Anthrax Toxin Receptor 1/Tumor Endothelium Marker 8 Mediates Cell Spreading by Coupling Extracellular Ligands to the Actin Cytoskeleton*\textsuperscript{[S]}

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Tumor endothelial marker 8 (TEM8) is induced in tumor-associated vasculature and acts as a receptor for Protective Antigen (PA), the cell-binding component of the anthrax toxin determinant for toxin entrance into cells. However, the normal function for TEM8 remains unknown. We show that TEM8 functions as an adhesion molecule mediating cell spreading on immobilized PA and collagen I. The mechanism for TEM8 interaction with collagen I was cell type-specific, because binding to collagen I was abrogated by \textbeta1 integrin function blocking antibody in HEK293 cells, but not in primary synovial rabbit fibroblasts. Binding to PA remained unaffected by the addition of \textbeta1 integrin function blocking antibody. Whereas the extracellular and transmembrane domains of TEM8 were sufficient to provide cell attachment, the intracellular domain was critical for spreading. Fusion of the cytosolic domain of TEM8 to the IL-2 receptor, conferred cell-spreading capability on IL-2 receptor antibody substrates. The cytoplasmic domain mediated linkage with the actin cytoskeleton as it co-precipitated actin and determined partitioning of TEM8 to the actin-containing detergent insoluble cellular fraction. TEM8 anchorage to actin was relevant as spreading was inhibited by the cytoskeleton-disrupting drug cytochalasin D, but persisted in the presence of the microtubule-depolymerizing drug nocodazole, and in cells lacking intermediate filaments. Thus, our results indicate that TEM8 is a new adhesion molecule linking collagen I or PA to the actin cytoskeleton.

The rapid and fatal course of \textit{Bacillus anthracis} sepsis results from the massive release of the bacterial codified proteins, Protective Antigen (PA),\textsuperscript{2} Lethal Factor (LF), and Edema Factor (EF), which assemble into a toxic complex at the cell surface.

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\textsuperscript{2} The abbreviations used are: PA, Protective Antigen; IL, interleukin; ECM, extracellular matrix; TEM, tumor endothelium marker; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HA, hemagglutinin; LF, Lethal Factor; EF, Edema Factor; MA, Matrigel; BSA, bovine serum albumin; HEK, human embryonic kidney.

Susceptible cells express receptors that bind the PA component of the toxin. Upon binding, furin-like proteases at the cell surface process PA, triggering oligomerization and exposure of sites that bind the catalytic components of the toxin, Lethal Factor and Edema Factor (reviewed in Ref. 1). The tripartite toxic complex is internalized via lipid rafts and directed to endosomal compartments. In endosomes, the acidic pH triggers PA-heptamer insertion into the membrane to form a pore that translocates LF and EF into the cytosol (2). Although much is known about the mechanism by which the toxin enters the cell, less is known about the targets of LF and EF in the cytosol and the consequences of their activity for toxicity. Edema factor is a calmodulin-dependent adenyl cyclase, raising the intracellular concentration of cAMP and thereby leading to tissue edema (3). LF is a metalloprotease that cleaves most mitogen-activated protein kinase isoforms (4). The PA-binding receptors play a mayor role in toxin activity by directing assembly on target cells and insertion site of the oligomer into the membrane along endosomal compartments (5, 6). However, binding of PA to the receptors could have additional still unrecognized roles in anthrax pathogenesis associated with the normal, yet unexplored, function of these receptors.

