Actin-dependent regulation of connective tissue growth factor

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Expression of connective tissue growth factor (CTGF) in endothelial cells is regulated by the small GTPase RhoA and activation of serum response factor (SRF). Monomeric actin binds the transcription factor serum response factor; endothelial cells; RhoA; CArG box

IN THE VESSEL WALL, endothelial cells are exposed to different flow conditions and varying pressure load. These mechanical forces lead to the reorganization of the cytoskeleton and the adaptation of cell shape in endothelial cells and, interestingly, also affect gene expression as shown by in vitro and in vivo studies (4).

An example of a gene regulated by mechanical forces is connective tissue growth factor (CTGF). CTGF is an extracellular matrix-associated signaling molecule promoting endothelial cell growth, migration, adhesion, and survival. It participates in endothelial cell biology, being pro- or antiangiogenic depending on the cellular context (3). Expression of CTGF by endothelial cells is regulated by various soluble stimuli, such as VEGF, or bioactive lipids (15, 28). CTGF is upregulated in endothelial cells exposed to nonuniform shear stress in vitro (31). In vivo, increased CTGF is detectable in endothelial cells lining atherosclerotic plaques, i.e., areas where endothelial cells are exposed not only to altered flow conditions but also to deformation due to plaque development (7, 16). Regulation of CTGF protein processing may vary depending on the vascular bed from which endothelial cells are derived. Differences in CTGF stability were detected between cells derived from large vessels and small vessels (2). Thus far, human umbilical vein endothelial cells (HUVEC) or other endothelial cells obtained from large vessels have been best studied regarding CTGF expression, whereas few data are available for microvascular endothelial cells, which are exposed to different hemodynamic conditions and thus mechanical forces in vivo.

Adaptation of endothelial cells to flow is accompanied by activation of integrins, small GTPases of the Rho family, as well as restructuring of focal adhesions and the actin cytoskeleton (29). These observations prompted us to investigate how CTGF expression is affected by alterations in the cytoskeleton resulting from mechanical forces. RhoA-mediated alterations of the actin cytoskeleton are related to CTGF expression in fibroblasts (17) or vascular smooth muscle cells (6). Furthermore, activation of RhoA by soluble mediators such as sphingosine-1-phosphate and lysophosphatidic acid is critical for CTGF induction in endothelial cells (15). Using actin-binding drugs such as cytochalasin D or latrunculin B, these studies suggested that downstream of RhoA monomeric G-actin might be involved in the regulation of CTGF. However, the signaling pathway from RhoA to CTGF has remained unclear.

Recent studies demonstrated that RhoA, via its ability to induce actin polymerization, regulates the transcription factor serum response factor (SRF). Monomeric actin binds the transcriptional coactivator megakaryocytic acute leukemia (MAL; MKL-1, MRTF-A) in the cytoplasm of resting cells, thereby preventing activation of SRF (14). Expression of actin mutants whose expression either favors F-actin formation (actin S14C) or that are no longer polymerizable into filamentous actin (actin R62D) has proven to be a valuable tool in these studies performed in fibroblasts (20, 21). SRF associates with CarG boxes in the promoter of various growth factor–regulated and muscle-specific genes. However, the 800-bp CTGF core promoter, which is responsive to transforming growth factor (TGF)-β, does not contain a CarG or CarG-like box. Therefore, it is not clear so far whether SRF is involved in actin-dependent regulation of CTGF.

To clarify this question we used primary cultures of HUVEC and a renal microvascular endothelial cell line (giEND.2) as a
model system. The comparison of these endothelial cell types appeared well suited to examine the mechanosensitive regulation of CTGF. We provide evidence that CTGF transcription is modulated by interference with the actin cycle, suppressed by monomeric actin, and upregulated by SRF.

