Angiotensin II–Induced Transactivation of Epidermal Growth Factor Receptor Regulates Fibronectin and Transforming Growth Factor-β Synthesis via Transcriptional and Posttranscriptional Mechanisms

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Abstract—The signaling cascade elicited by angiotensin II (Ang II) resembles that characteristic of a growth factor, and recent evidence indicates transactivation of epidermal growth factor receptor (EGF-R) by G protein–coupled receptors. Here, we report the involvement of EGF-R in Ang II–induced synthesis of fibronectin and transforming growth factor-β (TGF-β) in cardiac fibroblasts. Ang II stimulated fibronectin mRNA levels dose dependently, with a maximal increase (∼5-fold) observed after 12 hours of incubation. Fibronectin synthesis induced by Ang II or calcium ionophore was completely abolished by tyrosine kinase inhibitors and intracellular Ca²⁺ chelating agents. Ang II–induced fibronectin mRNA was not affected by protein kinase C inhibitors or protein kinase C depletion, whereas specific inhibition of EGF-R function by a dominant negative EGF-R mutant and tyrphostin AG1478 abolished induction of fibronectin mRNA. We isolated the rat fibronectin gene, including the 5′-flanking region, and found that the activator protein-1 (AP-1) binding site present in the promoter region was responsible for the Ang II responsiveness of this gene. A gel retardation assay revealed the binding of nuclear protein to the AP-1 site, which was supershifted with anti–c-fos and anti–c-jun but not anti–activating transcription factor (ATF)-2 antibodies. Conditioned medium from Ang II–treated cells contained TGF-β bioactivity, and addition of neutralizing TGF-β antibody modestly (46%) inhibited induction of fibronectin. Ang II–induced synthesis of TGF-β was also abolished by inhibition of EGF-R function. The effect of TGF-β was exerted by stabilizing fibronectin mRNA without affecting the promoter activity and required de novo protein synthesis. We concluded that Ang II–induced expression of fibronectin and TGF-β is mediated by downstream signaling of EGF-R transactivated by Ca²⁺–dependent tyrosine kinase and that Ang II–induced fibronectin mRNA expression is regulated by 2 different mechanisms, which are transcriptional control by binding of the c-fos/c-jun complex to the AP-1 site and posttranscriptional control by mRNA stabilization due to autocrine or paracrine effects of TGF-β. Thus, this study suggests that the action of Ang II on extracellular matrix formation should be interpreted in association with the EGF-R signaling cascade. (Circ Res. 1999;84:1073-1084.)

Key Words: angiotensin II receptor ■ angiotensin II type 1 receptor ■ angiotensin II type 2 receptor ■ angiotensin II ■ epidermal growth factor receptor

Cardiac fibroblasts isolated from neonatal rat hearts have abundant high-affinity angiotensin II (Ang II) receptors, which are classified pharmacologically as belonging to the Ang II type 1 receptor (AT₁) subtype.¹,² These cells have been used to examine AT₁-mediated signaling³–⁴ and extracellular matrix remodeling in heart failure.⁵ AT₁ stimulation was found to stimulate DNA synthesis and cell proliferation⁴ and also to increase the synthesis of extracellular matrix proteins,⁴,⁶ which suggests that cardiac fibroblasts contribute to remodeling of the cardiac interstitium under a variety of physiological and pathological conditions. Ang II, acting via AT₁, initiates early biochemical events, including rapid production of diacylglycerol and inositol 1,4,5-triphosphate by phospholipase C–mediated hydrolysis of inositol phospholipids and activation of protein kinase C (PKC).⁷–¹⁰ Ang II also induces a rapid increase in expression of the growth-associated nuclear proto-oncogenes in a manner similar to that of cellular events mediated by peptide growth factors and stimulates tyrosine phosphorylation of multiple substrates, including p44 and p42 mitogen-activated protein/extracellular signal-regulated kinases (ERKs).¹¹–¹⁶

