

Evidence for Annexin II-S100A10 Complex and Plasmin in Mobilization of Cytokine Activity of Human TrpRS*^[S]

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In mammalian cells, specific aminoacyl-transfer RNA (tRNA) synthetases have cytokine functions that require interactions with partners outside of the translation apparatus. Little is known about these interactions and how they facilitate expanded functions that link protein translation to other cellular pathways. For example, an alternative splice fragment of tryptophanyl-tRNA synthetase (TrpRS) and a similar natural proteolytic fragment are potent angiostatic factors that act through the vascular endothelial-cadherin receptor and Akt signaling pathway. Here we demonstrate mobilization of TrpRS for exocytosis from endothelial cells and the potential for plasmin to activate the cytokine function of the extracellular synthetase. Direct physical evidence showed that the annexin II-S100A10 complex, which regulates exocytosis, forms a ternary complex with TrpRS. Functional studies demonstrate that both annexin II and S100A10 regulate trafficking of TrpRS. Thus, complexes of mammalian tRNA synthetases with seemingly disparate proteins may in general be relevant to understanding how their expanded functions are implemented.

Aminoacyl-tRNA² synthetases (AARSs) arose early in evolution and established the genetic code by attaching specific amino acids to their cognate tRNAs as the first step in protein synthesis. Over their long evolution, AARSs acquired additional functions, including RNA splicing, RNA trafficking, tran-

scriptional and translational regulation, apoptosis, and cell signaling (1, 2). Thus, AARSs and aminoacyl-tRNA synthetase-interacting multifunctional proteins (AIMP1, AIMP2, and AIMP3) are active in signaling pathways outside of aminoacylation and even of protein synthesis (3–11). The cell signaling function has been demonstrated for naturally occurring fragments of two closely related human AARSs, tyrosyl- (TyrRS) and tryptophanyl-tRNA synthetase (TrpRS) (11–16). Here we report on the cellular interactions of one of these, TrpRS, as a required step in its gaining access to its extracellular receptor for a signaling pathway in angiogenesis. The work also offers potential insight into the many connections that are needed to build up biological systems in higher organisms.

Of the different mechanisms by which proteins can switch between functions, one way is by interacting with other factor(s) as part of a larger complex (17). Thus, AARSs and their auxiliary factors form complexes with other proteins both within and outside of the translational machinery that relates these proteins to their noncanonical functions. For example, glutamyl-prolyl-tRNA synthetase is a member of the GAIT (interferon (IFN)- γ -activated inhibitor of translation) complex that binds to a structural element in the 3'-untranslated region of ceruloplasmin mRNA and regulates its translation (10). Glutamyl-tRNA synthetase has an anti-apoptotic role by interacting with and inhibiting the apoptosis signal regulating kinase-1 in a glutamine-dependent manner (7). Human lysyl-tRNA synthetase is involved in the packaging of HIV virion via the interaction of its N-terminal motif with the C-terminal capsid region of the HIV Gag protein (3). AIMP1, one of three AARS auxiliary factors, interacts with α -subunit of ATP synthase and plays a role in proliferation of endothelial cells (18). Recently, AIMP1 has been suggested to control the retention of an endoplasmic reticulum-resident member of the HSP90 family, gp96, by enhancing gp96 dimerization and its interaction with KDEL receptor-1 that, in turn, mediates retrieval of KDEL-containing proteins from the Golgi to the endoplasmic reticulum (19).

Mammalian TrpRSs contain an N-terminal appended domain that is alternatively spliced to create mini-TrpRS (20, 21). The anti-angiogenic activity of mini-TrpRS and a mini-TrpRS-like proteolytic variant, T2-TrpRS, was demonstrated in several *in vitro* cell-based assays and, in addition, *in vivo* in the three different animal models (chick chorioallantoic membrane, mouse Matrigel model, and retinal tissues of the neonatal mouse) (14, 16). T2-TrpRS interferes with cellular responses

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² The abbreviations used are: tRNA, transfer RNA; TrpRS, tryptophanyl-tRNA synthetase; AARS, aminoacyl-tRNA synthetase; IFN, interferon; HUVEC, human umbilical vein endothelial cell; HLMVEC, human lung microvascular endothelial cell; Allt, heterotetrameric complex of S100A10 and annexin II; TGF, transforming growth factor; VE, vascular endothelial; HIV, human immunodeficiency virus; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IL, interleukin; TNF, tumor necrosis factor; siRNA, small interfering RNA; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; FGF, fibroblast growth factor; uPA, urokinase-type plasminogen activator.

to angiogenic signals, such as proliferation and migration (16), and cell signaling events that include activation of extracellular signal-regulated kinase, protein kinase B (Akt), and nitric-oxide synthase (22). In a shear stress model, T2-TrpRS inhibited canonical responses to mechanical force, such as endothelial cell alignment in direction of fluid flow (22). Subsequent work established vascular endothelial (VE)-cadherin as a receptor for T2-TrpRS that mediated its anti-angiogenic activity (23). This potent activity can be elucidated through administration of exogenous T2-TrpRS or delivery via gene therapy and has found applications to angiogenesis-related disease of the eye and to oncology (12, 14, 24).

Overexpression of TrpRS was observed during development of the salivary gland in *Drosophila* (25), during delayed type hypersensitivity reactions in guinea pigs (26) and in aged human epidermis (27). The stimulus for this overexpression in these instances was not established. However, earlier work demonstrated that the antiproliferative cytokine IFN- γ up-regulated levels of TrpRS and mini-TrpRS mRNA (20, 28, 29). IFN- γ regulates expression of hundreds of genes, some of which are involved in host defense or inflammation (30), whereas others are anti-angiogenic, such as IP-10 (interferon-inducible protein-10) and MIG (monokine induced by γ -interferon) (31, 32). Based on induction of TrpRS expression by IFN- γ , and because fragments of TrpRS show angiostatic activity, we wondered whether IFN- γ played a role in up-regulating TrpRS to poise it for mobilization and elaboration of its cytokine activity. With this perspective, we started by investigating the possibility of detecting secretion from cells that were stimulated with IFN- γ and, more broadly, to identify the interacting partners of TrpRS that were needed for its mobilization and trafficking.