MATERIALS AND METHODS

Materials. Cell culture reagents were from Gibco (Eggenstein, Germany), Promocell (Heidelberg, Germany), or Biochrom (Berlin, Germany). Accutase and fetal calf serum (FCS) were from PAA Laboratories (Pasching, Austria). Latrunculin B was obtained from Merck Biosciences (Bad Soden, Germany). Protease inhibitor cocktail was from Roche Diagnostics (Mannheim, Germany). For quantification purposes, blots were quantified with a chemiluminescence (ECL-Plus, Amersham, Freiburg, Germany). The cells were grown in high-glucose DMEM from GIBCO (Eggenstein, Germany; Ref. 12). The cells were grown in high-glucose DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin, 1% nonessential amino acids, and 5 mM β-mercaptoethanol. Experiments were performed 24 h after seeding of the cells at 80% confluence in the aforementioned medium. Human embryonal kidney cells (HEK 293) were maintained in MEM-Earle with 10% FCS, 1 mM sodium pyruvate, 1% nonessential amino acids, and 100 U/ml penicillin and 100 μg/ml streptomycin and detached for expansion by citrate saline (KCI 0.14 M, sodium citrate 15 mM). HEK cells were seeded in 24-well plates at a concentration that resulted in 80% confluence and 24 h later were subjected to transfection experiments for the times indicated. HUVEC were isolated from freshly delivered umbilical cords and grown on 0.1% gelatin-coated dishes as described previously (15). In brief, cells were cultured in endothelial cell growth medium (Promo Cell) supplemented with endothelial cell growth supplement (Promo Cell) in a humidified 5% CO2 atmosphere. All experiments utilized HUVEC at passage 5 or less that were expanded after detachment with accutase and split at a 1:2 ratio. For experiments, nearly confluent HUVEC cells were seeded the day before in supplemented endothelial cell growth medium.

Western blot analysis. Western blots were performed essentially as described previously (15). In brief, cellular proteins were isolated with radioimmunoprecipitation assay buffer [50 mM Tris-HCl, pH 7.5, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) deoxycholic acid, 0.1% (wt/vol) SDS, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 14 μg/ml aprotinin]. Cell culture supernatants were collected without addition of heparin and analyzed after concentration by ethanol precipitation. Incubation with the primary anti-CTGF antibody (1:250) was for 2 h at room temperature followed by incubation with a horseradish peroxidase-coupled secondary antibody (1:50,000) for 1 h. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL-Plus, Amersham, Freiburg, Germany). For quantification purposes, blots were quantified with a luminescent imager (LAS-1000 Image Analyzer, Fujifilm, Berlin, Germany) and Aida 2.1 image analysis software (Raytest, Berlin, Germany).

Immunocytochemistry. Endothelial cells were fixed with 3.5% paraformaldehyde in PBS for 10 min, followed by extraction in 0.2% Triton X-100 in PBS for 7 min. Actin filaments were stained with rhodamine phalloidin as described previously (17). Primary antibodies were diluted 1:100; anti-mouse and anti-goat secondary antibodies coupled to Alexa Fluor 488 and 555 were used at 1:500. Cells were washed four times in PBS after antibody incubations and before mounting. Images were obtained with a Leica microscope and processed as PICT files with Adobe Photoshop CS. Immunofluorescence was quantified with MetaVue software.

Transient transfections and gene expression assays. The following constructs were used for transient transfections. A CTGF promoter construct (−418 to +42) cloned into pEGFP-1 was kindly provided by R. Goldschmeding (Dept. Pathology, University Medical Center, Utrecht, The Netherlands). It was recloned into a pGL3 luciferase vector. pEGFP and RhoA-V14 were kindly provided by K. Giehl (Institute of Pharmacology and Toxicology; Univ. Ulm Medical Center, Ulm, Germany; Ref. 26). A 4.5-kb CTGF promoter cloned into pGL3 was kindly provided by D. Abraham (University College London, London, UK). The putative SRF binding site at −3791 bp (5′CCATATACGG) was mutated to 5′CCAGGGACGG with the site-directed mutagenesis kit from Stratagene (La Jolla, CA). pSRE-SEAP was from Clontech (Palto Alto, CA), pEFP-actin S14C, pEFP-actin R62D, and SRF-VP16 were described previously (21, 25).

