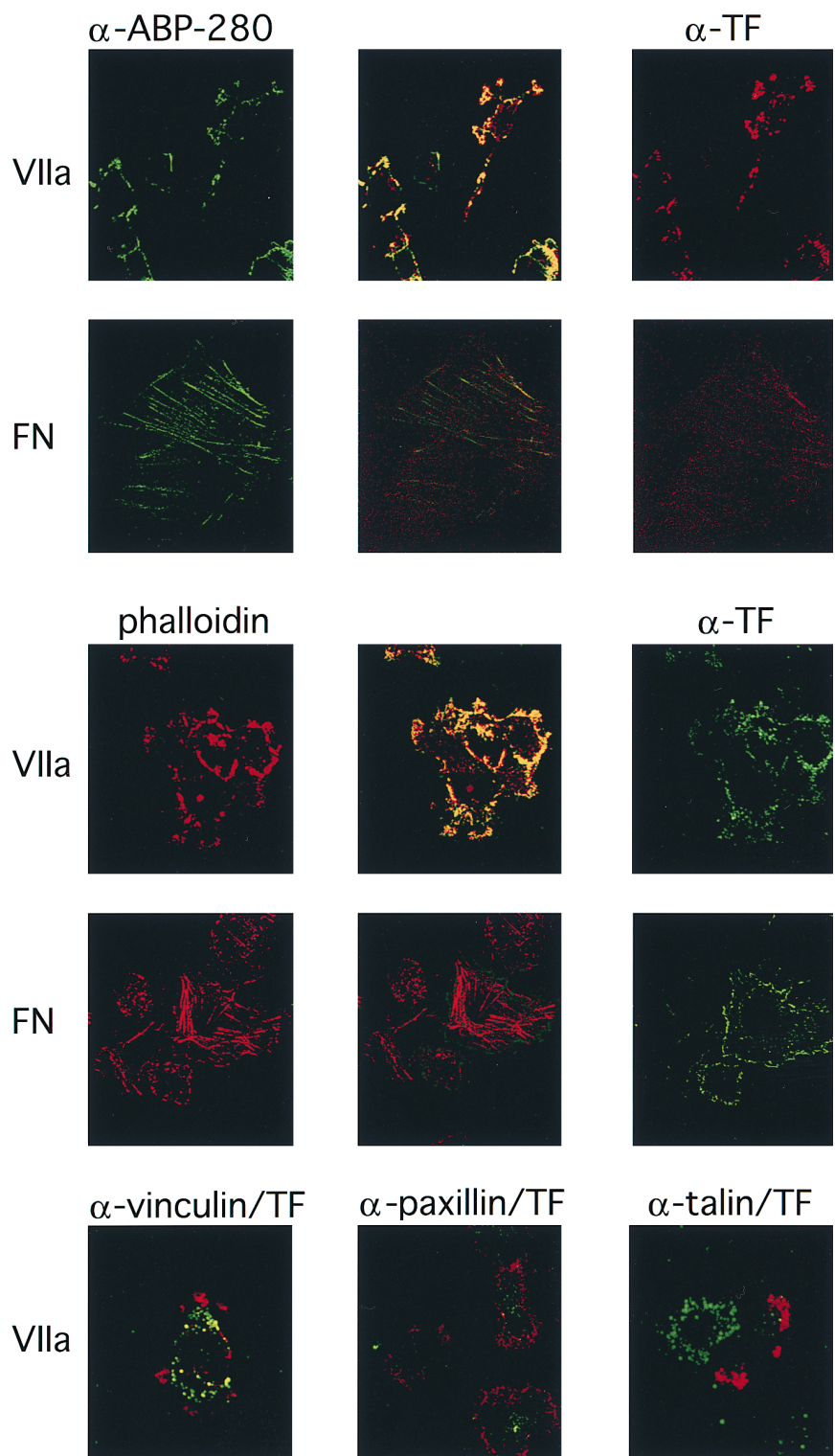
Figure 7. Cellular localization of proteins by confocal microscopy. Cells were seeded either on fibronectin or VIIa coated coverslips for 1 h, and stained for TF (*right column*) and ABP-280 or actin (*phalloidin*, *left column*). The center column shows the merged images. In experiments not depicted, ABP-280 and phalloidin staining were found to show almost complete overlap on cells adherent to both fibronectin and VIIa. In the bottom row, merged images only are shown for double staining for TF (*red*) and talin, paxillin, or vinculin, respectively, (*green*) in cells spreading on VIIa.

tein preparation under nonreducing conditions (Fig. 6 *A*). It is possible that the disulfide-linked species of TF does not bind ABP-280, but we had no monomeric preparation of TF of sufficient purity available to address this possibility. Notwithstanding these limitations, the presented data provide evidence that purified ABP-280 interacts with TF in vitro.