EXPERIMENTAL PROCEDURES

Cell Culture—Human umbilical vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HLMVECs) were obtained from Lonza (Basel, Switzerland) and maintained in endothelial growth medium (EGM) (Lonza) supplemented to 10% FBS (Invitrogen) and EGM-MV (Lonza), respectively, and cultivated on gelatin-coated surfaces in an atmosphere of 5% CO₂ in air at 37 °C according to the supplier's instructions. C8161 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS and 1% solution of penicillin and streptomycin.

TrpRS Expression and Secretion—HUVECs were cultured to 60% confluence, washed twice with phosphate-buffered saline (PBS) (Invitrogen) and further cultured in medium containing 2% FBS, with or without various concentrations of IFN- γ (Roche Applied Science). At specific time points, the cells were washed with PBS and collected in 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA. The cells were centrifuged at 1500 rpm for 10 min at 4 °C and lysed in 25 mM HEPES, pH 8.0, containing 5 mM EDTA and 0.1% CHAPS. The lysates were separated using a 4–12% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA), and probed with antibodies against TrpRS, TyrRS, and actin and sequential treatment with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch,

West Grove, PA). Western blots were developed using enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). To determine the effect of TGF- β (Roche Applied Science), IL-4 (R & D Systems, Minneapolis, MN), and TNF- α (Roche Applied Science) on TrpRS expression, HUVECs were transferred to 2% FBS containing endothelial growth medium and stimulated with various concentrations of these cytokines with or without IFN- γ (100 units/ml). TrpRS expression was determined in whole cell lysates by Western blot analysis.

To investigate whether TrpRS was secreted from HUVECs, culture supernatants from IFN- γ -treated cells were collected, concentrated using Centricon-10 (Millipore), and analyzed by Western blot analysis. Further, concentrated culture supernatants were precleared using protein G-Sepharose beads (Pierce). Cleared supernatants were then incubated with protein G-Sepharose previously conjugated with anti-TrpRS antibody (rabbit) for 3 h at 4 °C with continuous mixing. The samples were washed thoroughly in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture tablets (Roche Applied Science)), and the complexes were eluted using SDS-PAGE sample buffer. The samples were resolved on a 4–12% SDS-PAGE, and Western blots were performed using anti-TrpRS antibody (chicken) as primary and donkey anti-chicken horseradish peroxidase as the secondary antibody.

Yeast and Bacterial Strains—*Saccharomyces cerevisiae* strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4 Δ , gal80 Δ , LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ MEL 1) (Clontech, Mountain View, CA) was used for all yeast two-hybrid analyses. *Escherichia coli* strain DH5 α was used for plasmid amplification and other molecular biology protocols. *E. coli* strain BL21(DE3) was used for protein expression.

Yeast Two-hybrid Analysis—Yeast two-hybrid analysis was performed with the MATCHMAKER two-hybrid system (Clontech) using a mouse 7-day embryo cDNA library (Clontech), which was amplified in *E. coli* as per the manufacturer's protocols. Human Library transformations were made using the Yeastmaker Yeast Transformation System 2 (Clontech). The experimental setup aimed to reduce false positives by relying on three reporter genes: two auxotrophic markers (ADE2 and HIS3) and lacZ (which allows for measurement of β -galactosidase activity). TrpRS and T2-TrpRS were used as baits to screen the library. Under high stringency conditions, $\sim 3\text{--}5 \times 10^7$ yeast colonies were screened in four separate library transformations. Positive clones were selected on selective plates lacking leucine and tryptophan to allow for selection of the bait and target plasmids that carried a Leu⁻ and Trp⁻ marker, respectively, as well as histidine and adenine to enable selection for interacting proteins. From these screens, 12 positive colonies were isolated and further examined for β -galactosidase activity, which results from a protein-protein interaction that activates the Lac promoter. Positive yeast colonies were grown in liquid medium overnight, and DNA was extracted by treating yeast cells with 400 μ l of yeast cracking buffer (40 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, and 1 mM EDTA) and an equivalent volume of glass beads. The cells were vigorously vortexed for 5

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min, and DNA was extracted in phenol-chloroform-isoamylalcohol (25:24:1). DNA was concentrated with ethanol, and the insert region of target plasmid amplified by PCR using the MATCHMAKER insert screening primers. PCR products were sequenced, and the results were analyzed using BLAST (33). Once identified, positive clones were reintroduced into the vector pACT2 (Clontech) by ligation into the *Sma*I/*Bam*HI sites, cotransformed into yeast with plasmids encoding TrpRS and T2-TrpRS as bait. These experiments checked for the ability of the transformed yeast to grow on high stringency plates and also for induction of the lacZ reporter gene. *In vitro* binding to T2-TrpRS was tested using MATCHMAKER coimmunoprecipitation kit (Clontech). [³⁵S]Methionine-labeled protein products were prepared for pull-down using the TNT coupled reticulocyte lysate systems (Promega, Madison, WI).

