VE-cadherin Links tRNA Synthetase Cytokine to Anti-angiogenic Function*

Received for publication, September 14, 2004, and in revised form, November 30, 2004
Published, JBC Papers in Press, December 3, 2004,
DOI 10.1074/jbc.C400431200

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A natural fragment of an enzyme that catalyzes the first step of protein synthesis—human tryptophanyl-tRNA synthetase (T2-TrpRS) has potent anti-angiogenic activity. A cellular receptor through which T2-TrpRS exerts its anti-angiogenic activity has not previously been identified. Here T2-TrpRS was shown to bind at intercellular junctions of endothelial cells (ECs). Using genetic knock-outs, binding was established to depend on VE-cadherin, a calcium-dependent adhesion molecule, which is selectively expressed in ECs, concentrated at adherens junctions, and is essential for normal vascular development. In contrast, T2-TrpRS binding to EC junctions was not dependent on platelet endothelial cell adhesion molecule type-1, another adhesion molecule found at EC junctions. Pull-down assays confirmed direct complex formation between T2-TrpRS and VE-cadherin. Binding of T2-TrpRS inhibited VEGF-induced ERK activation and EC migration. Thus, a VE-cadherin-dependent pathway is proposed to link T2-TrpRS to inhibition of new blood vessel formation.

During their long evolution, aminoacyl-tRNA synthetases, enzymes that catalyze the first step of protein synthesis—acquired additional functions, including regulation of transcription and translation, RNA splicing, and cytokine activities in inflammatory and angiogenic signaling pathways (1, 2). Recently, fragments of the closely related human tyrosyl-tRNA and tryptophanyl-tRNA synthetase (TrpRS)† were discovered to regulate angiogenesis (2–6). In mammalian cells, TrpRS is activated for anti-angiogenic signaling by proteolysis or alternative splicing to give two natural isoforms—mini-TrpRS and T2-TrpRS (6). Expression of mini-TrpRS is strongly induced (along with other angiostatic factors such as IP-10 and MIG) by the anti-proliferative cytokine interferon-γ (7–9). Mini-TrpRS and T2-TrpRS inhibit development of new vessels without affecting pre-established vasculature (4, 6, 10). Regions within mini-TrpRS that contribute to angiostatic activity have been proposed (11, 12).

The anti-angiogenic activity of T2-TrpRS and mini-TrpRS was demonstrated in several cell-based assays in vitro and also in vivo in the chick embryo and in the neonatal and adult mouse (4, 6). More recently, in vitro studies on endothelial cells (ECs) have shown that the inhibitory activity of mini-TrpRS and T2-TrpRS abrogates cellular responses involved in angiogenesis, such as motility and migration of ECs (4, 6) and activation of extracellular signal-regulated kinase (ERK1/2) and Akt (13). T2-TrpRS inhibited several additional EC responses to the mechanical force of shear stress including endothelial nitric-oxide synthase activation, shear stress-induced gene expression, and cell alignment (13). While the protein binds specifically to the ends of newly formed vessels, the connection of this binding to the inhibition of angiogenic signal transduction pathways remains unknown. To further elucidate the mechanism of T2-TrpRS, we sought to identify the EC target responsible for the potent anti-angiogenic activity.

EXPERIMENTAL PROCEDURES

Cell Culture, Shear Stress, and Transfections—Bovine aortic endothelial cells (BAECs) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, 1 penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen) in a humidified 5% CO2/95% air incubator at 37 °C. VE-cadherin and VE-cadherin RC were prepared as described (14) and grown in DMEM containing 10% FBS, 5 μg/ml EC growth serum, and 100 μg/ml heparin. PECAM-1 and PECAM-1 RC cells were prepared as described (15) and grown in DMEM containing 10% FBS, 10 μg/ml HEPES, 2 mM L-glutamine, 1% non-essential amino acids, pyruvate, 10 μM 2-mercaptoethanol. For transfections, cells at ~50% confluence in 60-mm tissue culture dishes were transfected with the 0.5 μg of DNA using Effectene according to the manufacturer’s instructions (Qiagen, San Diego, CA). After 10 h in growth medium, cells were starved overnight in 0.5% serum prior to binding of T2-TrpRS.