Localization of ABP-280 in TF-mediated Adhesion Contacts

The association of ABP-280 with TF in J82 cells was analyzed by dual labeling and confocal microscopy. We determined the concentration of ABP-280 and TF in J82 cells by Western blotting of a defined number of cells along with known amounts of each purified protein. J82 cells ex-

pressed 0.23 pmol TF and 1.1 pmol ABP-280 per 10^5 cells, corresponding to a 1:5 molar ratio of the proteins. When suspension cells were plated on VIIa, TF and ABP-280 colocalized at the adhesive contacts (Fig. 7). In contrast, only a minor fraction of ABP-280 showed colocalization with TF in cells that spread on fibronectin. ABP-280 in these cells generally did not localize to adhesion plaques, but predominantly associated with filaments in the interior of the cell (Fig. 7). This typical localization of ABP-280 was seen at different optical sections of cells adherent to fibronectin (Fig. 8). In cells that spread on VIIa, ABP-280 was abundant in proximity to the adhesion plane, whereas staining was sparse in more apical sections which showed ABP-280 in a predominant subcortical localization (Fig. 8).

Adhesion through TF thus results in recruitment of a significant portion of the cellular ABP-280 to the contact sites. At these sites, actin reorganized into a submembraneous network that colocalized with TF. Typical stress fibers, as observed in fibronectin-adherent cells, were not seen (Fig. 7). In cells adherent on fibronectin, TF did not colocalize with actin or proteins that localize to focal contacts, including talin and paxillin (data not shown). Talin and paxillin did not localize to TF adhesion contacts (Fig. 7), suggesting that the actin-filament organization is not mediated by the typical cytoskeleton adaptor molecules that organize in focal contacts. There was partial colocalization of vinculin with TF independent of the adhesive substrate, but adhesion to VIIa did not appear to promote recruitment of vinculin to the adhesion contact as in the case of ABP-280 (Fig. 7). The specific recruitment of ABP-280, the lack of typical integrin adaptor molecules in the contact sites, and the different actin-filament morphology indicate that TF-mediated spreading is distinct from integrin-mediated focal contacts.

TF Ligation-dependent Recruitment of ABP-280

The confocal analysis suggested that extracellular ligation of TF is necessary for recruitment of ABP-280. We were unable to coimmunoprecipitate ABP-280 with TF or TF with ABP-280 from J82 cell lysates (data not shown). Antibody-coated beads can recapitulate extracellular adhesive interactions, as demonstrated in studies of integrin function (Miyamoto et al., 1995). We used paramagnetic beads coated with anti-TF antibody 10H10 to induce extracellular ligation of TF on J82 cells in suspension. After cell lysis in buffers that contained DNAse I to disrupt the cytoskeleton and to avoid coprecipitation of nonspecifically associated proteins, TF and bound proteins were precipitated with the antibody coated beads. When intact J82 cells were incubated with antibody-coated beads for 30 min at 4°C before cell lysis, ABP-280 was not detected in the anti-TF immunoprecipitate. Incubation of cells with antibody beads at 37°C, in contrast, resulted in a time-dependent association of ABP-280 with TF (Fig. 9). Similarly, ABP-280 was recruited to TF on TNFα-stimulated ECV304 endothelial cells, when the cells were incubated at 37°C with antibody coated beads (Fig. 9). The immunoprecipation of TF under control and experimental conditions was similar in each case. These data thus demonstrate association of ABP-280 with TF that is dependent on extracellular ligation of TF. Whereas ABP-280 did not
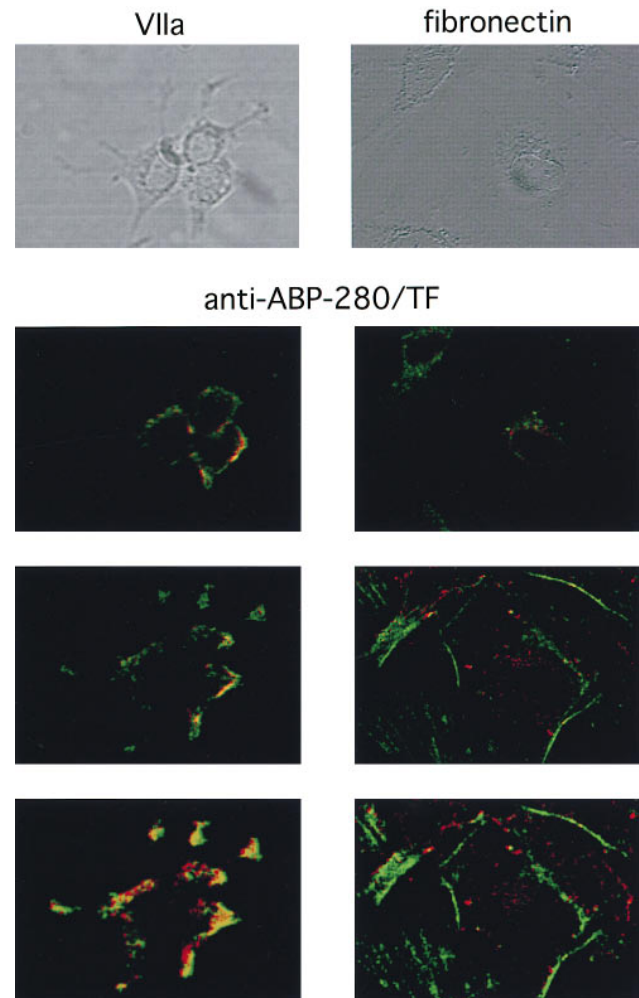