HUVEC were seeded at 70% confluence in supplemented endothelial cell growth medium the day before transfection. Shortly before transfection, medium was replaced with supplement-depleted endothelial cell growth medium. HUVEC were transfected with negatively charged liposomes composed of egg phosphatidylcholine, cholesterol, and soybean phosphatidylserine, produced and kindly provided by C. Rothkopf (University of Jena, Germany; Ref. 22). Transient transfections of HEK 293 cells were carried out with magnet-assisted transfection (MATra; IBA, Gottingen, Germany), following the manufacturer’s instructions. gliEND.2 cells were transfected with jetPEI (Qiogene, Heidelberg, Germany).

 Luciferase activity was determined in cellular homogenates with a luciferase assay kit (Promega, Madison, WI) and a luminometer (Bios-Fix Lumi-10, Macherey-Nagel, Duren, Germany) and normalized to either protein content or β-galactosidase activity determined by OPNG assay (Sigma, Munich, Germany). Secreted alkaline phosphatase (SEAP) was detected with CSPD as chemiluminescent substrate (Phospha-Light-Chemiluminescence Reporter Gene Assay, Tropix, Bedford, UK).

Flow experiments. For flow experiments, gliEND.2 at 7 × 104/ml were seeded inside flow-through cell culture slides (Ibidi, Munich, Germany), which enable direct fluorescence staining and microscopy of cells subjected to flow. gliEND.2 cells were allowed to attach for 24 h, followed by transfection with R62D actin. After 24 h cells were perfused for 4 h at 9.6 ml/min with a peristaltic pump (Ismatec, Wertheim, Germany). The flow system was kept at 37°C and ventilated with 95% humidified air-5% CO2. Immunofluorescence staining was carried out as described above. Individual transfected cells were identified within the endothelial monolayer with anti-flag antibodies.

Statistical analysis. Statistical analysis was done by ANOVA with Bonferroni post hoc test to compare several groups. A value of P < 0.05 was considered statistically significant.

RESULTS

Mechanosensitive regulation of CTGF in endothelial cells. Renal microvascular endothelial cells (gliEND.2) were seeded in flow chambers and after adherence were exposed to flow for 4 h. While the basal expression of CTGF was very low in gliEND.2 cells, it was strongly induced on mechanical stimulation (Fig. 1A). Expression of CTGF was observed in a perinuclear localization as described previously in fibroblasts. Since mechanical stimulation affects the cytoskeletal architecture, we sought to evaluate the role of the actin cytoskeleton in CTGF regulation. gliEND.2 cells cultured under static con-
ditions were transfected with actin mutants, actin S14C, whose expression favored F-actin formation, or actin R62D, which was no longer able to polymerize into F-actin (21). Tagged actin proteins allowed the identification of transfected cells by immunocytochemistry. Actin S14C colocalized with strengthened cell-spanning F-actin fibers (Fig. 1B). A prominent induction of CTGF was detectable in cells overexpressing actin S14C (Fig. 1B). The barely detectable expression of CTGF in gliEND.2 cells cultured under static conditions was not altered by overexpression of the mutant R62D (data not shown). Induction of CTGF by flow, however, was suppressed in cells transfected with actin R62D, but not with actin S14C (Fig. 1C). Together, these data indicated that CTGF expression was sensitive to changes in the relative levels of G-actin and F-actin.

A link between the actin cytoskeleton and CTGF expression was confirmed by pharmacological disruption or stabilization of the actin cytoskeleton. Stabilization of F-actin fibers by jasplakinolide increased CTGF expression within 2 h (Fig. 1D). On the other hand, disruption of F-actin fibers by latrunculin B interfered with the continuous de novo synthesis of CTGF. Therefore, reduction of CTGF levels was delayed and became detectable only after 4–6 h (Fig. 1D).

Functional role of SRF in CTGF gene expression. To further define the regulation of CTGF gene expression by actin, gliEND.2 cells were transfected with CTGF promoter constructs of different lengths, 4.5 kb, 3 kb, and 418 bp, coupled to luciferase, together with expression vectors for mutant actins S14C or R62D. All three promoter constructs showed higher activities in the presence of S14C actin compared with the nonpolymerizing mutant R62D. The difference between actin S14C and R62D was significantly more pronounced with the 4.5-kb construct than with the 3-kb and the 418-bp promoter constructs (Fig. 2A). This indicated an actin-sensitive site between 3 and 4.5 kb.