Coimmunoprecipitation—Following stimulation of HUVECs with IFN- γ for 24 h, the cells were washed with PBS and extracted in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Nonidet P-40, 1 mM EGTA, 50 mM octyl glucoside, 10 mM sodium fluoride, 1 mM sodium vanadate, and protease inhibitors) by passing several times through 22-gauge needle. The samples (10^6 cells) were precleared with 50 μ l of protein G-Sepharose at 4 °C. The supernatants were then incubated with protein G-Sepharose previously conjugated with anti-TrpRS antibody for 3 h at 4 °C with continuous mixing. The immunoprecipitates were washed thoroughly with lysis buffer and eluted using SDS-PAGE sample buffer. Western blot analysis was performed using anti-annexin II (Invitrogen) and anti-S100A10 (BD Biosciences, San Jose, CA) antibodies and horseradish peroxidase-conjugated goat anti-mouse secondary antibody. Similar immunoprecipitations were performed using anti-annexin II and anti-S100A10 antibodies.

RNA Interference—RNA nucleotides were obtained from Dharmacon Research (Lafayette, CO). A mix of four small interfering RNA (siRNA) duplexes derived from different regions of human annexin II (5'-AGACCAAAGGUGUGGAUGAUU-3', 5'-CUGUCAAGCCUAUACUAAUU-3', 5'-CGACGAGACUCUCUUCUAAUUU-3', and 5'-AUACUAAUUGAUGCUGAUU-3') and human S100A10 (5'-GAUAAAGGCUACUUAACAAUU-3', 5'-GAAAAGGAGUCCUGGAUUU-3', 5'-GAACACGCCAUGGAAACCAUU-3', and 5'-GGACCAGUGUAGAGAUGGCUU-3') was used for gene silencing. For siRNA transfections, HLMVEC, HUVEC, and C8161 cells were grown to 80% confluence and transfected with 2 μ g of each annealed siRNA duplex using a nucleofection kit (Amaxa, Gaithersburg, MD). 24 h after transfection, the cells were washed with PBS and incubated in medium containing 2% FBS with or without human IFN- γ . The cells were incubated for another 20 h, following which cells and culture supernatants were collected and analyzed for annexin II, S100A10, and TrpRS by Western blot analyses.

TrpRS Purification and Plasmin Digestion—Human full-length TrpRS was purified as described earlier (16). Briefly, DNA encoding TrpRS (residues 1–471) was cloned in *Nde*I/*Hind*III sites of pET-20b vector (Novagen, Madison, WI). The expressed TrpRS (484 amino acids) has 13 extra residues at the C terminus, including a six-histidine tag, from the vector sequence. TrpRS was expressed in *E. coli* strain BL21(DE3)

(Novagen) by induction with 1 mM isopropyl β -D-thiogalactopyranoside for 4 h. The protein was purified from the supernatants of the lysed cells using nickel-nitrilotriacetic acid-agarose column chromatography (Qiagen) according to the manufacturer's instructions.

Plasmin from human plasma (Sigma) was used to digest TrpRS. The digestions were performed at a TrpRS:Plasmin ratio of 10:1 at 25 °C for 0.5, 1, 2, 4, 6, and 22 h in PBS. Protein fragments so produced were resolved on a 10% Bis-Tris gel. The 4-h sample was dialyzed against water and characterized using MALDI-TOF mass spectrometry. The sample was also run on SDS-PAGE and transferred to polyvinylidene difluoride membrane, and the N termini of five prominent fragments were characterized by Edman degradation.

RESULTS

TrpRS Expression in Endothelial Cells—IFN- γ up-regulates expression of TrpRS and mini-TrpRS mRNA in several cell types (20, 28). In HUVECs, the level of TrpRS mRNA increased by ~30-fold following IFN- γ treatment (29). Because previous work only investigated the effect of IFN- γ on expression of TrpRS mRNA, we treated HUVEC monolayers with increasing concentrations of IFN- γ (50–250 units/ml) for 12 h and analyzed the levels of expressed protein. From Western blots of whole cell lysates, IFN- γ was seen to stimulate expression of full-length TrpRS and of lesser amounts of mini- and T2-TrpRS (Fig. 1A). Induction of TrpRS was time-dependent, with a continuous increase from 6 to 36 h, after which it reached a stable plateau (Fig. 1B). In contrast, IFN- γ stimulation had no effect on expression of actin or of TyrRS, a close homolog of TrpRS (Fig. 1A). (Induction of TrpRS was observed at concentration as low as 0.3 unit/ml IFN- γ , and TrpRS expression increased by 5-fold with 3 units/ml (0.4 ng/ml) IFN- γ . Even greater expression was observed at higher concentrations of IFN- γ , beyond the physiological range (supplemental Fig. S1).)

In addition, none of three other cytokines that were tested, TNF- α , TGF- β , and IL-4, had any effect on TrpRS expression in HUVECs (Fig. 1C). These experiments tested the three cytokines individually and in combination with IFN- γ . Collectively, these data demonstrate strong, specific, and dose-dependent stimulation of TrpRS expression in HUVECs.

TrpRS Secretion from Endothelial Cells—As stated earlier, full-length TrpRS is inactive in angiogenesis. Only the alternative splice fragment mini-TrpRS and the closely similar proteolytic fragment T2-TrpRS are potent angiostatic factors. The strong up-regulation of expression by IFN- γ treatment was most pronounced for full-length TrpRS. Thus, we surmised that if the biological rationale for up-regulation was for signaling in an angiogenesis pathway, then TrpRS itself, in addition to mini-TrpRS, would have to be secreted. Consistent with the idea that TrpRS would be expected to be secreted, Favorova *et al.* (34) showed that mammalian TrpRS is present in the exocrine part of the pancreas and in the pancreatic juice. After secretion, TrpRS could be converted by cleavage (with an extracellular protease such as plasmin) to T2-TrpRS. Indeed, in addition to native TrpRS, Favorova *et al.* (34) also detected, in the pancreatic juice, fragments of TrpRS that correspond to the molecular size of T2-TrpRS.