VIIa   fibronectin

anti-ABP-280/TF

Figure 8. Serial optical section of J82 cells adherent on fibronectin or VIIa. Cells were stained for TF (*red*) and ABP-280 (*green*). Only pseudocolor merged images are shown. The optical sections of the fluorescence images were in the adhesion plane (*bottom*), and ~1.2 (*middle*) or ~3.0 μm (*top*) from the adhesion plane. A DIC image of the cells is shown in the top row.

coprecipitate with endogenously expressed TF, we were able to precipitate ABP-280 with immobilized cytoplasmic domain peptide of TF (Fig. 5). We interpret this result as an indication that the high immobilization density of the peptide on the Ni-NTA resin mimicked to some extent the clustered state of the TF cytoplasmic domain.

Cell Spreading Mediated by the TF Cytoplasmic Domain

To evaluate contributions of the extracellular and transmembrane domain to TF-mediated adhesion and spreading, we used chimeric molecules consisting of the extracellular and transmembrane domain of the interleukin 2 (IL-2) receptor (LaFlamme et al., 1992) and wild type or mutant TF cytoplasmic domains. J82 cells were transiently transfected with the chimeras and, after 48 h, tested for adhesion to immobilized antibody directed to the IL-2 receptor extracellular domain. Untransfected J82 cells did not adhere to the antibody-coated surfaces. Expression of the
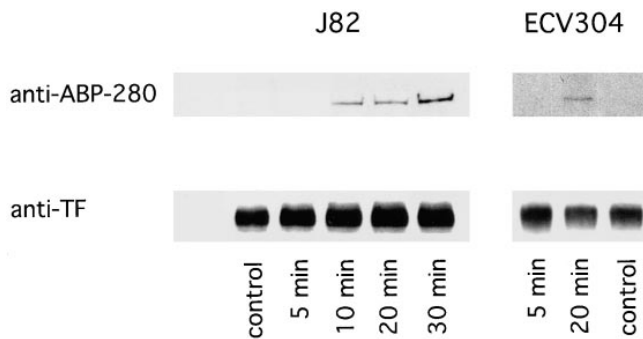
Figure 9. Ligation-dependent recruitment of ABP-280 to TF in J82 cells and TNFα-stimulated ECV304 cells. 10^7 cells were incubated with a 3×10^7 antibody-modified Dynabeads M450 for the indicated times at 37°C with gentle agitation. The controls were incubated for 30 min on ice under the same conditions. Cells were lysed and the anti-TF immunoprecipitate was extensively washed before SDS-PAGE and Western blotting for ABP-280 and TF as a control for equal loading and efficient immunoprecipitation.