Actin polymerization is regulated by the small GTPase RhoA and has been linked to the activation of the transcription factor SRF (14, 25). To assess whether SRF acts as a transcriptional regulator of CTGF gene expression, the impact of overexpression of constitutively active RhoA (RhoA-V14) or SRF (SRF-VP16) on CTGF promoter activity was investigated. gliEND.2 cells were transfected with plasmids expressing RhoA-V14 or SRF-VP16 together with CTGF promoter constructs of different lengths (Fig. 2B). Only the 4.5-kb construct was significantly activated by overexpression of either RhoA or SRF. The shorter constructs of 3-kb and 418-bp length were not significantly activated, which indicated the location of an SRF-responsive site between 4.5 and 3 kb. The 4.5-kb construct was also activated in HEK cells by RhoA or

Fig. 1. Modulation of connective tissue growth factor (CTGF) expression by mechanical stimulation. A: gliEND.2 cells were cultured in flow-through cell culture slides for 24 h under static conditions (left) and then exposed to flow for 4 h (right). CTGF expression was visualized in situ by immunocytochemical staining. Immunofluorescence images are representative of 3 experiments with comparable results. B: gliEND.2 cells were transfected with flag-tagged actin S14C for 24 h. Overexpressed actin was detected by anti-flag antibodies (Flag-actin-S14C); F-actin was visualized by Alexa Fluor phalloidin; and CTGF was detected by specific antibodies. Arrows indicate examples of transfected cells; arrowheads indicate nontransfected cells. C: inside the flow-through chambers gliEND.2 cells were transfected with flag-tagged actin S14C or actin R62D. After 24 h the cells were exposed to flow for 4 h. Arrows indicate examples of transfected cells visualized by anti-flag antibodies; arrowheads indicate nontransfected cells. The results shown are representative of 3 independent experiments. D: gliEND.2 cells were treated with latrunculin B (Lat B, 1 or 0.1 μM) or jasplakinolide (J, 0.1 μM) for 2 and 6 h. Cell-associated CTGF protein was detected by Western blot analysis. Detection of tubulin was used to confirm equal loading and blotting.
SRF (Fig. 2C), whereas the shorter constructs were not activated (data not shown). In these cells, characterized by >90% transfectability, induction of endogenous CTGF could be detected also at the protein level, supporting a functional role of SRF in endogenous CTGF expression (Fig. 2D).

Role of CArG box in CTGF activation. A binding site for SRF has been described at position −3791 upstream of the transcription start site of CTGF (19). To investigate the functional role of the CArG box-like element, the site was mutated from 5’ CCATATACGG to 5’CCAGGGACGG. The mutated construct was barely induced by RhoA-V14. A residual activation of the mutated construct by SRF-VP16 was observed, which followed slower kinetics and was significantly less effective than the activation of the wild-type construct (Fig. 3A).

Coexpression of the mutant actin R62D only slightly reduced the basal activity of either the original or the mutated 4.5-kb promoter constructs. However, overexpression of the SRF-activating mutant S14C significantly activated the 4.5-kb construct, whereas there was little effect on the mutated promoter (Fig. 3B). In line with these findings, actin S14C strongly activated a consensus serum response element, the basal expression of which was barely affected by actin R62D (data not shown). These data indicate that the CArG box at position −3791 is essential for the actin sensitivity of the CTGF promoter.

Regulation of CTGF by monomeric actin in HUVEC. In contrast to the microvascular endothelial cell line, primary cultures of HUVEC express high basal levels of CTGF in vitro