ERK Activation Assays—ERK activation was detected by Western blotting using a monoclonal antibody against phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling); total ERK was detected using a polyclonal antibody against p44/42 MAPK (Cell Signaling).

Fluorescence Microscopy—Alexa488-T2-TrpRS and Alexa546-T2-TrpRS were prepared using AlexaFluor protein labeling kits according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). Cell binding was performed by incubating Alexa488-T2-TrpRS or Alexa546-T2-TrpRS at 30 μg/ml for 15 min. Cells were fixed for 30 min in 2% formaldehyde, rinsed twice with PBS, and mounted in immunofluorescence mounting medium (ICN). Images of fixed cells were acquired using a Bio-Rad 1024 confocal microscope.

Immunoprecipitations and Western Blotting—Following binding of T2-TrpRS, cells were harvested in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Na2VO4, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 1 mM aprotinin, 1 mM PMFS, 1 mg/ml leupeptin, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate). Samples (2 × 106 cells) were preclarified with 50 μl of protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden) for 1 h at 4 °C. Supernatant was then incubated with 50 μl of dium; FBS, fetal bovine serum; MAPK, mitogen-activated protein ki-

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protein A-Sepharose previously conjugated with anti-His Ab (Santa Cruz Biotechnology) for 1 h at 4 °C with continuous mixing. Samples were washed three times in lysis buffer, and immune complexes were eluted in SDS sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with anti-VE-cadherin Ab (Alexis Biochemicals) and horseradish peroxidase-goat anti-rabbit IgG (Jackson Immunochemicals). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham Biosciences). For direct binding of VE-cadherin to T2-TrpRS, a recombinant Fc/VE-cadherin chimera (Novagen) (1 μg/ml) was immobilized on magnetic anti-human IgG beads (50 μl) (Novagen) for 3 h at 4 °C and incubated with (30 μg/ml) T2-TrpRS for 3 h at 4 °C. Bound proteins were resolved by SDS-PAGE and analyzed by Western blot analysis.

Flow Cytometry—Live cells were incubated with 30 μg/ml Alexa488-T2-TrpRS for 30 min at 4 °C and then washed twice with PBS. Cell surface-bound fluorescence was determined in a FACScan® flow cytometer (BD Biosciences), using CellQuest® software.

Migration Assays—Migration assays were performed using the CHEMICON® QCM™ cell migration assay, based on the Boyden chamber principle. Briefly, BAECs that had been serum-starved were allowed to migrate using a chemotactic stimulus, VEGF_{165} (0.5 nM), for 6 h at 37 °C in a 5% CO₂ incubator. For inhibition assays, full-length TrpRS (1 μM) or T2-TrpRS (1 μM) was added 15 min before placement in the chamber. Migrated cells on the bottom of the insert membrane were dissociated from the membrane, lysed, and detected by the CyQUANT™ GR dye.

RESULTS AND DISCUSSION

Binding of T2-TrpRS to Endothelial Cells—Previous studies identified T2-TrpRS as an inhibitor of angiogenesis in vivo, localizing it to blood vessels, and as an inhibitor of EC proliferation and migration in response to VEGF in vitro (4, 6). Further studies revealed that T2-TrpRS was effective at inhibiting EC responses stimulated by either growth factors or mechanical stress (13), indicating that T2-TrpRS may interrupt these signaling systems by acting on a point common to both mechanisms. Shear stress responses inhibited by T2-TrpRS included activation of intracellular signaling pathways involving ERK and Akt, gene expression, and cell alignment (13). These fundamental activities, as well as those stimulated by growth factors, are needed during angiogenesis, and therefore, inhibition of EC responses to either VEGF or shear stress could explain how T2-TrpRS inhibits angiogenesis in vivo.