IL-2 receptor extracellular domain resulted in adhesion to the antibody-coated plates with each of the chimeras tested, indicating similar transfection efficiency and expression levels of the chimeras, also confirmed by flow cytometry (data not shown). The IL-2 receptor extracellular and transmembrane domain without a cytoplasmic domain, or the chimera with the GP Ibα cytoplasmic domain did not support spreading (Fig. 10 *A*), excluding that the IL-2 receptor extracellular domain recruits adhesion receptors that support spreading of J82 cells. The inability of glycoprotein Ibα to induce spreading may reflect lack of specific intracellular adaptor molecules in J82 cells or may indicate that binding to repeat 17–19 of ABP-280 has biological effects distinct from the TF interaction with the very carboxyl terminus of ABP-280.

Chimeras in which phosphorylation of the cytoplasmic domain Ser residues was mimicked by Asp replacement supported spreading on anti-IL-2 receptor antibody coated plates (Fig. 10 *A*). A similar percentage of cells spread on chimeras with the TF wild-type cytoplasmic domain, whereas Ala mutation of Ser^{253} and Ser^{258} resulted in a significantly lower percentage of cells that spread on anti–IL-2 receptor antibody. These data are consistent with biochemical two-hybrid analysis suggesting that affinity of ABP-280 for the TF cytoplasmic domain is dependent on Ser^{253} and Ser^{258} and possibly phosphorylation of these residues. The morphology of cells spreading through IL-2 receptor chimera–mediated adhesion contacts was similar to cells spread on the TF ligand VIIa, including a similar actin-filament staining with no evidence of stress fibers (Fig. 10 *B*). These data thus demonstrate that the TF extracellular and transmembrane domain play no role in TF-dependent spreading, which is adequately supported by the cytoplasmic domain of TF.

To test whether the TF cytoplasmic domain is sufficient to support cell migration, we established a stably transfected cell line that expressed the wild-type TF cytoplasmic domain as chimera with the IL-2 receptor extracellular and transmembrane domain. Based on flow cytometry, cell surface expression of the IL-2 receptor chimera was
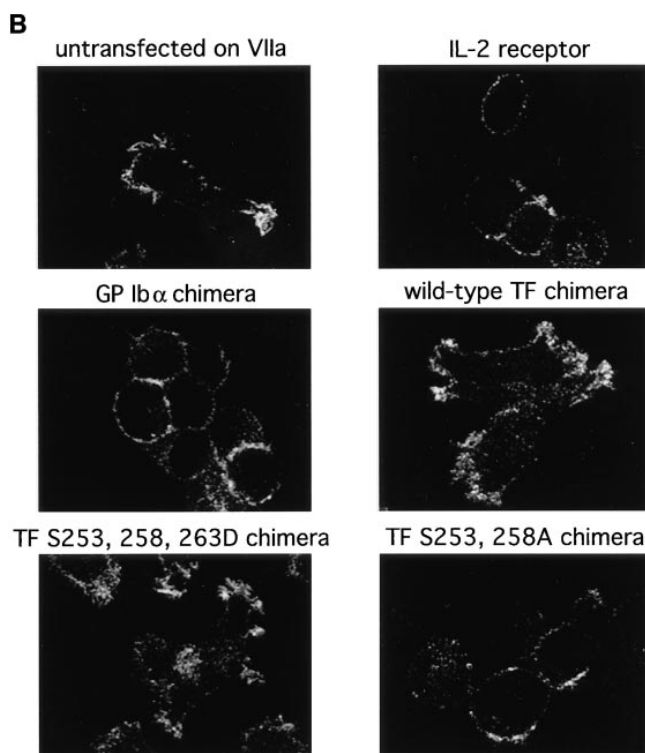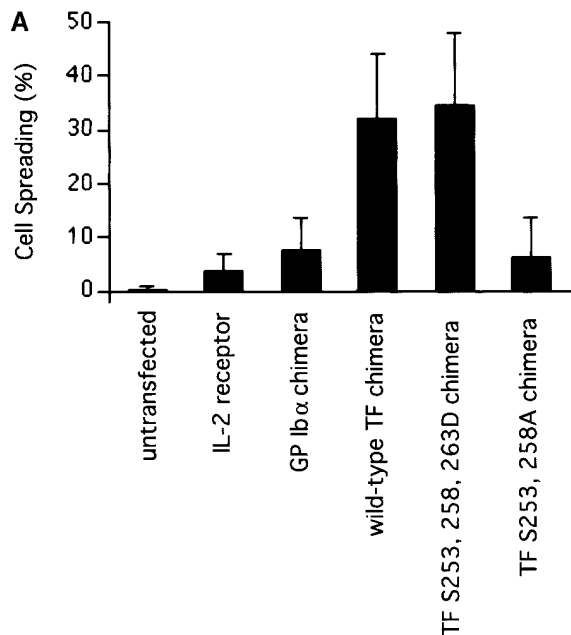