Two different cellular receptors mediate anthrax toxin entry to the cells: anthrax receptor 1 (ATR1), also called tumor endothelium marker 8 (TEM8) (7); and ATR2, also known as capillary morphogenetic protein 2 (CMG2) (8). TEM8 was identified by its increased expression in tumor-associated endothelial cells (9) and, therefore, has been proposed as a candidate molecule to target antitumor therapies (10). Antibody-based evidence suggests that TEM8 is also expressed in tissues where the bacterium spores enter the organism, such as skin, lungs, and small intestine (11). The soluble extracellular domain of TEM8 binds to collagen type 1 and gelatin (12) and co-immunoprecipitates with the C5 domain of collagen \alpha3 (VI) (13). This evidence suggests interaction with molecules of the extracellular matrix (ECM); however the implications of these interactions for cell adhesion remain undetermined. TEM8 also increases endothelial cell chemotactic activity on collagen substrata; however the mechanism for this function remains unclear (12). The second receptor, CMG2, is robustly induced during capillary tube formation in collagen gels and has a more ubiquitous pattern of expression than TEM8 (14). The CMG2 extracellular domain shows binding activity to collagen type IV and laminin (14). Interestingly, genetic mutations identified in juvenile hyaline fibromatosis and infantile systemic hyalinosis show reduced fibroblast adhesion to laminin substrates but not...
to collagen I or collagen IV (15). Both receptors are type I transmembrane proteins sharing a 60% homology at the amino acid level, concentrated in a von Willebrand factor type A (VWA) domain similar to the inserted (I) domain present in a subset of integrins. This domain includes a metal ion-dependent adhesion site (MIDAS) motif necessary for cation-dependent binding of ECM molecules and TEM8 modulation of cell chemotaxis or contribute to the host response triggered by toxin internalization.

In contrast, much less is known about the function of the cytosolic domain of both receptors, for which at least three different variants can be generated by differential splicing (7, 8). In silico analysis reveals that TEM8 has a consensus signal for basolateral sorting and for phosphorylation. The cytosolic domain is palmitoylated and ubiquitinated, modifications controlling endocytosis of the receptor (17). However, no other known protein motifs or interacting proteins have been described so far that could give additional clues about the function of the receptor cytosolic domain. Importantly, whereas this domain is not required for toxin delivery (18), it could potentially play a determinant role in regulating receptor localization or contribute to the host response triggered by toxin internalization.

Given the interaction of the extracellular domain of TEM8 with ECM molecules and TEM8 modulation of cell chemotactic activity, we decided to test the hypothesis that TEM8 functions as an adhesion molecule, directly linking extracellular ligands with the cytoskeleton. Using engineered chimeric receptors containing either the extracellular or intracellular domains of TEM8, we analyzed the role of these domains in linking proteins of the extracellular milieu with the actin cytoskeleton. Our results indicate that the cytosolic domain of TEM8 mediates actin-dependent cell spreading when binding to the immobilized ligand.

**EXPERIMENTAL PROCEDURES**

**Cells, Plasmids, Transfections, and Adenoviral Infections**—Stably full-length TEM8 or extracellular domain TEM8-1 (18) expressing cell lines were obtained by selection of hygromycin-resistant HEK293 cells (QB1–293 from Qbiogene, Carlsbad, CA). HEK293 cells transiently expressing TEM8, IL-2R, IL-2RVE cadherin CD or IL-2R/TEM8-CD were obtained by 14-h infection with recombinant adenoviruses. The recombinant adenoviruses were prepared according to published methods (19) by subcloning TEM8 HA from pIRES using the 5′-primer CACCAA-

**PCR product was cloned into pcDNA3 and then subcloned into pAdTrack vector containing IL-2R with an engineered HindIII site at the 3′-end (23). The construct was sequenced, and expression was verified by Western blot using anti-TEM8 antibody (Imgenex, San Diego, CA) and anti-IL-2R (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit synovial fibroblasts were isolated and used as described before (20).**

**Substrate Coating**—Plastic dishes and glass coverslips were coated with 1% heat-inactivated bovine serum albumin, 10 µg/ml PA, 10 µg/ml anti-IL-2 receptor monoclonal antibody (BIOSOURCE, Camarillo, CA) in PBS, 20 µg/ml of collagen I in 0.02 m HAc or Matrigel (MA, BD Biosciences) diluted 1:40 in DME (Cellgro, Hendon, VA) for 4 h at room temperature. After a PBS wash, plates were blocked for 20 min with heat-inactivated 0.3% BSA in PBS. Excess of BSA and other proteins was removed by three washes with PBS.