Interestingly, BAECs that had been pretreated with T2-TrpRS and were subjected to long periods of shear stress failed to align their stress fibers in the direction of flow (13). Eventually cells detached from the substratum and their cell-cell contacts were visibly altered, compared with cells not treated with T2-TrpRS (13). This observation suggested that the cellular target for T2-TrpRS was involved either directly or indirectly in cell-cell and cell-substratum contacts.

Thus, we examined the specific binding of T2-TrpRS (Alexa488-T2-TrpRS) to live BAECs and localized it at foci near intercellular contacts (Fig. 1a). The binding was specific, as it was blocked by preincubation with unlabeled T2-TrpRS (Fig. 1a). The peripheral localization suggested that T2-TrpRS bound to a component of endothelial intercellular junctions. We examined binding to ECs with genetic knock-outs of two prominent cell junction proteins, vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule (PE-
T2-TrpRS Binds to VE-cadherin—At adherens junctions, VE-cadherin interacts with three armadillo proteins, β-catenin, plakoglobin, and p120. Phosphorylation of VE-cadherin regulates the composition of these junctional complexes and release of β-catenin may contribute to cell signaling as it is a transcription cofactor in the Wnt pathway (19). VE-cadherin also acts as a scaffolding protein able to associate dynamically with vascular endothelial cell growth factor receptor (VEGFR2) and to promote its signaling (reviewed in Ref. 20). In several studies the association of proteins to VE-cadherin has been investigated through immunoprecipitation and Western blotting (21–23). Using this approach, we examined whether T2-TrpRS could be used to isolate VE-cadherin from BAEC extracts. T2-TrpRS was immunoprecipitated with an antibody directed against the His-tag of recombinant T2-TrpRS and bound VE-cadherin was detected by Western blot. As a control another nonspecific antibody was used for immunoprecipitation of T2-TrpRS but not with nonspecific IgG.

Because several proteins associate in adherens junctions, it was possible that T2-TrpRS binding to VE-cadherin was dependent on the presence of another junction component. To determine whether T2-TrpRS binds directly to the extracellular domains of VE-cadherin in the absence of adherens junctions, a recombinant VE-cadherin/Fc chimera containing the extracellular domain of VE-cadherin immobilized on magnetic anti-human IgG beads was incubated with T2-TrpRS. As shown in Fig. 2b, T2-TrpRS bound to VE-cadherin immobilized beads. In contrast, T2-TrpRS did not bind to beads alone. Thus, the interaction between T2-TrpRS and VE-cadherin appeared to be direct.

VE-cadherin Reconstitutes Binding of T2-TrpRS—The direct interaction of T2-TrpRS with VE-cadherin indicated that this junctional receptor was the essential endothelial-specific component that facilitated binding of T2-TrpRS to ECs. On this point, VE-cadherin is only expressed by ECs, suggesting that it exerts cell specific function. To test this hypothesis, we examined the binding of T2-TrpRS to a non-EC line, COS7, after transfection with VE-cadherin. COS7 cells lack VE-cadherin and as such do not bind T2-TrpRS. However, COS7 cells do contain a homologue N-cadherin, as well as catenins, which may provide an appropriate cellular context for overexpressing VE-cadherin. COS7 cells were transiently co-transfected with expression plasmids for human VE-cadherin cDNA plus GFP or GFP alone to show specific binding of Alexa488-T2-TrpRS only to VE-cadherin-expressing cells.