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**Fig. 2.** Functional role of serum response factor (SRF) in CTGF gene expression. A: glEND.2 cells were cotransfected with actin R62D (gray bars) or actin S14C (black bars) and CTGF promoter constructs of varying length. In each experiment, the promoter activity of cells transfected with actin R62D was set to 1. Data are means ± SD of 3 experiments performed in triplicate. *P < 0.05. B: glEND.2 cells were cotransfected with CTGF promoter constructs of different length (418 bp, 3 kb, or 4.5 kb) coupled to luciferase and expression plasmids encoding RhoA-V14 (R), SRF-VP16 (S), or an empty vector (C). In each set of experiments, the promoter activity of control cells was set to 1. Data are means ± SD of 4 experiments performed with duplicate biological samples. *P < 0.05. **P < 0.01. C: HEK 293 cells were cotransfected with the 4.5-kb CTGF promoter construct and expression plasmids encoding RhoA-V14, SRF-VP16, or an empty vector (C). Data are means ± SD of 5 experiments with duplicate biological samples. *P < 0.01. **P < 0.001. D: HEK 293 cells were transfected with plasmids encoding RhoA-V14, SRF-VP16, or an empty vector (C). After 9 h medium was replaced with serum-depleted medium, and after another 15 h aliquots of the supernatant were analyzed for CTGF protein expression. Data are means ± SD of 4 experiments with duplicate biological samples. *P < 0.05. **P < 0.01.

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**Fig. 3.** Activation of the CTGF promoter by SRF and actin via the CArG box. A: glEND.2 cells were transfected with a 4.5-kb CTGF promoter construct or a construct mutated in the CArG box (4.5-mut) and expression plasmids encoding RhoA-V14 (RhoA), SRF-VP16 (SRF), or an empty vector (Co). Data are means ± SD of 4 experiments performed with triplicate biological samples. *P < 0.05, 4.5-mut vs. 4.5-kb promoter construct. **P < 0.01. B: glEND.2 cells were cotransfected with actin S14C or actin R62D and the 4.5-kb CTGF promoter construct (4.5 kb) or the CArG box-mutated construct (4.5-mut). In each experiment, the promoter activity of cells transfected with control vector (C) was set to 1. Data are means ± SD of 4 experiments performed in triplicate. **P < 0.01, 4.5-mut vs. 4.5-kb promoter construct.
(15). Disruption of the F-actin cytoskeleton by latrunculin B (1 μM) reduced CTGF protein expression within 2 h, indicating that an intact cytoskeleton is essential for the basal CTGF expression in HUVEC (Fig. 4A).

To analyze the role of monomeric actin in the regulation of endogenous CTGF expression of HUVEC, we transfected the cells with mutant actin S14C or R62D for 24 h. Compared with neighboring nontransfected cells, overexpression of S14C actin did not alter the structure of the F-actin cytoskeleton, which was strongly expressed in these cells. Nonpolymerizable actin R62D, in contrast, prevented stress fiber formation (Fig. 4B). Compared with nontransfected cells, CTGF expression appeared unchanged in cells transfected with actin S14C, but was reduced in cells transfected with actin R62D (Fig. 4B). Immunocytochemical staining was analyzed by a quantitative approach. In three experiments with duplicate transfections, the fluorescence intensities of 15 transfected cells each were compared with their neighboring cells with MetaVue software. The fluorescence intensity of the actin R62D-transfected cells was reduced by 80% compared with nontransfected cells (Fig. 4C). These data indicated that CTGF expression was reduced by increased levels of monomeric actin in HUVEC.

**DISCUSSION**

SRF has been implicated in the regulation of a large variety of immediate-early and muscle-specific genes. In this study, evidence for SRF as an important transcriptional activator of
CTGF expression is provided. We show that CTGF induction or repression occurs as a consequence of G- and F-actin levels. To alter actin dynamics, we used actin-binding drugs such as latrunculin B and actin mutants that either are nonpolymerizable (R62D) or alter actin dynamics in favor of filamentous actin (S14C) (21).

Overexpression of mutant actin differentially affected HUVEC and the microvascular endothelial cells because of the distinct structures of the actin cytoskeleton in these cells in culture. Expression of the actin mutant R62D in HUVEC significantly decreased the cell-spanning F-actin stress fibers, whereas actin S14C did not obviously alter the actin cytoskeleton in HUVEC. This is indicative of a dominant-negative action of actin R62D in these cells, which could potentially act as a suppressor on endogenous, SRF-driven cytoskeletal components such as the β-actin gene itself. Because of the very low amount of F-actin stress fibers in glEND.2, there was no further reduction on expression of mutant actin R62D, whereas overexpression of actin S14C apparently colocalized with and increased filamentous actin. Consistent with the different pattern of changes in F-actin, CTGF expression was differentially modulated on overexpression of the mutant actin. Overexpression of actin S14C increased CTGF expression in glEND.2 cells, whereas flow-mediated upregulation of CTGF was prevented by actin R62D. Similarly, overexpression of monomeric actin R62D significantly reduced CTGF protein in HUVEC, which show high basal levels of CTGF indicative of ongoing CTGF synthesis on in vitro culture. These data are consistent with a model in which high levels of monomeric actin interfere with CTGF synthesis, whereas reduction of monomeric actin leads to an activation of CTGF transcription.