**Cell Attachment Assay**—Cells were released with trypsin, resuspended in serum-free DME (if not stated otherwise) at a concentration ranging 350,000 to 500,000 cells/ml and incubated for 15 min at 37 °C before adding 100 µl of cell suspension to substrate-coated MaxiSorb 96 wells Immuno Plate (Nunc, Rochester, NY) for the indicated time at 37 °C. After binding, the plate was washed three times by immersion in PBS supplemented with Ca2+ and Mg2+. The attached cells were quantitated by crystal violet staining and dye solubilization. Percentage of attachment was calculated considering 0% the readings obtained by binding to BSA and 100% the readings obtained using Matrigel. Binding to Matrigel was high, unaltered by transfection or infection and directly related to the amount of cells seeded. Thus these readings were used to correct for the number of cells competent to bind between the different cell groups. The cells were preincubated for 5 min with the blocking antibodies before adding them to the coated plates. The anti-human β1 blocking antibody clone P5D2 was obtained from R&D Systems (Minneapolis, MN). The antibodies AIIb2 and BIIg2 were developed by Dr. Damsky and were used protein G-purified (in spreading assay) or from concentrate obtained from the Developmental Studies Hybridoma Bank (Iowa University) in the cell attachment assay.

**Cell Spreading**—Cells were trypsinized, resuspended in DME, and incubated for the indicated time on substrate-coated glass coverslips. The drugs cytochalasin D and nocodazole were obtained from Sigma and Calbiochem (EMD Biosciences, La Jolla, CA), respectively. Unattached cells were washed with PBS, and the remaining cells were fixed with 4% PFA for 20 min and embedded in gelvatol. Fields selected at random were photographed at ×20 magnification under phase contrast in a Leica fluorescence microscope (model DMR-E) equipped with narrow band pass filters and a camera (model Orca; Hamamatsu). Images were captured and processed using Open Lab software (Improvision Inc.). Spreading area of 100 cells selected at random, was measured by registering the pixel area covered by the cell body, excluding filopodia and thin protrusions using Metamorph software (Universal Imaging, Downingtown, PA). The distribution plots were obtained by determining the number of cells included in 500 pixels sized bins ranging from 0 to the maximum pixel area measured in the experiment. Step plots were produced in Kaleidagraph 3.6 (Synergy Software, Reading, PA). The arrows mark the value of the median.

**MEK1 Cleavage Assay**—Cells were incubated for the indicated time with a mixture of 1 µg/ml of PA and 0.1 µg/ml of LF (List Biological Laboratories, Inc., Campbell, CA) in DME supplemented with 10% fetal bovine serum. The selective cleavage of the N terminus domain of MEK1 (MAPKK-1) was detected by Western blot analysis with antibodies directed to the N terminus (Nt MEK1) (Upstate Biotechnology, Charlottesville, VA)
and compared with the existing MEK1 detected by probing after blot stripping with an antibody recognizing the C terminus (Ct MEK1) (Santa Cruz Biotechnology).

**Cytoskeleton Association Assay**—Cells were plated on substrate-coated dishes for the indicated time, and the soluble proteins were extracted following the procedure described by Ref. 21, using a 5-min extraction with 0.15% Triton X-100 in 360 mM piperazine diethanesulfonic acid, 25 mM HEPES, 10 mM ethylene glycol bis(3-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 2 mM MgCl2, pH 6.9 buffer and antiproteases on ice. Laemmli sample buffer with antiproteases was used to recover Triton-insoluble proteins (I) and to equalize the volume of the Triton-solubilized proteins (S) to the (I) fraction. The proteins present in comparable proportion of each fraction were analyzed by Western blot using antibodies anti-actin, tubulin (Sigma-Aldrich), and IL-2R (Santa Cruz Biotechnology). Solubility index was determined by densitometric analysis of the bands using NIH Image Software and defined as (Triton-soluble/Triton-insoluble) ± 1.