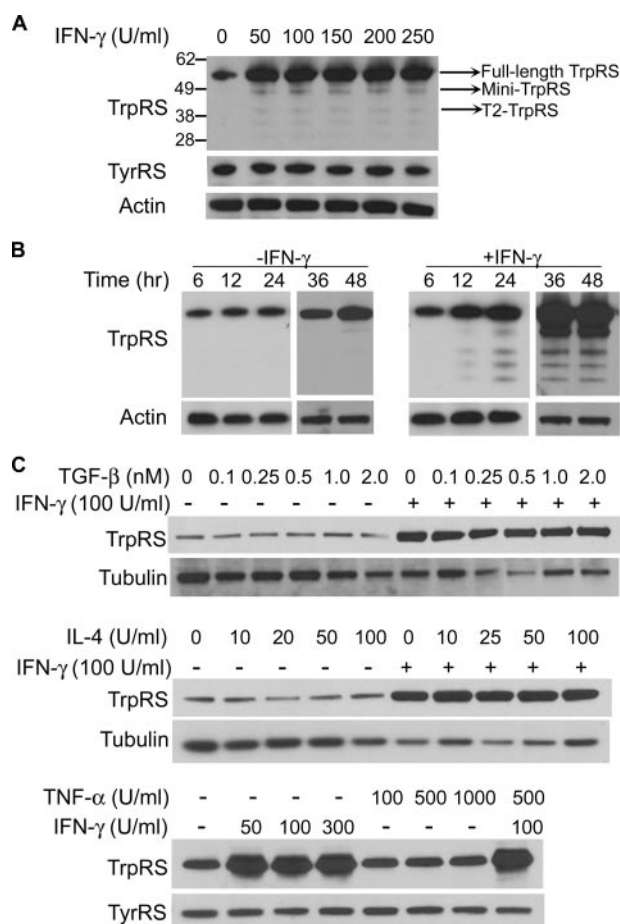


FIGURE 1. IFN- γ stimulates expression of TrpRS in HUVECs. *A*, concentration-dependent up-regulation of TrpRS in HUVECs stimulated with IFN- γ . HUVEC monolayers were stimulated with 50–250 units/ml IFN- γ in 2% FBS containing medium for 12 h, and protein expression in whole cell lysates was determined by Western blotting with the indicated antibody. Bands corresponding to positions of full-length (53 kDa), mini (48 kDa), and T2-TrpRS (43 kDa) are indicated. The numbers on left side of the panel indicate the positions of molecular mass markers in kDa. IFN- γ stimulation did not affect levels of TyrRS, a homolog of TrpRS. Actin was used as a loading control. *B*, time-dependent up-regulation of TrpRS in HUVECs stimulated with IFN- γ . Western blot of HUVEC whole cell lysates stimulated with 200 units/ml IFN- γ for 6, 12, 24, 36, and 48 h. A continuous increase in TrpRS levels, upon IFN- γ stimulation, was observed from 6 to 36 h, after which it reached a stable plateau. *C*, TGF- β , IL-4, and TNF- α do not stimulate expression of TrpRS in HUVECs. HUVEC cultures were stimulated with TGF- β (0.1–2 nM) and IL-4 (10–100 units/ml), with and without stimulation with 100 units/ml IFN- γ , and protein expression in whole cell lysates was determined by Western blot analysis with the indicated antibody. Tubulin was used as a loading control. In a similar experiment, whole cell lysates from HUVECs stimulated with TNF- α (100–1000 units/ml) and TNF- α (500 units/ml), together with IFN- γ (100 units/ml), were probed for TrpRS by Western blot analysis.

To detect secretion of TrpRS from cultured cells, we investigated culture supernatants of IFN- γ (200 units/ml)-stimulated HUVECs at 6, 12, 18, 24, and 36 h. As compared with unstimulated cultures, a sharp increase in secreted protein at 12 and 18 h was observed (Fig. 2A). This increase was detectable right up to 36 h. Based on a quantitative analysis, the percentage of secreted TrpRS at 24 h was 0.44 and 1.88%, without and with IFN- γ induction, respectively. As shown by anti-actin (or anti-GAPDH in supplemental Fig. S2) blots of culture supernatants, no cell lysis was observed at any of these time points. (In separate experiments we showed that, had cell lysis occurred, we could have detected less than 0.2% of the total cellular GAPDH.

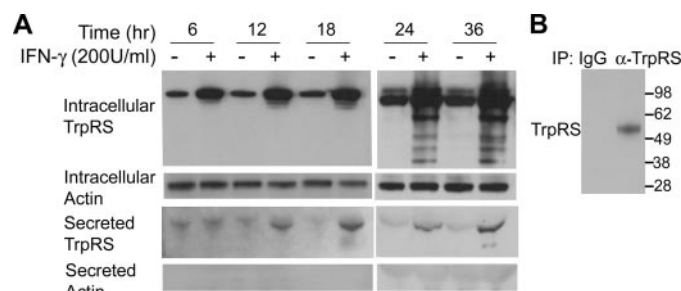


FIGURE 2. IFN- γ stimulates secretion of TrpRS in HUVECs. *A*, HUVEC monolayers were stimulated with 200 units/ml IFN- γ , and TrpRS levels were examined in whole cell lysates and supernatants at 6, 12, 18, 24, and 36 h after stimulation. Secreted TrpRS was detected at 6 h, with an increase in secreted protein at 12 and 18 h. The lack of actin in the culture supernatant (secreted actin) indicates absence of cell lysis. *B*, supernatants from IFN- γ stimulated cultures were further subjected to immunoprecipitation (IP) using anti-TrpRS antibody. The immunoprecipitate was subjected to electrophoresis, and a single band at \sim 53 kDa, corresponding to the full-length TrpRS, was observed in a Western blot using anti-TrpRS antibody (lane 2). Lane IgG side of the panel indicate the positions of molecular mass markers in kDa.