Figure 10. Spreading mediated by IL-2 receptor chimeras. (*A*) J82 cells transfected with IL-2 receptor chimeras were tested for spreading 1 h after seeding onto anti–IL-2 receptor 7G7B6-coated plates. Spreading was visually assessed by inspection of 100 cells of each transfectant. Cell spreading of untransfected J82 on VIIa under the same experimental conditions was ~50%. Mean and standard deviation ($n \geq 3$). (*B*) Morphology of cells that are transiently transfected with the IL-2 receptor that lacks a cytoplasmic domain or the indicated chimeras are shown on anti–IL-2 receptor 7G7B6-coated plates 1 h after seeding. Actin-filament organization is visualized by phalloidin staining. The morphology of untransfected cells spreading on VIIa is shown for comparison.
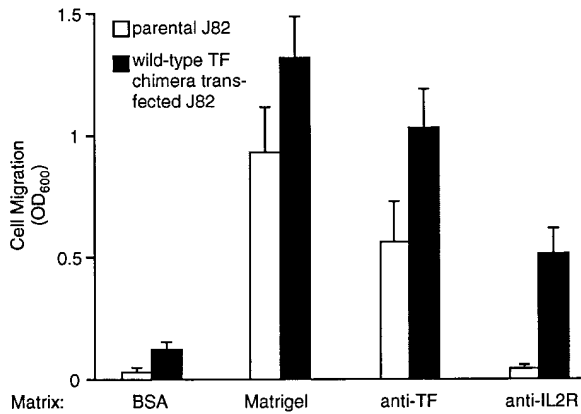
Figure 11. Migration of nontransfected, parental J82 cells, and J82 cells stably transfected with chimeric constructs encoding for the IL-2 receptor, extracellular and transmembrane domain, and the TF cytoplasmic domain on various matrices. Cell migration was determined in a modified Boyden chamber assay. Wells were coated with blocking protein BSA (*BSA*) as control, with reconstituted basement membrane (*Matrigel*) for integrin-dependent migration, anti-TF antibody 6B4 (*anti-TF*) for migration dependent on endogenous TF, or with (Fab′)$_2$ fragments of antibody 7G7B6 to the IL-2 receptor (anti-IL2R) for migration dependent on the transfected chimera. Mean and standard deviation for triplicates of a typical experiment (*n* = 3) are shown.

30% of the expression levels of endogenous TF on J82 cells. Whereas untransfected, parental J82 cells did not migrate on immobilized anti–IL-2 receptor antibody, the cell line transfected with the chimera supported migration to a level that was comparable to the migration rate of both cell lines on anti-TF antibody that is mediated by endogenous TF. These data thus demonstrate that the cytoplasmic domain of TF is not only sufficient to allow for cell adhesion and spreading, but also to support migratory functions of cells.

Discussion

Protease receptors play a critical role in regulating adhesive and migratory properties of cells. In part, these effects are mediated by virtue of the activation of protease cascades that result in matrix degradation and remodeling (Mignatti and Rifkin, 1993; Brooks et al., 1996). Signaling and adhesive functions of this class of cell surface receptors have recently been shown to play an equally important role in extracellular interactions. The urokinase receptor (uPAR), a GPI-anchored receptor involved in cell-associated fibrinolysis, supports adhesion and migration on vitronectin (Wei et al., 1994). Stabilized through an interaction with caveolin that binds GPI-anchors, uPAR is thought to associate with the extracellular domain of the integrin β$_1$-subunit, thereby modulating integrin-dependent adhesion (Wei et al., 1996). Our results demonstrate that the cytoplasmic domain of TF serves to reorganize the cortical actin–filament network, providing a novel mechanism by which a protease receptor influences the adhesive and migratory functions of cells. We found neither an influence of RGD-containing peptides on TF-dependent adhesion, nor recruitment of intracellular cytoskeletal adaptor pro-

teins which associate with integrins at the TF-adhesion contacts, suggesting that the extracellular clustering of TF is sufficient to reorganize the cytoplasmic actin network independent of integrin function.