**Co-immunoprecipitation and Immunofluorescence**—Cells were lysed in buffer A (22), and equal amounts of protein were added to protein G beads preincubated with monoclonal anti-IL-2R or anti-HA (Roche Applied Science). After washing, the immunoprecipitates were separated by SDS-PAGE using non-reducing sample buffer to avoid interference of the immunoglobulin chains with actin and tubulin detection. Cells were processed for immunofluorescence as described before (22). Actin filaments were stained with Alexa 568-conjugated Phalloidin (Invitrogen, Carlsbad, CA). The specimens were viewed using an Axiovert 100 M confocal microscope (Carl Zeiss) coupled to a HeNe1 and Argon ion lasers and equipped with BP 565-615 and BP 500-550IR filters and a Plan Apochromat ×63/1.4 oil DIC objective with filters. Images were acquired using LSM 500 sp1 software (Carl Zeiss).
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Statistics—Error bars in the graphs represent S.E. The distribution of the spreading area data and cell attachment were considered to be parametric; therefore, significance was determined applying a two-tailed Student's t test assuming equal variance.

RESULTS

To determine whether TEM8 could function as an adhesion molecule, we used PA as a known TEM8 ligand to engage its extracellular domain. PA interacts with the MIDAS motif in the integrin-like inserted domain of TEM8 in a similar manner as ligands of ECM interact with integrins (16), thus we tested whether PA would engage TEM8 when immobilized on a surface. HA-tagged TEM8 was expressed in HEK293 cells, which lack detectable levels of endogenous full-length TEM8 (13) and Fig. 1A, compare lanes 1 and 2). When added in soluble form together with LF, PA binding triggers lethal toxin internalization and LF translocation into the cytosol, resulting in the N-terminal cleavage of MEK1. MEK1 cleavage occurred earlier in TEM8-expressing cells than in non-transfected cells (Control, Fig. 1B). This result confirms the expression of a functional PA receptor at the cell surface that is competent to deliver toxin into cells. When PA was used to coat plastic dishes, mimicking an ECM substrate, TEM8-expressing cells showed increased attachment activity modulated by the presence of divalent cation (Fig. 1C). Maximum attachment to PA occurred in the presence of 1 mM Mn\(^{2+}\), both in PBS or in DME media, whereas Mg\(^{2+}\) and Ca\(^{2+}\) had less effect, results consistent with the known cation selectivity profile for PA binding to TEM8 (16). Non-transfected cells attachment to PA increased by Mn\(^{2+}\) addition, which could be caused by a low level expression of short variants of TEM8 or by CMG2, not detected with our antibodies. However, this background activity did not become evident in the spreading assay (see below).

Upon attachment to PA tethered to a surface, TEM8 induced widespread cell shape changes and cell body extension. Spreading area was quantitated as the number of pixels covered by the cell body and expressed in frequency distribution histograms. Within 30 min of plating on PA or collagen type I, a recently described endogenous ligand for the extracellular domain of TEM8 (12), either stably expressing cell lines (Fig. 1D) or transiently TEM8-expressing cells (Fig. 3, C and D) covered a significantly larger area than non-transfected cells (Fig. 1D, compare dashed versus continuous lines, p < 0.001). Collagen I promoted cell spreading with efficiency comparable to PA, as the cell areas did not significantly differ when spread on either substrate (p = 0.5). The fact that cell spreading occurs upon attachment to two different immobilized ligands, indicates that this attribute is not unique to the interaction of TEM8 with PA.

Spreading on tethered ligands is a hallmark of adhesion molecules associated with the cytoskeleton. Linkage to the cytoskeleton could occur directly through the cytosolic domain or indirectly through association with other transmembrane molecules. Many cell surface proteins cooperate and modulate the adhesive function of integrins (reviewed in Refs. 23–25). Therefore, to define whether there is a possible functional cooperation between TEM8 and integrins to accomplish cell spreading, we sought to disrupt the function of collagen binding