There is good evidence that the G-actin level negatively correlates with the activation of the transcription factor SRF (24). Myocardin-related transcription factors (MAL/MKL-1/MRTF-A), that are functional coactivators of SRF and are involved in the regulation of certain SRF dependent genes can bind to monomeric actin (14). Liberation of MAL from G-actin resulted in binding of an SRF-MAL complex to the promoter of cyr61, a protein closely related to CTGF (14). Microarray and chromatin immunoprecipitation analysis of cells overexpressing dnMKL-1 showed upregulation of CTGF mRNA that was attributed to CarG elements located in an intron (23) or 3.9 kb upstream of the transcription start site (19). Consistent with these analyses, the first 800 bp of the CTGF promoter, which are sufficient to mediate TGF-β-induced activation of CTGF, do not contain a CarG box (1, 9). These data pointed to a link between actin, SRF, and CTGF, but thus far, the experimental evidence has been lacking.

With the results of this study, we provide evidence for a functional role of the CarG box at −3.9 kb. Mutation of this element significantly reduced the activation of the CTGF promoter by SRF or RhoA in glEND.2 cells. Furthermore, activation by actin S14C was detected with the 4.5-kb promoter construct and significantly reduced when the construct containing a mutated CarG box was tested. As expected from the protein data, only a small decrease in the basal promoter activity was observed when the cells were transfected with actin R62D. The sequence of the CarG box at −3791 bp of the CTGF promoter does not correspond to the classic CarG box, but contains one mismatch (19, 27). While SRF binds with high affinity to consensus CarG boxes, it shows weaker affinity to CarG-like elements with 1-bp deviation from the consensus sequence (13). Similar mismatches have been characterized in smooth muscle cells and shown to play a role in myocardin-regulated binding of SRF to these sites (10). Functionally, the degenerate sequences in α-smooth muscle actin are involved in the control of gene expression in response to injury. It is tempting to speculate that the CarG box in the CTGF promoter plays a similar functionally confined role.

The activity of promoter constructs, which did not contain the CarG box, was less affected by actin expression but not inert to changes in the actin content of the cells. This indicated that regulation of CTGF gene expression goes beyond the modulation of SRF activity in endothelial cells. Given the multiple actin monomer-binding proteins, which are functionally active (18), it is likely that high levels of monomeric G-actin modulate CTGF gene transcription by additional SRF-independent mechanisms. The NF-kB element, located within the core promoter, has been linked to mechanical stimulation of CTGF in smooth muscle cells (5). Furthermore, recent data indicate that actin plays a direct role in transcription by RNA polymerases (8, 11). In a very recent study by Wu et al. (30), formation of F-actin within the nucleus was related to transcription elongation. Overexpression of different forms of actin may thus regulate gene expression in multiple ways, not only by shifting the cytosolic equilibrium between monomeric and filamentous actin. The impact of nuclear actin on CTGF expression will require further investigation.

Our data provide the molecular basis whereby changes in cell architecture are translated into CTGF gene expression. Furthermore, actin-SRF signaling also plays a role in CTGF induction by soluble stimuli that activate the RhoA-Rho kinase pathway. The singular signal transduction pathway allows a variable degree of interaction with other signaling pathways such as activation by TGF-β, endothelin, angiotensin, or lysophosphatidic acid. This implies that the response of endothelial cells to soluble mediators is modulated by the physical state of the cells, which is altered depending on the flow conditions to which the cells are exposed. In line with this interpretation, vascular CTGF expression is primarily induced in areas of disturbed flow, where nonuniform shear stress together with soluble mediators contributes to CTGF expression.

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