However, even at this level of sensitivity, no GAPDH was detected in cell supernatants.) To confirm that the major secreted protein was full-length TrpRS, anti-TrpRS antibody was used to immunoprecipitate culture supernatants from IFN- γ stimulated cultures. The immunoprecipitate was dissolved in SDS-PAGE loading dye and subjected to electrophoresis and Western blot analysis using anti-TrpRS antibody. A single band at \sim 53 kDa, corresponding to the full-length TrpRS, was observed (Fig. 2B).

TrpRS secretion was not inhibited by brefeldin A (supplemental Fig. S3), which is known to inhibit the classical secretion pathway by causing disintegration of the Golgi apparatus and redistribution of Golgi enzymes into the endoplasmic reticulum. In the same experiment, brefeldin A inhibited secretion of matrix metalloproteinase-2. A23187 stimulates calcium-dependent exocytosis and enhances the release of thioredoxin, IL-1 β , and galectin-3 (proteins known to be secreted by non-classical pathway). TrpRS secretion was not affected by A23187, thus suggesting that TrpRS may use a nonclassical secretion pathway that is different from any of these proteins.

Identification of S100A10 as a TrpRS-interacting Protein— The polypeptide sequence of native TrpRS does not contain secretion signatures. This absence raised the possibility that TrpRS associated with one or more proteins that are known to be secreted and thereby was carried out of the cell. To identify proteins that interact with TrpRS *in vivo* and possibly aid its secretion, a mouse cDNA expression library, derived from 7-day embryos, was screened using the Clontech Matchmaker yeast two-hybrid system. Both a cDNA for TrpRS and, separately, for T2-TrpRS were used as baits. Portions of the following genes were identified in the positive clones: glycosylphosphatidylinositol-specific phospholipase D1, lymphocyte antigen 6 complex, TGF- α , Nieman Pick type C2 protein, and S100A10 (Table 1).

Subsequently, positive clones were individually retransformed into yeast with plasmids encoding TrpRS and T2-TrpRS as bait. As shown in Table 1, in the retrieval of the yeast two-hybrid system, upon cotransformation of various positive clones with TrpRS and T2-TrpRS-containing plasmids, only

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TABLE 1

List of clones identified by yeast two-hybrid analyses and the subsequent characterization of their interaction based on three assays

Clones	Growth	β -Galactosidase	Coimmunoprecipitation
Glycosylphosphatidylinositol-specific phospholipase D1	–	–	–
Lymphocyte antigen 6 complex	–	–	–
Niemen Pick type C2 protein	–	–	–
TGF- α	+	–	–
S100A10	+	+	+

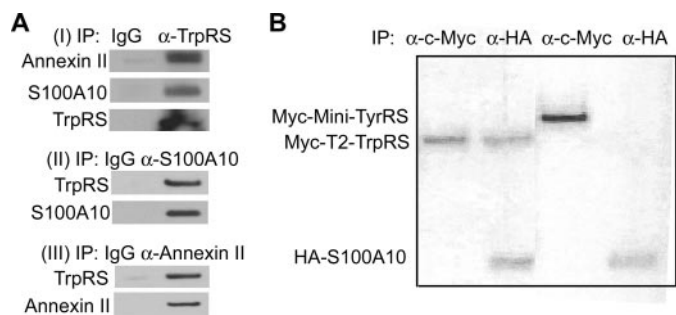


FIGURE 3. S100A10 interacts with TrpRS. *A*, coimmunoprecipitation of annexin II and S100A10 with TrpRS. HUVEC lysates were subjected to immunoprecipitation using anti-TrpRS (*I*), anti-S100A10 (*II*), and anti-annexin II (*III*) antibody coated on protein G beads. The precipitates were analyzed by Western blots using indicated antibodies as probes. Lane IgG was loaded with proteins precipitated by rabbit IgG-coated protein G beads. *B*, *in vitro* pull-down assay. [35 S]Methionine-labeled proteins were prepared by *in vitro* transcription and translation, and immunoprecipitation was performed. The samples were run on SDS-PAGE and analyzed by autoradiography. Lane 1, immunoprecipitation (IP) of Myc-T2-TrpRS with anti-c-Myc antibody; lane 2, coimmunoprecipitation of Myc-T2-TrpRS and HA-S100A10 with anti-HA antibody; lane 3, immunoprecipitation of Myc-mini-TyrRS with anti-c-Myc antibody; lane 4, coimmunoprecipitation of Myc-mini-TyrRS and HA-S100A10 with anti-HA antibody. Myc-T2-TrpRS coimmunoprecipitated with HA-S100A10 (lane 2); Myc-mini-TyrRS, a similar size fragment of TyrRS, did not coimmunoprecipitate with HA-S100A10 (lane 4).

cells harboring the cDNA encoding mouse TGF- α or S100A10 grew in the absence of selective nutrients. Further, only the S100A10 protein was able to stimulate production of β -galactosidase. Sequencing of the TGF- α -encoding clone revealed it to be a portion of the 3'-untranslated region of the gene. BLAST searches using the predicted amino acid sequence of this clone returned no significant homology to any known protein.

TrpRS Interacts with S100A10-Annexin II Complex—To further confirm the interaction between S100A10 and TrpRS in cultured cells, we performed coimmunoprecipitation experiments using HUVEC lysates. As observed by Western blot analysis using anti-S100A10 antibodies, anti-TrpRS antibody coimmunoprecipitated S100A10 (Fig. 3A). Aware that S100A10 forms a complex with annexin II, we probed for and experimentally confirmed the trimeric complex containing annexin II, S100A10, and TrpRS. Thus, in our anti-TrpRS pull-down samples that demonstrated an interaction between S100A10 and TrpRS, we probed for and easily detected annexin II.