FIGURE 2. Role of β1 integrins in TEM8-mediated binding and spreading on collagen I is cell type-specific. A, cell attachment of TEM8 adenovirus-infected or uninfected (control) HEK293 cells plated for 20 min on collagen I, PA, or MA-coated dishes without or with 1 mM Mn\(^{2+}\) in the absence or presence of 25 μg/ml anti-β1 integrin blocking antibody (PSD2) or 25 μg/ml anti-α5β1 integrin blocking antibody (BIIG2), (n = 2). B, cell attachment of TEM8 adenovirus-infected primary rabbit synovial fibroblasts or control cells plated for 1 h on collagen I-, PA-, or MA-coated dishes without or with 1 mM Mn\(^{2+}\) in the absence or presence of 25 μg/ml anti-β1 integrin blocking antibody (PSD2), 2.5 μg/ml anti-β1 integrin blocking antibody (BIIG2), or 2.5 μg/ml anti-α5β1 integrin blocking antibody (BIIG2), (n = 2). C, spreading area distribution plots of TEM8-expressing primary rabbit synovial fibroblasts or non-transfected cells (Control) plated in cell culture medium supplemented with 1 mM Mn\(^{2+}\) or Mg\(^{2+}\) respectively, on collagen I-precoated dishes for 1 h without (No antibody) or with 5 μg/ml of BIIG2 or BIIG2 antibodies. Arrows indicate the median (n = 2).
integrins, which mostly belong to β1 family of integrins, with β1 integrin-blocking antibodies (26). TEM8 expression in HEK293 did not increase overall cell attachment to collagen (Fig. 2A), most likely because of the high endogenous collagen attachment activity, which was evident even in the absence of added Mn²⁺. Interestingly, addition of a β1 function-blocking antibody (P5D2) abrogated completely attachment to collagen, without modifying binding activity to PA. As a control, we used BIIG2, which is a rat monoclonal antibody that interferes with human α5β1 integrin binding to fibronectin (27) without affecting binding to collagen. To examine further whether TEM8 expression can increase cell attachment to collagen, we used next a different cell type, primary rabbit synovial fibroblasts, because they are efficiently infected with adenovirus and this cell type uses α1 and α2β1 integrins to bind collagen I when integrins are activated with divalent cations, (26). TEM8 expression increased cell attachment to collagen in the absence of added Mn²⁺ (Fig. 2B). Upon Mn²⁺ addition, binding was elevated with or without TEM8 expression. Addition of the β1-blocking antibodies P5D2 and AIIB2, competed for attachment of control cells in a small but significant proportion (p < 0.05), without affecting attachment of TEM8-expressing cells to collagen or to PA. BIIG2 did not affect attachment. These contrasting effects of the β1 blocking antibodies were more apparent in spreading assays. We measured spreading of control or TEM8 adenovirus infected cells on collagen I (Fig. 2C) in the absence or presence of the β1 integrin function-perturbing antibody AIIB2 (Fig. 2C). After 1 h, TEM8-expressing fibroblasts spread significantly more efficiently than non-infected cells (p < 0.001). Interference with β1 integrin function using AIIB2, reduced cell spreading of non-infected (control) cells (compare full line versus dashed line in left panel of Fig. 2C, p < 0.001), but did not affect TEM8-expressing fibroblast spreading on collagen I, in right panel of Fig. 2C (p > 0.1). Furthermore, selectively modifying α5β1 integrin (fibronectin receptor) function with BIIG2 (light line) did not affect spreading of control cells (p > 0.1) nor TEM8-expressing cells (p > 0.1), indicating that the inhibition by the anti-β1 antibody is integrin-selective. Thus, these experiments suggest that the participation of β1 integrins can be excluded in TEM8-mediated cell spreading on collagen in primary fibroblasts. However, collectively, they indicate that the participation of these integrins in TEM8 spreading on collagen vary with cell type.

The activity of TEM8 as an adhesion molecule predicts that TEM8 lacking the cytosolic domain will not be sufficient to mediate cell spreading. HEK293 cells expressing a construct

![Figure 3. The extracellular domain of TEM8 (ED-TEM8) is insufficient to mediate cell spreading.](image-url)
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A

B

C

D

Attachment (%)