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Although it was reported that PKC played a dominant role in the Ang II–induced activation of ERK in vascular smooth muscle cells (VSMCs)\(^\text{17,18}\) and in cardiac myocytes,\(^\text{13}\) other studies indicated that calcium signaling rather than PKC plays a critical role in ERK activation in these cells.\(^\text{14–16}\) Certain aspects of signal transduction characteristic of Ang II stimulation resemble those evoked by growth factors. Activation of phospholipase C-\(\gamma\), tyrosine kinases, and ERK and expression of nuclear proto-oncogenes exemplify phenomena common to Ang II and growth factor signaling.\(^\text{9,12}\) Recently, cross-talk between G protein–coupled receptors and growth factor receptors with intrinsic tyrosine kinase activity was reported. In VSMCs, stimulation of the AT\(_1\) receptor causes transactivation of platelet-derived growth factor \(\beta\) receptor\(^\text{19}\) or epidermal growth factor receptor (EGF-R)\(^\text{20}\) resulting in expression of AT\(_1\)-mediated ERK signaling in association with formation of an adapter protein complex containing the SH2 domain, such as Grb2 or Shc. Stimulation of Rat-1 cells with endothelin-1, lysophosphatidic acid, or thrombin induced a rapid increase in tyrosine phosphorylation of EGF-R and p185\(^\text{2\(\beta\text{II}\)}\), leading to activation of the ERK.\(^\text{21}\) Stimulation of Cos-7 cells with Gi- or G\(_q\)-coupled receptors caused phosphorylation of EGF-R associated with assembly of Shc and Grb2\(^\text{22,23}\) and calcium-dependent EGF-R activation by stimulation of voltage-sensitive calcium channels was also found in PC12 cells.\(^\text{24,25}\) Ang II activated tyrosine phosphorylation of insulin-like growth factor-1 receptor and insulin receptor substrate-1 in VSMCs.\(^\text{26}\) In addition, both murine acetylcholine receptor was also shown to transactivate EGF-R and its downstream signaling, resulting in modulation of potassium channel function in human embryonic kidney 293 cells.\(^\text{27}\) Thus, additional tyrosine kinases that phosphorylate receptor tyrosine kinases ligand independently appear to contribute in a general or cell type–specific way to mitogenic signaling mediated through G protein–coupled receptors. We\(^\text{28}\) and Eguchi et al\(^\text{20}\) recently showed that Ang II–induced ERK activation and its mitogenic signals are dominantly mediated by downstream signaling of transactivated EGF-R.

Two thirds of the myocardial cell population is composed of nonmyocyte cells, the majority of which are fibroblasts.\(^\text{5}\) Cardiac fibroblasts are responsible for the production and deposition of extracellular matrix proteins such as fibronectin and collagen types 1 and 3.\(^\text{5,6}\) Fibronectin is an extracellular matrix and plasma protein with various functions that plays important roles in cardiac remodeling as well as cell adhesion and migration.\(^\text{29}\) Although fibronectin synthesis in cardiac fibroblasts was reported to be induced in response to Ang II,\(^\text{2,6}\) the signal transduction mechanism responsible for this induction remains to be determined. In the present study, we found that Ang II–induced synthesis of fibronectin and transforming growth factor-\(\beta\) (TGF-\(\beta\)) is regulated through downstream signaling of EGF-R transactivated by Ca\(^{2+}\)/calmodulin-dependent tyrosine kinases. Induction of fibronectin mRNA by Ang II was regulated by 2 different mechanisms, which are transcriptional control by binding of the \(c\)-fos/c-jun complex to the activator protein-1 (AP-1) site and posttranscriptional control by autocrine or paracrine effects of TGF-\(\beta\), which is exerted by increasing the mRNA stability via de novo protein synthesis and which upregulates fibronectin mRNA levels by \(\sim 46\%\).

**Materials and Methods**

GF109203X, BAPTA-AM, genistein, ST638, and W7 were purchased from Calbiochem. TMB-8, nifedipine, and calmidazolium chloride were purchased from Calbiochem. A23187, phorbol 12-myristate 13-acetate (PMA), and EGTA were purchased from Sigma. Losartan was kindly provided by DuPont Merck Pharmaceutical. PD123319 was kindly provided by Parke-Davis and Warner-Lambert Co. Fibronectin antibody was purchased from Telios Pharm.

**Cell Culture**

Cardiac fibroblasts were prepared from ventricles of 1- to 2-day-old Wistar rats and grown as previously described.\(^\text{30,31}\) Subcultured fibroblasts from passages 4 and 5, used in this experiment, were >99% positive for immunostaining with vimentin antibody and were negative for desmin (for myocytes), smooth muscle \(\alpha\)-actin (for VSMCs), and a polyclonal antibody against von Willebrand factor (for endothelial cells) (Sigma). Subconfluent cells were serum starved for 24 hours and used for the experiments.

**Northern Blotting**

Total RNA was extracted by guanidinium isothiocyanate–cesium chloride centrifugation, fractionated on 1% agarose-formaldehyde gels, and transferred to nylon membranes as previously reported.\(^\text{30,31}\) Blots were then hybridized with random-primed \(^{32}\)P-labeled cDNA probes consisting of fibronectin, TGF-\(\beta\), and GAPDH as an internal control. Hybridized signals were measured by scanning densitometry, and fibronectin mRNA levels were arbitrarily normalized relative to the GAPDH mRNA levels.

**Stable Transfection and Chloramphenicol Acetyltransferase (CAT) Assay**

Cardiac fibroblasts were cotransfected with a 5:1 molar ratio of the fibronectin promoter-CAT chimeric constructs and pSV2neo DNA\(^\text{15}\) via de novo protein synthesis and which upregulates fibronectin mRNA levels by \(\sim 46\%\).