To further confirm the complex formed from a S100A10, annexin II, TrpRS interaction, we used anti-S100A10 and anti-annexin II antibodies for pull-downs with HUVEC lysates. These immunoprecipitates were then probed with anti-TrpRS antibodies. A band corresponding to the size of TrpRS was clearly detected in Western blots using anti-TrpRS antibodies (Fig. 3A). Based on quantitation of Western blots, 0.64 and 0.85% of total TrpRS was associated with annexin II and S100A10, respectively (supplemental Fig. S4).

Our results with the yeast two-hybrid system suggested that the vertebrate-specific N-terminal extension (of ~60 amino acids) that regulates the cytokine function of TrpRS was not needed for the interaction with S100A10. (As stated above, the cytokine function is activated by removal of the vertebrate-specific N-terminal extension, through alternative splicing to give mini-TrpRS (47 amino acids removed from the N terminus) or by natural proteolysis to give the closely similar fragment T2-TrpRS (93 amino acids removed from the N terminus.) To further investigate the interaction of S100A10 with the angiostatic form of TrpRS, T2-TrpRS, we used pull-down of *in vitro* translation products to study the ability of proteins to interact. A natural fragment of human TyrRS, known as mini-TyrRS, is the closest homolog of human T2-TrpRS. For both T2-TrpRS and mini-TyrRS, the extraneous domains that are idiosyncratic to TrpRS and TyrRS, respectively, have been stripped away, thereby leaving the homologous core proteins. Thus, the potential interaction of S100A10 with *in vitro* translated mini-TyrRS was thought to be a good control.

T2-TrpRS (with c-Myc tag) and S100A10 (with HA tag) were prepared by *in vitro* transcription/translation. The translation products were immunoprecipitated with anti-HA antibodies, and the precipitate was then dissolved and subsequently run on an SDS-PAGE gel. A band corresponding to the size of T2-TrpRS was readily detected, thus confirming the interaction of S100A10 with T2-TrpRS (Fig. 3B). In contrast, following the same protocol with a c-Myc tag for the mini-TyrRS translation product, no interaction of S100A10 with mini-TyrRS was seen (Fig. 3B). Other members of the S100 family, including S100A8, A9, and A11, did not interact with T2-TrpRS in either the yeast two-hybrid assay or pull-down experiments (data not shown). Thus, the interaction of S100A10 with TrpRS appears to be specific and to require a region of TrpRS that is common to T2-TrpRS.

Functional Test for Annexin II and S100A10 as Interacting Partners for TrpRS—The annexin II-S100A10 complex regulates exocytosis in endothelial cells (35) and is crucial in routing epithelial calcium channels, TRPV5 and TRPV6, to the plasma membrane (36). To demonstrate a functional link that tested for and expanded on the above stated genetic and physical evidence for an interaction between annexin II, S100A10 and TrpRS, we suppressed cellular levels of annexin II and S100A10, using siRNAs. As depicted in Fig. 4A, transfection of primary HLMVECs, with either a mix of four duplex annexin II or of S100A10 siRNAs, resulted in a 90–95% decrease in levels of annexin II and S100A10. In both cases, intracellular TrpRS levels were not affected by the siRNA treatments. However, Western blots of cell culture supernatants showed that upon IFN- γ administration, knocking down S100A10 expression led to higher levels of TrpRS in culture supernatants. Although not as

dramatic, depletion of annexin II in the same cells also caused an elevation in levels of TrpRS. (As shown by anti-GAPDH blots of culture supernatants (Fig. 4A and supplemental Fig. S2), no cell lysis was observed during these experiments.) Thus, the increased levels of TrpRS in the culture supernatants corresponded to its increased exocytosis from HLMVECs.

To investigate whether these observations could be extended to other endothelial cell lines, we knocked down levels of S100A10 and annexin II in HUVECs and in the C8161 melanoma cell line. When S100A10 expression was knocked down (in the presence of IFN- γ), an increase in secretion of TrpRS was observed in supernatants of HUVECs (Fig. 4B). In C8161 cells, although knocking down S100A10 levels slightly elevated TrpRS secretion, a reduction in the expression of annexin II

caused a marked (in presence of IFN- γ) increase in secretion of TrpRS (Fig. 4C). Thus, depending on the specific cell type, knocking down expression of S100A10 (HUVECs) and of annexin II (C8161 cells) significantly increased exocytosis of TrpRS. (Quantitative analysis of the data showed that the amount of secreted TrpRS is significantly increased in the presence of siRNA directed against annexin II and S100A10, with or without IFN- γ in HLMVECs. In HUVECs, siRNA against S100A10 significantly enhanced TrpRS secretion, and in C8161 cells, siRNA against annexin II increased TrpRS secretion (supplemental Table S2).)

These results further strengthen the connection between S100A10 and annexin II with TrpRS, which was established by direct genetic (two-hybrid selections) and physical (pull-down) assays. Part of this connection seems to involve a role for S100A10 alone or in complex with annexin II in the cytoplasmic retention of TrpRS, possibly as part of an endocytosis-exocytosis cycling system. In this connection, the recent work of Renigunta *et al.* (37) showed that S100A10 plays a similar role as a cytoplasmic retention factor for TASK-1, an endoplasmic reticulum potassium channel.

Processing of TrpRS into Its Angiostatic Forms by Plasmin—

The aforementioned results show that native, full-length TrpRS is secreted and that annexin II and S100A10 affect cellular export. Because angiogenesis is dependent upon the ability of proteases to create locally new signaling molecules, major proteases required for angiogenesis must cleave secreted full-length TrpRS into angiostatic, truncated forms like T2-TrpRS. Several proteases that are essential for angiogenesis release from the extracellular matrix regulatory fragments like endostatin, canstatin, and tumstatin by generating activated fragments from inactive full-length proteins (38). Because the angiostatic activity of TrpRS can only be seen when the enzyme is split, one or more proteases are needed to process the secreted native enzyme. With these considerations in mind,

plasmin is of particular interest because of its extracellular location on endothelial cells (39).