anti IL-2R  PA  MA

anti IL-2R  PA  MA

Matrigel  anti IL-2R  PA

IL-2R  

IL-2R/TEM8-CD  

TEM8  

On Matrigel  On Antibody  On PA

Spreading Area (pixels x 10^-3)
comprising the extracellular and transmembrane domains only (ED-TEM8, 1–343 amino acids), were more susceptible to lethal toxin than non-transfected cells as assessed by MEK1 cleavage (Fig. 3A). These results are consistent with published studies showing that the extracellular domain tethered to the plasma membrane is sufficient to mediate toxin internalization (18). In fact, MEK1 was cleaved at a similar rate as in full-length TEM8-expressing cells (Fig. 3A), suggesting comparable expression levels of both receptors. Like TEM8, ED-TEM8 expression increased cell attachment to PA-coated plates (Fig. 3B). However, ED-TEM8 failed to promote cell spreading as assessed by direct observation by phase contrast microscopy (Fig. 3C). ED-TEM8-expressing cells did not extend on PA-coated dishes after 1 h of plating, although they were fully capable of spreading on MA, a complex mixture of ECM proteins found in basement membranes that engages multiple adhesion molecules (Fig. 3D). These experiments indicate that the extracellular domain of TEM8 is sufficient to mediate cell attachment and toxin internalization, but not cell spreading, thus implicating the essential role for the cytosolic domain in cell spreading.

To directly test the role of the intracellular domain in cell spreading, the cytosolic domain of TEM8 (TEM8-CD) was fused to the extracellular domain of the IL-2 receptor α-subunit (IL-2R). IL-2R is a cell surface molecule involved in signal transduction, with no reported role in adhesion or cell shape control; thus, it has been widely used to study the role of cytosolic domains of other adhesion molecules (28–30). The addition of TEM8-CD to the IL-2 receptor shifted the molecular weight of IL-2R from 55 to 80 kDa (Fig. 4A) and imparted immunoreactivity to the anti-TEM8 cytosolic epitope antibody (Fig. 4A). Expression of IL-2R, as well as IL-2R/TEM8-CD chimera, conferred specific cell attachment to dishes coated with a monovalent antibody against the extracellular domain of the IL-2R, without affecting basal cell attachment to PA (Fig. 4B). Attachment of infected cells to Matrigel remained similar to non-infected cells (mock), thus excluding a detrimental effect of the adenovirus infection. Whereas attachment to anti-IL-2R antibody was similar in wild-type IL-2R and IL-2R/TEM8-CD expressing cells, spreading on this substrate occurred selectively when the cytosolic domain of TEM8 was present (Fig. 4C). IL-2R/TEM8-CD-expressing cells spreading on IL-2R-antibody covered a median area of 3983 pixels (dashed arrow in middle panel of Fig. 4D), area comparable to the median area of 3027 pixels (black arrow in right panel of Fig. 4D) covered by TEM8-expressing cells spreading on PA (Fig. 4D). Thus, these experiments demonstrate that the cytosolic domain is competent to promote cell spreading when linked to a molecule providing cell attachment to an immobilized ligand.

Whereas the direct engagement of the cytoskeleton is not necessary for adherence, as several ECM binding molecules including the 67-kDa extracellular laminin receptor (31) and collagen type I-binding discoidin receptor (32) do not directly interact with the cytoskeleton, association with the cytoskeleton is essential to drive cell spreading. Thus, we next sought evidence for TEM8 association with cytoskeletal proteins by co-immunoprecipitation assays (Fig. 5A). We found a small, but significant, amount of actin co-immunoprecipitating with IL-2R/TEM8-CD but not with the wild-type IL-2R (Fig. 5A). Under identical conditions, no tubulin was immunoprecipitated with IL-2R/TEM8-CD. We could not detect actin associated with full-length TEM8 using antibodies directed to cytosolic epitope or the HA tag (not shown). Besides a possible steric hindrance of the epitopes recognized by the antibodies, an alternative explanation for the low efficiency of actin recovery is the potential association of these receptors with the insoluble cytoskeleton, as is the case with E-cadherin (33). Therefore, we analyzed whether TEM8 and IL2R/TEM8-CD remain associated with the insoluble cytoskeletal fraction in cells undergoing spreading. After 5 min of extraction with 0.15% Triton X-100 at 4 °C, detergent extractable proteins, such as tubulin and IL-2R, distributed to the soluble fraction (S in Fig. 5B), whereas actin, IL-2R/TEM8-CD and TEM8, as well as IL-2R/VE cadherin cytosolic domain chimera remained in large part associated with the insoluble fraction (I in Fig. 5B). Treatment with the actin depolymerizing drug, Latrunculin A, for 5 min before the extraction, promoted solubilization of this group of proteins, indicating that their association with the detergent insoluble fraction is dependent on the integrity of the actin cytoskeleton. The attachment to the cytoskeleton was quantitated by densitometry of the bands in two experiments, and assessed by calculating a solubility index (Fig. 5C). Readily extractable proteins like IL-2R and tubulin had positive values while insoluble proteins, including TEM8, had a negative solubility index. Latrunculin A treatment changed the solubility index of these proteins into positive values as they partitioned like soluble proteins upon disruption of the actin cytoskeleton.