Our data demonstrate the interaction of the TF cytoplasmic domain with ABP-280 as the molecular link by which TF-mediated adhesion influences cytoskeleton reorganization. This link may explain the observations that TF localizes to cellular processes and membrane ruffles at the leading edge of cells (Müller et al., 1993; Carson et al., 1994; Carson et al., 1996). The comparison with other receptors that interact with ABP-280 illustrate the unique properties of the interaction with TF. Based on its association with the β$_2$-integrin subunit, ABP-280 has been suggested to associate with other β-integrin subunits, due to sequence conversation in their cytoplasmic domains (Sharma et al., 1995). The epithelial J82 cells presumably adhere through β$_1$ integrins to fibronectin. On this matrix, we found that ABP-280 is predominantly associated with cytoplasmic filaments, which contrasted with the clustered recruitment to sites of TF-dependent adhesion. Because the β$_2$ integrin cytoplasmic domain by two-hybrid assay did not interact with the binding region for TF in the carboxyl terminus of ABP-280, we suggest that the clustering of ABP-280 on TF ligands results from the specific interaction of the TF cytoplasmic domain with the very carboxyl terminus of ABP-280.

Unlike the ligation-dependent recruitment of ABP-280 to TF adhesions, the immunoglobulin G Fc receptor I (FcγRI) is preferentially associated with ABP-280 in the absence of ligand (Ohta et al., 1991). ABP-280 also constitutively associates with GP Ibα through a binding site in repeats 17–19 (Meyer et al., 1997). The binding sites for GP Ibα and TF localize to distinct domains of ABP-280, as defined by cleavage with calpain in two hinge regions of ABP-280 (Gorlin et al., 1990), suggesting agonist-induced posttranslational processing of ABP-280 as a regulatory mechanism to direct association with a specific receptor, if TF or GP Ibα are coexpressed on the same cell. When associated with TF through the dimerized carboxyl terminus, ABP-280 would orient the amino-terminal actin–binding domains towards the cytoplasm, thus providing a nucleation point for actin-filament assembly. This specific orientation of ABP-280 may be provided uniquely by TF as compared to other receptors with which ABP-280 associates.

We here demonstrate TF-dependent adhesion to specific antibodies and the physiological ligand protease VIIa. Because VIIa is a monomeric, soluble protein in the blood, we consider it unlikely that it functions as an immobilized ligand in TF-mediated adhesive events. However, the complex of TF and VIIa will form with high affinity in the circulating blood or at extravascular sites of increased vascular permeability, suggesting that interactions with the binary TF·VIIa complex may be of importance. Indeed, we found that hematogenous metastasis is dependent on high affinity binding of VIIa by TF, indicating that the TF·VIIa complex may engage in higher order assemblies in the process of TF-dependent tumor cell dissemination (Mueller and Ruf, 1998). Known ligands for the TF·VIIa complex are good candidates for these interactions that could modulate cell adhesion and migration. First, specific

inhibitors for the TF·VIIa complex are known to be associated with cell surfaces and the extracellular matrix. A Kunitz-type inhibitor homologous to TFPI, designated TFPI-2, interacts with TF·VIIa (Sprecher et al., 1994) and is predominantly associated with the extracellular matrix (Rao et al., 1996). TFPI, the major vascular inhibitor for TF·VIIa, binds to heparin-sulfated proteoglycans, which can be located either in matrices or on the endothelial cell surface (Broze, 1995), providing a ligand for cell–cell contacts mediated through the active site of VIIa. This interaction is further stabilized by factor Xa, which is readily formed upon contact of TF-expressing cells with the blood. In the context of vascular hyperpermeability induced by VEGF (Dvorak et al., 1995), these interactions may become relevant for migratory and adhesive properties of endothelial cells during angiogenesis or tumor cells during metastatic spread.