**[\(^{35}\)S]Methionine Labeling of Cells and Immunoprecipitation**

Quantification of fibronectin secreted into medium was performed as previously reported.\(^\text{6}\) Briefly, fibroblasts were made quiescent by serum depletion for 12 hours. After 12 hours of incubation with or without Ang II (0.1 \(\mu\text{mol/L}\)), 100 \(\mu\text{Ci/mL}\) of \([^{35}\text{S}]\) methionine was added for another 12 hours. For immunoprecipitation, aliquots (100 \(\mu\text{L}\)) of the medium were diluted with 900 \(\mu\text{L}\) of radioimmunoprecipitation assay buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) and treated with normal rabbit serum (10 \(\mu\text{L}\)) to remove nonspecific binding, followed by incubation with rabbit antibody to fibronectin for 16 hours at 4°C. Immune complexes were removed by protein A–Sepharose beads (Pharmacia LKB) and analyzed by SDS-PAGE.\(^\text{6}\)
Creation of Mutations in the AP-1 Site
Mutation in the AP-1 site at –453 present in the 5′ flanking region was created by polymerase chain reaction (PCR) overlap extension mutagenesis as previously reported. Briefly, 2 DNA fragments with overlapping ends were first amplified with 2 sets of primers (A and B, C and D) by using p504-CAT as a template for making the mutations. Primers A and D were T3 and T7 primers for the pBS vector, respectively. Primers B and C had the following DNA sequence: B: 5′-GTACCGTTGGTGGTTGTTGAACCATTTATC-CA-3′ (antisense, nucleotides –453 to –421), and C: 5′-TCAAACACCACCGGTAC-3′ (sense, nucleotides –439 to –421). Primer B contained a mutation in the AP-1 site at the 3′ end, and primers B and C were designed to overlap at the 5′ end. The 2 respective PCR products were mixed and amplified again with primers A and D. After confirming the DNA sequence, the PCR product was subcloned into the 5′ end of the CAT gene.

Gel Retardation Assay
The gel retardation assay and preparation of nuclear extract from cells exposed to Ang II (100 nmol/L) for 45 minutes was performed as previously reported. The final protein concentration was 0.5 to 1 mg/mL. The oligonucleotides corresponding to the DNA fragment containing AP-1 and the mutated AP-1 sequence (between nucleotides –438 and –473) or the ATF/CRE motif (nucleotides –143 to –178) were synthesized, labeled with [γ-32P]ATP using T4 kinase (Takara Shuzo), and annealed to make double-strand DNA. Nuclear extracts were incubated with the probe for 30 minutes at room temperature in the binding buffer, consisting of (in mmol/L) HEPES 0.5, KCl 60, EDTA 0.1, DTT 0.5, and phenylmethylsulfonyl fluoride 0.5; 12% glycerol; and 2 μg of poly(dI-dC). The mixture was loaded on a 6% polyacrylamide gel and electrophoresed as previously reported. Supershift assays were performed with rabbit polyclonal anti-c-fos IgG raised against the portion of c-fos containing amino acids 128 to 152 and rabbit anti-c-jun IgG raised against the portion of c-jun containing amino acids 247 to 263 (Santa Cruz Biotechnology). Each antibody (1 μg each) was added to the samples after the initial binding reaction and then incubated for 1 hour at room temperature.

Quantification of Latent and Active TGF-β
The bioassay for TGF-β released into medium was performed by measuring proliferation inhibition of mink lung epithelial cell line CCL-64 (American Type Culture Collection), as reported elsewhere. Briefly, CCL-64 cells were seeded in 24-well plates in DMEM containing 10% FCS. After an additional incubation for 24 hours, control or test samples were added, and cells were incubated for another 24 hours. Subsequently, 1 μCi/mL of [3H]thymidine (NEN) was pulsed for 6 hours, and the amount of [3H]thymidine incorporated into trichloroacetic acid–precipitable materials was measured by a liquid scintillation counter. To determine whether cells were producing latent TGF-β, latent TGF-β was converted to the active form by treating culture supernatant with 10N HCl (final pH, 2.0) for 30 minutes at room temperature, followed by neutralization with equimolar NaOH. TGF-β activities were determined on the basis of the standard curve (TGF-β concentration versus percentage of inhibition), which was drawn by using TGF-β1 (R&D Systems, Inc). Culture supernatants were serially diluted to fall within the range of the standard curve. Specificity of the observed effects was proven by competition of the TGF-β activity with a purified rabbit anti–TGF-β IgG and a control IgG (R&D Systems, Inc).

Measurement of Lactate Dehydrogenase (LDH) Activities
A spectrophotometric enzyme assay (DRI-CHEM slide LDH-P, Fuji Film) was performed to measure LDH release in the medium. In brief, 10 μL of triplicate medium and reagent were mixed for 2 minutes at room temperature. Absorbance at 540 nm was determined, and LDH activity (U/L) was automatically calculated by FD3030 analyzer. One unit of LDH activity is defined as that amount of enzyme that will catalyze the formation of 1 μmol of NADH per minute.