We analyzed the contribution of intermediate filaments in the cytoskeletal association of transfected TEM8 by examining TEM8 association with the insoluble fraction of SW13 carcinoma cells, where vimentin is the sole intermediate filament protein. We compared TEM8 association with the insoluble fraction of SW13 control cells and of a vimentin deficient SW13 cell variant (22). TEM8 associated with the insoluble cytoskeleton regardless of the presence or absence of vimentin, under conditions where actin remained insoluble and most of the cellular tubulin was solubilized (Fig. 5D). Thus, these results exclude intermediate filament participation in TEM8 association with the cytoskeleton. Collectively, they support the interaction of the cytosolic domain of TEM8 with the actin cytoskeleton and suggest that actin cytoskeleton reorganization drives cell shape changes and spreading.

We determined next whether the association with the actin cytoskeleton is necessary for cell spreading. Analysis of actin

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**FIGURE 4. TEM8 cytosolic domain (TEM-CD) is sufficient to confer spreading activity.** A, Western blot analysis for HA tag, TEM8 cytosolic epitope, and IL-2 receptor of mock-infected (lane 1), IL-2 receptor (lane 2), IL-2R/TEM8-CD chimera (lane 3), and TEM8-HA tag (lane 4) delivered into HEK293 cells by adenoviral infection. B, Attachment activity of cells expressing the indicated constructs after 15 min of incubation in cell culture media (DME) to anti-IL-2 receptor, PA, or Matrigel-coated dishes. C, Phase contrast microscopy of cells expressing IL-2 receptor, the chimera IL-2R/TEM8-CD, or TEM8 spreading on Matrigel, anti-IL-2R antibody, or Protective Antigen-coated dishes for 30 min. Scale bar: 50 μm. D, Distribution plots of the quantitation of the experiment shown in C. Arrows indicate the median. (n = 2 independent infections.)
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 filament staining during cell spreading of non-transfected cells (control) on immobilized substrates revealed filopodia-like projections rich in actin (Fig. 6A). In TEM8-expressing cells, the filopodia-like projections lead to lamellipodia formation and membrane extension, increasing the area covered by the cell body. Immunofluorescence of TEM8 showed plasma membrane and a small punctuate intracellular distribution. No distribution reminiscent of focal-adhesion complexes or strong co-localization with stress fibers was observed, as would be expected for a molecule functioning like an integrin. However, TEM8 was localized at the tip of actin-rich filopodia and was enriched at the membrane of lamellipodia. Punctuated co-localization with actin was observed at the base of the lamellipodia (arrowhead, Fig. 6A) and along the actin filaments extending into the lamellipodia (arrows, Fig. 6A). The extensive reorganization of the actin cytoskeleton during spreading predicts the requirement for an intact microfilament cytoskeleton. Interference with microfilament polymerization by cytochalasin D abrogated cell spreading on PA as well as on Matrigel (Fig. 6B). In contrast, disruption of the microtubule cytoskeleton with nocodazole did not affect cell spreading on either substrate. These experiments indicate that TEM8-mediated cell spreading proceeds through the selective reorganization of the actin cytoskeleton, similar to spreading mediated by integrin interaction with the extracellular matrix.

DISCUSSION

In this study, we have shown that TEM8 functions as an autonomous adhesion molecule coupling binding of an immobilized extracellular ligand and cell spreading through association to the actin cytoskeleton. Our results extend to the proposed structural and functional similarity of TEM8 with integrins. Previous studies demonstrated that the interaction of TEM8 with PA is similar to integrin ligands interactions because of divalent cation dependence (16, 34, 35). Furthermore, PA mimics integrin ligands, sharing a carboxylate group-containing residue necessary for binding to the receptor (16). Our studies further indicate that TEM8 can mediate cell-matrix interactions and linkage to the cytoskeleton.