In addition to TF-dependent interactions with cell or matrix-associated ligands, adhesive and migratory properties of cells may be influenced by extracellular complex formation involving TF and possible subsequent TF-dependent signaling. Complex formation involving cell-associated TFPI regulates the subcellular localization of TF. On endothelial cells, one mechanism of anchoring TFPI to the cell is through a GPI-linkage (Sevinsky et al., 1996). After initial protease generation and formation of the quaternary complex of TF·VIIa with factor Xa and TFPI, the GPI-anchoring of TFPI directs translocation of TF to caveolae, cellular subdomains with crucial functions in signaling (Lisanti et al., 1994). By a second mechanism, TFPI becomes cell associated through interaction of its basic third Kunitz-type domain and carboxyl terminus with heparan-sulfated proteoglycans, most notably syndecan 4 (Kojima et al., 1996). Syndecan 4 is a component of focal contacts and critically important for stress fiber formation in cells adhering to fibronectin (Couchman and Woods, 1996). Complex formation of TF with TFPI bound to syndecan 4 may affect syndecan 4 localization and thereby integrin function. Consistent with this hypothesis, in vitro migration assays have demonstrated that TFPI can regulate TF·FVIIa complex-dependent migration of smooth muscle cells (Sato et al., 1997).

The reorganization of the actin cytoskeleton by the TF-dependent recruitment of ABP-280 suggests specific pathways that are influenced by extracellular interactions of TF. We here demonstrate that actin-filament assembly induced by adhesion to TF leads to phosphorylation of FAK, a central nonreceptor tyrosine kinase involved in cytoskeleton-dependent signaling. We take these data as evidence that the TF-mediated recruitment of ABP-280 and the subsequent actin reorganization provide a suitable template for the assembly of signaling complexes that depend on an intact cytoskeleton. More intriguing is the fact that the carboxyl terminus of ABP-280 itself is critical for activation of the MAP kinase pathway in response to TNFα or lysophosphatidic-acid stimulation (Marti et al., 1997). Whereas ABP-280 that includes repeats 21–23 restores TNFα signaling, the G protein–coupled signaling, induced by lysophosphatidic acid, requires the dimerization domain in the 24th repeat with which TF interacts. Because lysophosphatidic acid is a key regulator of the assembly of focal contacts and stress fibers by the small GTPase rho (Nobes and Hall, 1995), these data emphasize a potential signaling role of TF specifically related to adhesive and migratory functions of cells.

Although speculative in the absence of ABP-280 deficient mice, the molecular link of TF to the cytoskeleton may explain its poorly understood role in early embryogenic vessel development. A common theme for knockouts that produce phenotypes similar to the TF deletion is their role in cell adhesion, migration, and differentiation, which may be crucial for the remodeling of the maturing vascular bed at this embryonic stage. TGFβ (Dickson et al., 1995) regulates integrin expression (Ignotz and Massagué, 1987) and it is activated in co-cultures of endothelial cells and pericytes that are thought to mimic interactions during remodeling of the vessel wall (Beck and D'Amore, 1997). The importance of migratory functions is illustrated by the knockout of $G_{\alpha 13}$ subunit that is critical for cell migration (Offermanns et al., 1997) and by the deletion of factor Va (Cui et al., 1996) possibly acting on the thrombin receptor (Connolly et al., 1996) that affects actin-filament polymerization (Hartwig et al., 1995). TF may function to transiently stabilize the subcortical actin-filament network of endothelium and visceral endoderm during separation when mesenchymal cells migrate to reorganize the vessel wall into mature vitelline vessels of the yolk sac. Analogous functions may be required in the process of tumor angiogenesis where endothelial cells detach from an existing vessel and migrate into the tumor stroma to establish a sprouting capillary.

Whereas the specific role of TF's intracellular interactions in vascular remodeling and angiogenesis has not been formally tested, in vivo experiments have established an essential role for the TF cytoplasmic domain in tumor cell metastasis (Bromberg et al., 1995; Mueller and Ruf, 1998). The metastatic process requires that tumor cells home into the target vasculature, transmigrate through the endothelial lining, and establish growing tumor nodules. The specific molecular interaction between TF and ABP-280 provides a mechanism by which the TF cytoplasmic domain functions in the metastatic process. Because extracellular ligation of TF recruits ABP-280, which is critical for cell migration (Cunningham et al., 1992), interactions of TF may support directionality of migration, regulated adhesion, and deadhesion during transmigration of tumor cells through the endothelium. ABP-280 further regulates MAP kinase signaling pathways (Marti et al., 1997) which may cooperate with the TF-dependent activation of FAK to provide essential signals that allow the tumor cells to survive and proliferate into an established metastatic nodule. The identified molecular pathway by which the TF cytoplasmic domain influences adhesive and migratory functions of cells may further prove crucial for the complex biological functions of TF in tumor angiogenesis and embryonic vascular development.

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