To pursue whether plasmin could produce the T2-/mini-TrpRS-like fragments needed for angiostatic activity, recombinant TrpRS (484 amino acids, including 13 extra residues at the C terminus from the vector) was cleaved with plasmin. Five major fragments of TrpRS were generated by plasmin digestion and their identities determined by N-terminal sequencing and MALDI-TOF mass spectrometry. Fig. 5A shows the pattern on a SDS gel of fragments of TrpRS that were obtained after incubation with plasmin. The mass of the fragments so generated was determined by MALDI mass spectrometry (Fig. 5B). Mini-TrpRS and T2-TrpRS begin at positions 48 and 94 in the

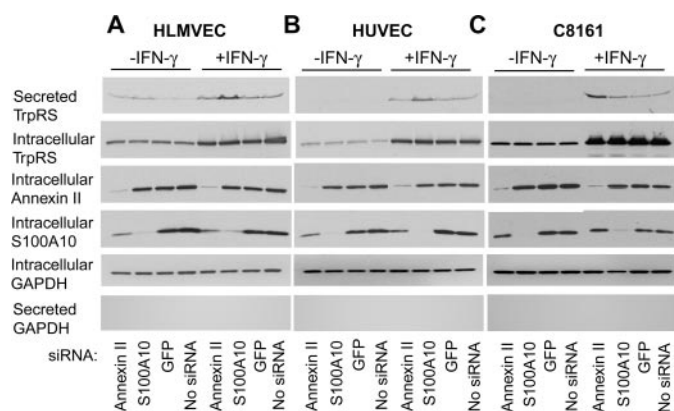


FIGURE 4. Knocking down annexin II and S100A10 facilitates TrpRS secretion from cells. Expression of annexin II and S100A10 in HLMVEC (A), HUVEC (B), and C8161 cells (C) was knocked down by either annexin II or S100A10 siRNAs. siRNA against green fluorescent protein and transfection medium without siRNA (No siRNA) were used as controls. The transfected cells were incubated for 20 h in culture medium supplemented with 200 units/ml human IFN- γ , and cells and culture supernatants were collected. TrpRS, annexin II, S100A10, and GAPDH were detected by Western blotting as described under "Experimental Procedures." The result is representative of three separate experiments.

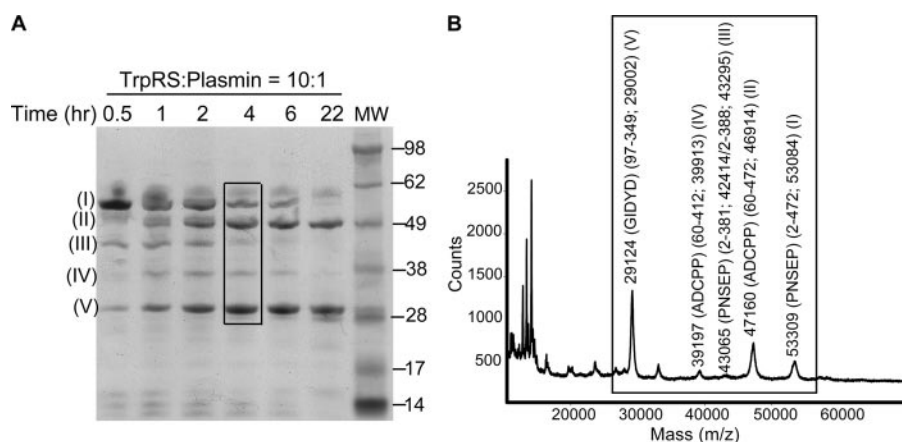


FIGURE 5. Cleavage of TrpRS by plasmin generates mini-TrpRS and T2-TrpRS-like fragments. A, SDS-PAGE of TrpRS cleaved with plasmin. 5 μ g of TrpRS was incubated with plasmin at a TrpRS:Plasmin ratio of 10:1 at 25 $^{\circ}$ C, and samples were collected at 0.5, 1, 2, 4, 6, and 22 h and resolved on a 10% Bis-Tris gel (lanes 1–6); molecular mass markers are shown in lane 7. The numbers on right side of the panel indicate the positions of molecular mass markers in kDa. B, MALDI-TOF mass spectrometry analysis of TrpRS incubated with plasmin for 4 h. The experimentally determined mass for each fragment is given on top of the corresponding peak. The N-terminal residues (based on Edman degradation) of five prominent fragments (I–V) are given in parentheses along with the predicted fragment size and expected mass based on amino acid sequence of the fragment. Fragments II and IV and fragment V are similar to the angiostatic forms of TrpRS: mini-TrpRS and T2-TrpRS, respectively.

Annexin II-S100A10 Complex Interacts with TrpRS

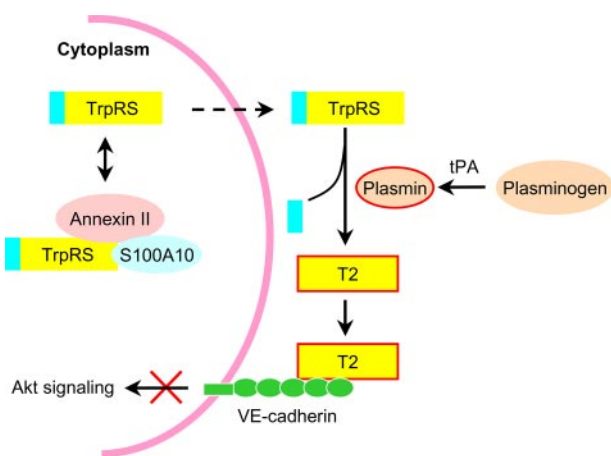


FIGURE 6. Schematic mechanism of export and activation of TrpRS during angiogenesis. Interaction of TrpRS with annexin II-S100A10 complex (AII) regulates its retention in the cytosol. Dissociation of AII from TrpRS allows export to the cell exterior. Proteases, such as plasmin, act on TrpRS to remove the eucaryote-specific extension (shown in cyan), to generate T2-TrpRS. T2-TrpRS then binds to cell surface receptor VE-cadherin and initiates the angiostatic signaling pathway. tPA, tissue plasminogen activator; T2, T2-TrpRS; Akt, protein kinase B.