Statistical Analysis
The results are expressed as mean±SE. Statistical analyses were performed with a 1-way ANOVA followed by pairwise contrasts (control versus conditions) using the Dunnett multiple comparison test. Data were considered statistically significant at P<0.05.

Results
Ang II–Induced Fibronectin mRNA Level Is Mediated by AT1 Stimulation
Ang II (100 nmol/L) significantly stimulated fibronectin mRNA accumulation after 6 hours of incubation, reaching a maximal increase after 12 hours, and thereafter maintained a similar level until 24 hours of incubation (Figure 1). Ang II–induced fibronectin mRNA levels were increased dose dependently with a maximal peak at 100 nmol/L (data not shown). Therefore, subsequent experiments were performed.
with 100 nmol/L Ang II stimulation for 12 hours. To determine which Ang II receptor subtypes mediate fibronectin mRNA accumulation, Ang II receptor densities were quantified in cardiac fibroblasts, and the effects of the AT1 antagonist losartan or the AT2 antagonist PD123319 were examined. Cardiac fibroblasts (passage 4) expressed only AT1 (Kd, 0.21±0.01 nmol/L; Bmax, 178±5 fmol/mg protein); AT2 expression was not detectable in these cells. Induction of fibronectin mRNA by Ang II was completely blocked by losartan but not by PD123319 (Figure 1), which demonstrated that Ang II–induced fibronectin mRNA accumulation is mainly mediated by AT1, and AT2 is not involved in this induction mechanism by Ang II. We previously reported that AT1 is substantially expressed in primary culture of cardiac fibroblasts from cardiomyopathic hamsters during heart failure, whereas AT2 expression is abruptly downregulated with repeated passages of cells. Given that the primary culture of neonatal rat cardiac fibroblasts also expresses detectable amounts of AT2, it is likely that downregulation of AT2 in the cells (passages 4 to 5) used in this study is due to repeated passages of cells.

**Phorbol Ester–Sensitive PKC Is Not Involved in Ang II–Induced Fibronectin mRNA Accumulation**

Stimulation of AT1 results in generation of inositol triphosphate and diacylglycerol, which induce the release of Ca2+ from intracellular stores and PKC activation, respectively. We next examined the effects of the PKC inhibitors GF109203X and calphostin C on Ang II–induced fibronectin mRNA levels. Pretreatment with GF109203X (1 μmol/L) or calphostin C (50 nmol/L) completely inhibited fibronectin mRNA accumulation in response to 100 nmol/L PMA, whereas no significant inhibition of Ang II–induced fibronectin mRNA level was observed (Figure 2). Depletion of phorbol ester–sensitive PKC by 24-hour incubation with 10 μmol/L of PMA also did not inhibit Ang II–induced accumulation of fibronectin mRNA (Figure 2). These results suggest that PKC-mediated pathways do not play a dominant role in Ang II–induced fibronectin mRNA accumulation, and that GF109203X–, calphostin C–, and phorbol ester–sensitive PKC are not involved in this mechanism.

**Ang II–Induced Fibronectin mRNA Accumulation Is Stimulated via Ca2+/Calmodulin-Dependent Pathway**

We have previously shown that exposure of fibroblasts to Ang II markedly increases intracellular Ca2+ levels, and this increase was blocked by the intracellular Ca2+ chelator BAPTA-AM (10 μmol/L) but not by extracellular Ca2+ chelation by EGTA. These observations indicated that Ca2+ mobilization after AT1 stimulation is mainly caused by release of Ca2+ from intracellular stores. As shown in Figure 3, Ang II–induced fibronectin mRNA levels were completely inhibited by pretreatment with BAPTA-AM (10 μmol/L) and TMB8 (100 μmol/L), which are commonly used as intracellular Ca2+ chelators, but not by pretreatment with EGTA. These Ca2+ chelators did not affect the basal expression level of fibronectin mRNA (data not shown). In agreement with these results, addition of the Ca2+ ionophore A23187 also stimulated fibronectin mRNA accumulation (Figure 3). Elevation of cytosolic Ca2+ activates a variety of enzymes through interaction with calmodulin. To examine whether calmodulin mediates fibronectin mRNA levels in response to Ang II, fibroblasts were preincubated with the calmodulin inhibitors W-7 (100 μmol/L) and calmidazolium (10 μmol/L). These drugs completely blocked Ang II–induced fibronectin mRNA accumulation (Figure 3). These calmodulin inhibitors at the concentrations used in the present study did not affect the basal level of fibronectin mRNA and did not have any effect on the PMA-induced increase in fibronectin mRNA level (data not shown), which suggests that Ang II stimulates fibronectin mRNA accumulation through a Ca2+/calmodulin-dependent mechanism.