When PA is used as a ligand, our results indicate that TEM8 can function as a single subunit receptor mediating the functions required for adhesion. This notion is supported by our experiments showing that the extracellular domain of TEM8 confers cell tethering when associated to the membrane and that the cytosolic domain imparts anchorage to the cytoskeleton. This would be a feature distinguishing TEM8 from heterodimeric integrins, but resembling other adhesion molecules such as cadherins and selectins. However, these experiments do not exclude cooperation with other proteins to carry out each of these functions.

Because previous studies have shown that the soluble extracellular domain of TEM8 binds to collagen I substrates and overexpression increases cell attachment to collagen I, we eval-
more, many extracellular ligands and transmembrane proteins cooperate with β1 integrins to promote cell spreading: immobilized ADAM12 binds to syndecan-1 to trigger cell spreading through β1 integrins (37), as well as uPAR (reviewed in Ref. 38) and C1q (39). Transmembrane proteins such as tetraspanins and IAP (reviewed in Refs. 24 and 25) also regulate many integrin functions, as well as many cytosolic proteins do. Thus, cell adhesion on either ligand could require a distinctive set of molecular interactions. The results observed in primary fibroblast allow us to exclude a cooperative mechanism with β1 integrins for ligand binding, as TEM8 increased cell attachment and spreading on collagen I in the presence of AIIB2 antibody, which blocks all integrins with affinity for collagen. Although this experiment does not exclude the participation of other β integrins, we deem this possibility unlikely, as we did not find localization of TEM8 into focal adhesion complexes during or after cell spreading. However, our experiments underscore the necessity of directly addressing whether collagen I binding directly to TEM8 with further experiments.

As our results with the extracellular domain of TEM8 illustrate, cell attachment to a substrate does not necessarily leads to spreading (40). Spreading is a multi-step process initiated by cell contact with a substrate resulting in filopodia and lamellipodia extension, which are independent of anchorage to the substrate (41). Progression to spreading requires mechanical coupling of the substrate with the cytoskeleton and concurrent signal transduction. Thus, cell attachment and spreading are dissociable events by defined molecular requirements elicited upon cell attachment and preceding spreading, such as arachidonic acid production (42), protein kinase C activation (43), and small GTPases activation (44). Therefore, our results not only show that TEM8 is capable of mechanically anchoring extracellular proteins to the cytoskeleton, but suggest that this receptor can trigger the necessary signaling events required for cell spreading to proceed in the absence of serum.

Linkage to the cellular cytoskeleton is essential to spreading. TEM8-dependent spreading is similar to integrin mediated spreading on conventional ECM substrates, as it is dependent on microfilaments and not microtubules (45). We found that actin co-immunoprecipitated with the cytosolic domain of TEM8, mediated association of the cytosolic domain to the insoluble cytoskeleton, and became extensively reorganized during TEM8-dependent cell spreading. We could not detect actin associated to the immunoprecipitate of full-length TEM8;
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however, this is common to many other adhesion molecules, which interact with actin indirectly. The complex of cadherins, catenins and actin has not been isolated in immunoprecipitates (46). In the case of integrin, it is necessary to introduce a spacer sequence to the cytosolic domain to circumvent the low affinity interaction with actin binding proteins and thus detect actin association (47). Even though the lack of any known interaction motifs in the cytosolic domain prevents us from speculating about the mechanism for linkage to actin, the productive association of TEM8 with the cytoskeleton is predicted to confer stress resistance and mechanical coupling of the extracellular matrix ligand with the microfilament network. This function provides a mechanistic framework for the previous observation showing increased endothelial cell migration upon TEM8 expression (12). In addition to cell motility, linkage with the cytoskeleton has functional implications for morphogenesis, homeostasis and signal transduction, all processes relevant to angiogenesis (reviewed in Refs. 48 and 49). In sum, our findings show that TEM8 functions as an adhesion molecule, independently of integrins, autonomously linking extracellular ligand and the actin cytoskeleton to support cell spreading.

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