full-length TrpRS sequence, respectively. Fragments similar to the angiostatic forms, mini-TrpRS (fragment II, cleavage after Lys⁵⁹ and Lys⁴⁷²; and fragment IV, cleavage after Lys⁵⁹ and Lys⁴¹²) and T2-TrpRS (fragment V, cleavage after Lys⁹⁶ and Lys³⁴⁹), were identified (Fig. 5B). Thus, once full-length TrpRS is secreted, it can in principle be cleaved into angiostatic active fragments by action of extracellular proteases such as plasmin. Whether additional proteases can also process TrpRS in a similar manner remains to be determined.

DISCUSSION

Based on our results, we infer that exocytosis of TrpRS requires dissociation of TrpRS from the annexin II-S100A10 complex. (In separate experiments, upon IFN- γ stimulated exocytosis of TrpRS, we did not observe an accompanying exocytosis of annexin II or S100A10 (data not shown).) Once outside the cell, plasmin or another protease cleaves the native enzyme into the angiostatic fragments. These fragments can then inhibit the Akt signaling pathway through their known interaction with VE-cadherin (Fig. 6).

In our experiments, on the order of 1% of TrpRS is tied up with annexin II and with S100A10. At the same time, the amount of secretion of TrpRS is also on the order of 1%. This raises the question of how the small amount of TrpRS that is associated with annexin II and S100A10 can prevent a greater secretion of TrpRS. Possibly, the nonclassical secretory mechanism used by TrpRS has kinetic and thermodynamic parameters that allow only a small fraction of the enzyme to be secreted. It is also at least a formal possibility that a greater percentage of TrpRS is complexed to annexin II and S100A10 *in vivo* than what we detected. The results of quantitation analysis can depend on experimental conditions of the immunoprecipitation procedure. (For example, dissociation of the complex caused by lysis buffer components can lead to reduction in percentage of TrpRS bound to S100A10 and annexin II. Limited binding of antibody to the antigen can also cause a reduction in

immunoprecipitation efficiency.) Additionally or alternatively, TrpRS could also be complexed to other, unidentified cellular proteins, including components of the translation apparatus, so that only a small portion of the total TrpRS is present in a free form that can be secreted from cells.

Protein export in mammalian cells usually occurs via the classical secretory pathway that traverses the endoplasmic reticulum and Golgi apparatus, where the secreted proteins contain a signal sequence with all the information required to target them for exocytosis. TrpRS appears not to follow this pathway and to be more like the nonclassical examples, such as basic fibroblast growth factor (FGF-2), FGF-1, interleukin-1 β , HIV-tat, galectin-3, and thioredoxin, which are secreted in a variety of nonclassical ways independent of the endoplasmic reticulum-Golgi pathway (40). Of all the well studied nonclassical export pathways, most is known about the secretion of FGF-1. Two intracellular cleavage products of synaptotagmin, p40-Syt1 and S100A13, associate with and are exported with FGF-1 (40). The association of FGF-1 with p40-Syt1-S100A13 is the first step in the FGF-1 export pathway and is followed by direct translocation of this protein complex across the plasma membrane.

In several other examples, the heterotetrameric complex (AII) of S100A10 and annexin II appears to play a role in nonclassical secretion. (Among other examples, down-regulation of AII in HUVECs is associated with reduction in von Willibrand factor secretion (41).) The data in Fig. 4 suggest that the AII complex is involved in retaining TrpRS in the cytosol. Previous work demonstrated a similar role for S100A10 as a cytoplasmic retention factor for TASK-1 (37). In that work, RNA interference directed against S100A10 caused enhanced trafficking of TASK-1 to the cell surface membrane. Binding of S100A10 to TASK-1 retarded the surface expression of the channel, most likely by virtue of a di-lysine retention signal at the C terminus of S100A10 that, in turn, is able to interact with coat protein complex, which surrounds transport vesicles in the early secretory pathway. Efficient forward trafficking of TASK-1 has been suggested to require either dissociation of S100A10 or masking of the retention signal on S100A10 by another protein.

The connection of TrpRS to S100A10 and AII and possibly the plasminogen-plasmin system invites speculation on one of the many possible extratranslational roles of TrpRS. Plasminogen activation by cancer cells is usually initiated by the release of urokinase-type plasminogen activator (uPA), which is tightly bound by its cell surface receptor, uPAR. The colocalization of plasminogen with uPA-PAR complex stimulates conversion of plasminogen to plasmin (42). Further, elevations in uPA and its receptor have been demonstrated in a variety of tumors including those of breast, bladder, colon, lung, ovary, and prostate (43). The expression of uPA by neoplastic cells at the invading front of cancerous tissue leads to a cascade of proteolytic activities critical to local tumor growth and metastasis. Human TrpRS secreted from endothelial cells could be cleaved by plasmin at the tumor-host interface, generating angiostatic fragments of TrpRS that could then inhibit tumor growth.

More generally, the results presented here offer insight into how mammalian tRNA synthetases are connected to cell sig-

naling events outside of translation. By associating with key factors in secretory and receptor signaling pathways, such as the annexin II-S100A10 complex and VE-cadherin, a specific tRNA synthetase is able to go from intracellular translation of mRNA to cell signaling through a surface receptor. Understanding the networks built by protein-protein interactions between seemingly disparate partners, such as those described here, offers a specific framework for understanding how the broad systems side of biology is assembled, starting with components of the translation apparatus.

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