**Ang II–Induced Fibronectin mRNA Accumulation Is Activated by Ca2+/Calmodulin-Dependent Protein Tyrosine Kinases**

To determine whether tyrosine kinase activity is required for Ca2+-dependent fibronectin mRNA accumulation, cells were pretreated with genistein (100 μmol/L) and ST638 (100 μmol/L), protein kinase inhibitors with a strong preference for tyrosine-specific kinases, and then stimulated by either Ang II or A23187. These inhibitors completely abolished both Ang II– and A23187-induced fibronectin mRNA accumulation with no effects on basal fibronectin mRNA levels.
levels (Figure 4). On the other hand, these tyrosine kinase inhibitors did not have any effect on PMA-induced increase in fibronectin mRNA level (data not shown). These findings suggest that protein tyrosine kinases activated downstream of the Ca\(^{2+}\)/calmodulin pathway are closely involved in Ang II–induced fibronectin mRNA accumulation.

We also examined the effects of these kinase inhibitors on cell toxicity at the concentrations used in the above experiments. Although we also measured LDH in culture medium from cells treated with these drugs, no LDH activities were detected. These results suggested that complete inhibition of Ang II– or A23187-induced fibronectin mRNA accumulation by these drugs was not due to nonspecific cytotoxicity.

Ang II–Induced Transactivation of EGF-R Is Involved in Induction of Fibronectin mRNA

We recently found that in cardiac fibroblasts Ca\(^{2+}\)/calmodulin-dependent tyrosine kinases activated by AT\(_1\) stimulation cause tyrosine phosphorylation of EGF-R but not platelet-derived growth factor receptor, which mediates AT\(_1\)/Ras/ERK signaling. Therefore, we next examined whether this EGF-R transactivation mechanism is involved in AT\(_1\)-mediated fibronectin mRNA accumulation. Specific inhibition of EGF-R function by the specific EGF-R antagonist tyrphostin AG1478 markedly inhibited both Ang II– and A23187-induced fibronectin mRNA accumulation (Figure 4). EGF also dose-dependently stimulated fibronectin mRNA levels, and this increase was blocked by AG1478 (Figure 4). However, AG1478 did not affect the basal expression level of fibronectin mRNA (data not shown).

The role of EGF-R in AT\(_1\)-mediated fibronectin mRNA accumulation was further analyzed by specific inhibition of the EGF-R signal. A dominant negative EGF-R mutant lacking the cytoplasmic domain of human EGF-R (HEGF-R 533del) was constructed and stably transfected...
into cardiac fibroblasts. This mutant inhibited the downstream signaling of rat EGF-R by formation of signaling-defective heterodimers with the wild-type receptor, and we have shown that cardiac fibroblasts stably expressing HEGF-R 533del abolished Ang II– or EGF-induced EGF-R phosphorylation. Using these cloned cells, we examined Ang II– or EGF-induced effects on fibronectin mRNA accumulation. In HEGF-R 533del cells, fibronectin mRNA accumulations at low and higher ligand concentrations (2 and 50 ng/mL EGF) were completely and partially attenuated, respectively, compared with that in the control transfected cells, whereas Ang II–induced expression of fibronectin mRNA was completely blocked (Figure 5A and 5B). We next examined the involvement of ERK activated downstream of EGF-R on Ang II–induced fibronectin mRNA levels. Inhibition of ERK activity by ERK kinase (MEK) inhibitor PD98059 greatly (74%; \( P < 0.05 \)) inhibited Ang II–induced fibronectin mRNA accumulation (Figure 5A), which suggests an important role of ERK activation in induction of fibronectin mRNA after Ang II stimulation. To test whether PD98059 causes cell injury at the concentrations used in this study, we measured LDH levels in culture medium. We did not detect LDH activity in culture medium from Ang II–stimulated cells pretreated with PD98059 (10 \( \mu \)mol/L), which indicates that the effect of this drug was not due to induction of apoptosis or nonspecific cytotoxicity.

**Figure 5.** Specific inhibition of Ang II–induced fibronectin mRNA induced by dominant negative EGF-R mutant and MEK inhibitor and quantification of fibronectin protein released into culture medium. A and B, Cardiac fibroblasts stably transfected with pcDNA expression vector alone (control) (A) or pcDNA containing HEGF-R 533del (panel B) were starved of serum for 12 hours and then stimulated with Ang II (100 nmol/L) or EGF (2 and 50 ng/mL) for 12 hours. The effect of MEK inhibitor PD98059 (10 \( \mu \)mol/L) was examined by pretreatment with PD98059 for 30 minutes. Quantification of mRNA levels was performed as described in Figure 1. Results shown are mean±SE of 4 separate experiments, and representative data are shown. \( * P < 0.01 \) vs control. C, Cardiac fibroblasts (control cells) or HEGF-R 533del cells incubated in the presence or absence of Ang II (100 nmol/L) for 12 hours were subsequently incubated with \( [35S] \)methionine for an additional 12 hours. AG1478 (250 nmol/L) and PD98059 (10 \( \mu \)mol/L) were added 20 minutes before exposure to Ang II. Culture supernatants were used for immunoprecipitation of fibronectin as described in Materials and Methods. Signals of 220 kDa representing fibronectin on SDS-PAGE were measured by densitometry, and the value in the control was arbitrarily expressed as 1 unit. Values represent mean±SE of 6 separate experiments. \( * P < 0.01 \) vs controls (unstimulated cells).