Inhibition of Angiogenesis Signaling—Angiogenesis involves a complex series of events including the proliferation, migration, differentiation, and apoptosis of capillary ECs (24). To show that the binding interactions of T2-TrpRS to ECs directly inhibit angiogenesis, we examined the transduction of two well characterized angiogenesis-related signals. VEGF activates specific intracellular survival pathways in ECs including ERK activation. Increased phosphorylation of p42/44 MAPK was observed after 10 min of exposure to VEGF; importantly T2-TrpRS down-regulates VEGF-induced ERK activation in BAECs (Fig. 3a). T2-TrpRS also inhibits angiogenesis by inhibiting VEGF-induced migration of ECs. As shown in Fig. 3b, T2-TrpRS inhibited VEGF-stimulated BAEC chemotaxis, whereas full-length TrpRS had no effect.

Conclusions—The absence of VE-cadherin or blocking of its adhesive activity prevents a normal organization of new vascular structures (16, 25, 26), suggesting that VE-cadherin may be a molecular target for anti-angiogenic therapy. The identification of VE-cadherin as a target for T2-TrpRS explains for the first time how the anti-angiogenic activity of T2-TrpRS is transduced through the inhibition of EC signaling in response to growth factors and shear stress. VE-cadherin has a well established, fundamental role in the growth, survival, and function of normal and pathological tissues (24, 27). During angiogenesis, vascular ECs form capillaries (29, 30) and the endothelial adherens junctions, which are mediated by VE-cadherin/catenins complexes, maintain the integrity of the endothelium, and facilitate capillary tube formation (31–35). In point of fact, targeted inactivation of the VE-cadherin gene caused lethality in mice at 9.5 days of gestation due to vascular insufficiency (16). In VE-cadherin−/− mice, ECs undergo apoptosis and lose the capacity to activate Akt in response to the survival signal of the VEGF (16).
Previous work established that T2-TrpRS inhibited activation of Akt in ECs and induced intercellular gap formation and cell detachment under shear stress (13). Also, VE-cadherin junctions have been shown to mediate the transduction of shear-stress signals in vascular ECs (22, 36–38). Consistent with our observations with T2-TrpRS, disruption of VE-cadherin-independent signaling by the use of blocking antibodies leads to inhibition of angiogenesis in vitro (26, 28, 39) and of tumor vascularization in vivo in mice. Unlike T2-TrpRS, which is selective for newly developing blood vessels (6), antibodies to VE-cadherin not only inhibit new capillary formation but can also induce disorganization within the formed capillaries causing vascular leak and hemorrhage (25, 26, 39). The specificity of T2-TrpRS for newly developing blood vessels in vivo suggests that its interaction is selective for those functions of VE-cadherin that support angiogenesis.

Acknowledgments—We are very grateful to Dr. Elizabetta Dejana for providing VE-cadherin knock-out cells and VE-cadherin cDNA.

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J. Biol. Chem. 2005, 280:2405-2408.
doi: 10.1074/jbc.C400431200 originally published online December 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.C400431200

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PAGE 2406:
Fig. 1: The incorrect images were printed for panels a and b. The correct images are shown below.

a

488-T2-TrpRS + +
T2-TrpRS - +

b

VE-RC

VE-Cadherin-/-

PECAM-1-RC

PECAM-1-/-

FIGURE 1. Panels a and b.

Arachidonate-derived dihomoprostaglandin production observed in endotoxin-stimulated macrophage-like cells.
Richard Harkewicz, Eoin Fahy, Alexander Andreyev, and Edward A. Dennis

PAGE 2910:
Although the authors are listed correctly, Ref. 14 refers to the incorrect paper in J. Lipid Res. The correct reference should read: Raetz, C. R. H., Garrett, T. A., Reynolds, C. M., Shaw, W. A., Moore, J. D., Smith, D. C., Riberio, A. A., Murphy, R. C., Ulevitch, R. J., Fears, C., Reichart, D., Glass, C. K., Benner, C., Subramaniam, S., Harkewicz, R., Bowers-Gentry, R. C., Buczynski, M. W., Cooper, J. A., Deems, R. A., and Dennis, E. A. (2006) J. Lipid Res. 47, 1097–1111.

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