**Ang II–Induced Fibronectin mRNA Expression Results in an Increase in Fibronectin Protein Release**

We also examined whether fibronectin mRNA accumulation by Ang II results in induction at the fibronectin protein level. Fibronectin protein secreted into the medium was analyzed by immunoprecipitation using anti-fibronectin antibody as previously described.\(^6\) Immunoprecipitated fibronectin secreted into medium was increased by 2.8-fold over the control by 0.1 \( \mu \)mol/L of Ang II. Pretreatment with AG1478 completely abolished Ang II–induced fibronectin secretion, whereas PD98059 modestly (67%; \( P < 0.05 \)) inhibited it (Figure 5C). To further confirm the role of EGF-R transactivation, we tested the effects of Ang II and EGF on the fibronectin release from cells expressing HEGF-R 533del. As shown in Figure 5C, fibronectin release induced by Ang II (100 nmol/L) and EGF (2 ng/mL) was completely abolished in HEGF-R 533del cells. Although we tested the effect of PD123319 on Ang II–stimulated release of fibronectin, no significant effect was observed in fibronectin release from cells pretreated with PD123319 (data not shown).

**AP-1 Binding Sequence in the Fibronectin Gene Promoter Confers Responsiveness to Ang II**

To investigate the effects of Ang II on fibronectin gene transcription, a rat genomic EMBL3 library was screened for the 5′-flanking region of the fibronectin gene with a \( ^{32} \)P end–labeled DNA fragment synthesized by PCR on the basis...
of the sequence data reported by Patel et al. 41 We isolated the rat fibronectin gene containing 1908 bp of 5′-flanking region from the rat genomic library. The sequence downstream of position –1080 relative to the transcriptional start site (+1) was identical to that reported by Patel et al. 41 Computer analysis of the sequence revealed the presence of binding sites for several transcription factors such as AP-1 (TGCAGCG at –453), ATF/CRE (TGACGTCA at –160), AP-2 (CCCCAGGC at –364), and PEA2 (GACCGCA at –257). To examine the promoter activity of the rat fibronectin gene in cardiac fibroblasts, we constructed 5′-flanking region and progressively shorter deletions between –1908 and –1079, –564 and –414, –202 and –123, and –123 and +0, which suggests the presence of several positive cis-regulatory elements (Figure 6A).

Figure 6. Ang II responsiveness of fibronectin promoter regions and binding of AP-1 complex analyzed by gel mobility shift assay. A, CAT constructs containing 1908 bp of the 5′-flanking region and progressively shorter promoter sequences were stably transfected into cardiac fibroblasts. Transfected cells were stimulated by Ang II (100 nmol/L) for 12 hours after 24 hours of serum depletion, and relative CAT activities were determined as described in Materials and Methods. AG1478 (250 nmol/L) and PD98059 (10 µmol/L) were added 30 minutes before exposure to Ang II. Values represent mean ± SE of 6 separate experiments. p564 m-CAT (564 mCAT) contained a mutation in the AP-1 site at nucleotide –453. *P < 0.05 vs the CAT activity of cells stably transfected with promoterless control construct; †P < 0.05 vs the CAT activity of unstimulated cells in the absence of Ang II. B, Double-stranded oligonucleotide containing an AP-1 site (nucleotides –438 to –473) was used as a probe in the gel shift assay. Nuclear extract was obtained from cells exposed to Ang II (100 nmol/L) for 45 minutes. AG1478 (250 nmol/L) and PD98059 (10 µmol/L) were added 20 minutes before exposure to Ang II. An unlabeled oligonucleotide corresponding to nucleotides –438 to –466 was used as the competitor at 25× and 100× molar excess. AP-1 and mutated AP-1 oligonucleotides were used at 200× molar excess. Oligonucleotide AP-1 indicates AP-1 sequence present at –453 (TGACGCA), and mutated oligonucleotide AP-1 indicates the mutated sequence TGGATAA. Supershift assays were performed with rabbit polyclonal anti-c-fos IgG raised against amino acids 247 to 263 of c-jun. Each antibody was added to the samples after the initial binding reaction and then incubated for 1 hour at room temperature. C, Oligonucleotides (–143 to –178) including the ATF/CRE motif were used as a probe in the gel shift assay. Nuclear extract was obtained from cells exposed to Ang II (100 nmol/L) or PMA (1 µmol/L) for 45 minutes. The unlabeled ATF/CRE oligonucleotide corresponding to –143 to –178 was used as a competitor at 200× molar excess. Cont indicates control.

Exposure to Ang II of cells stably transfected with p1079-CAT, p882-CAT, p746-CAT, or p564-CAT induced significant increases (≈3-fold) in CAT activity to an extent similar to that in p1908-CAT, whereas in cells transfected with p414-CAT, p202-CAT, or p123-CAT the Ang II–induced increases were abolished, which suggests the presence of an Ang II–responsive element in the promoter region between nucleotides –564 and –414. Because the AP-1 site (TGACGCA) was located at nucleotide –453 of the fibronectin gene and c-fos and c-jun mRNA expression was upregulated in response to Ang II in cardiac fibroblasts, 28 we tested the involvement of the AP-1 site by site-directed mutation (TGACGCA→TGGATAA) in this motif of p564-CAT (termed p564 m-CAT) followed by stable transfection into the cells. Interestingly, we found that the responsiveness to Ang II was completely inhibited in cells transfected with p564 m-CAT (Figure 6A), indicating a critical role of the AP-1 site in the Ang II–induced increase of fibronectin gene transcription. To further study the role of EGF-R transactivation in Ang II–stimulated fibronectin promoter activity, we tested the effects of EGF-R antagonist AG1478 or MEK...
inhibitor PD98059. As shown in Figure 5A, treatment of p1908-CAT with AG1478 completely abolished Ang II–stimulated fibronectin promoter activity, whereas its activity was partially inhibited by PD98059 (64% inhibition in promoter activity).

**c-fos and c-jun Heterodimer Induced by Ang II Binds to the AP-1 Site of the Fibronectin Gene Promoter**

We have previously shown that c-fos and c-jun mRNA expression is upregulated in response to Ang II in cardiac fibroblasts. To test whether Ang II–induced c-fos and c-jun complex binds the AP-1 binding site of the fibronectin gene promoter to enhance gene transcription, we performed a gel retardation assay using an oligonucleotide (nucleotides –438 to –463), including the AP-1 binding site as a probe. The nuclear extract from unstimulated control cells contained no protein capable of binding to the probe, whereas we found a retarded band when the probe was incubated with nuclear extract from cells stimulated with Ang II (Figure 6B). An oligonucleotide corresponding to the AP-1 (TGACGCA) sequence inhibited the binding of nuclear extract, whereas a mutated oligonucleotide (TGGATGAA) did not affect binding. Furthermore, protein that bound to the AP1 oligonucleotide was supershifted when preincubated with anti–c-fos and anti–c-jun antibodies, but not anti-ATF2 antibody (Figure 6B). To further study the role of EGF-R transactivation in Ang II–stimulated gel mobility of AP-1 complex, we tested the effects of EGF-R antagonist AG1478 or MEK inhibitor PD98059. As shown in Figure 6B, treatment with AG1478 abolished Ang II–stimulated binding of AP1 complex to the AP-1 sequence, whereas its binding was not completely inhibited by PD98059.

The ATF/CRE motif (TGACGTCA) present at nucleotide –160 is known to stimulate transcription of several genes. Promoter analyses using CAT reporter constructs (Figure 6A) revealed that the ATF/CRE motif present in the promoter region of the fibronectin gene did not confer Ang II responsiveness. To test whether nuclear extract from Ang II–treated cells binds the ATF/CRE motif, we performed a gel retardation assay using an oligonucleotide (nucleotides –143 to –178) including the ATF/CRE motif. As shown in Figure 6C, the binding activity of nuclear extract to the ATF/CRE motif was not enhanced by Ang II treatment, whereas PMA induces the binding protein specific to the ATF/CRE motif.

**TGF-β Secreted Into Medium After Ang II Treatment Induces Fibronectin Expression**

Because Ang II is known to induce the synthesis and secretion of TGF-β, resulting in stimulation of fibronectin expression in mesangial cells, we tested whether this autocrine or paracrine effect of TGF-β also occurs in cardiac fibroblasts. TGF-β activity was measured using the standard CCL-64 cell bioassay, in which TGF-β neutralizing antibody on Ang II–induced fibronectin mRNA. A, Cardiac fibroblasts or HEGF-R 533del cells incubated with serum-free medium for 12 hours were stimulated with Ang II (100 nmol/L) for 12 hours, and then TGF-β activity in culture supernatant was measured by bioassay as described in Materials and Methods. PD98059 (10 μmol/L) was added 20 minutes before exposure to Ang II. Open bars represent TGF-β activity in culture supernatant from 6 fibroblast cultures; gray bars, TGF-β activity of the same culture supernatants after transient acidification to activate latent TGF-β. *P<0.01 vs latent TGF-β activity in the control. B, Serum-deprived cells were preincubated with anti-TGF-β neutralizing antibody (100 μg/mL) or control IgG (100 μg/mL) and then exposed to Ang II (100 nmol/L) for 12 hours. Fibronectin mRNA levels were quantified as described in Figure 1. Values represent mean±SE of 4 separate experiments. *P<0.01 vs controls (unstimulated cells).

Activity was completely abrogated by neutralizing anti–TGF-β antibody, but not by nonspecific control rabbit IgG. Acid treatment of conditioned medium resulted in significant increases in active TGF-β in both control and Ang II–treated cultures. However, acid treatment of control conditioned medium resulted in 0.8 ng/mL active TGF-β, whereas active TGF-β in Ang II–treated culture medium increased to 2.9 ng/mL (gray bars). This result indicated that Ang II not only increased the production of both latent and active TGF-β but also stimulated the conversion of latent to active TGF-β.

To study the role of EGF-R transactivation and ERK activation in Ang II–stimulated release of TGF-β, we deter-
mined Ang II–stimulated TGF-β activity using HEGF-R 533del cells or the cells pretreated with MEK inhibitor PD98059. Ang II–stimulated TGF-β activities in the incubation medium were completely inhibited in HEGF-R 533del cells and by treatment with PD98059 (10 μmol/L) for 20 minutes and then challenged with 100 nmol/L Ang II for 12 hours. TGF-β mRNA was quantified as described in Figure 1. *P<0.01 vs controls. B, Cells stimulated for 12 hours with serum-free medium containing Ang II (100 nmol/L) or TGF-β (10 ng/mL) were treated with actinomycin D (5 μg/mL), and then time-dependent changes in fibronectin mRNA level were analyzed by Northern blotting as described in Figure 1. To examine the effects of cycloheximide (CHX), cells were pretreated for 4 hours with cycloheximide (5 μg/mL) and then exposed to Ang II (100 nmol/L) or TGF-β (10 ng/mL). Values (○) indicates Ang II; ●, TGF-β, control represent mean±SE of 6 separate experiments, and a representative Northern blot is shown.

Figure 8. Effects of Ang II–induced EGF-R transactivation and MEK inhibitor on TGF-β mRNA accumulation and mRNA stability. A, Serum-deprived cardiac fibroblasts or HEGF-R 533del cells were pretreated with or without AG1478 (250 nmol/L) and PD98059 (10 μmol/L) for 20 minutes and then challenged with 100 nmol/L Ang II for 12 hours. TGF-β mRNA was quantified as described in Figure 1. *P<0.01 vs controls. B, Cells stimulated for 12 hours with serum-free medium containing Ang II (100 nmol/L) or TGF-β (10 ng/mL) were treated with actinomycin D (5 μg/mL), and then time-dependent changes in fibronectin mRNA level were analyzed by Northern blotting as described in Figure 1. To examine the effects of cycloheximide (CHX), cells were pretreated for 4 hours with cycloheximide (5 μg/mL) and then exposed to Ang II (100 nmol/L) or TGF-β (10 ng/mL). Values (○) indicates Ang II; ●, TGF-β, control represent mean±SE of 6 separate experiments, and a representative Northern blot is shown.

Ang II–stimulated TGF-β activity using HEGF-R 533del cells or the cells pretreated with MEK inhibitor PD98059. Ang II–stimulated TGF-β activities in the incubation medium were completely inhibited in HEGF-R 533del cells and by treatment with PD98059 (Figure 7A), indicating that ERK activated downstream of EGF-R is a major mediator to regulate the release of TGF-β by Ang II.

To assess whether secreted TGF-β mediated the effect of Ang II on fibronectin mRNA accumulation, Ang II–treated cells were coincubated with neutralizing anti–TGF-β antibodies (100 μg/mL) or control IgG (100 μg/mL) (Figure 7B). Interestingly, treatment of cultures with neutralizing antibody significantly (46%; P<0.01) abolished the induction of fibronectin mRNA expression. Control IgG had no effect. Addition of a sufficient amount of anti–TGF-β neutralizing antibody was confirmed by the finding that the conditioned medium from cells incubated with Ang II and anti–TGF-β neutralizing antibody for 24 hours contained control levels of TGF-β activity (0.15 ng/mL) as measured by bioassay. These results suggested that Ang II–induced fibronectin mRNA synthesis is mediated by autocrine or paracrine effects of TGF-β and that this action of TGF-β is not sufficient to cause the full induction of fibronectin mRNA.

Ang II Stimulates TGF-β mRNA Expression Through EGF-R Transactivation, and TGF-β Regulates Fibronectin Expression by Stabilizing Its mRNA With No Effect on Transcriptional Activity

We next examined the effects of Ang II–induced EGF-R transactivation on TGF-β mRNA expression and the mechanism by which TGF-β regulates fibronectin synthesis. Addition of Ang II (100 nmol/L) stimulated TGF-β mRNA accumulation with a time course similar to that of fibronectin mRNA induction (significant increase at 6 hours and peak level at 12 hours with a 3-fold increase). Pretreatment with the EGF-R inhibitor AG1478 completely abolished Ang II–induced TGF-β mRNA accumulation (Figure 8A) without affecting the basal level of TGF-β mRNA expression (data not shown). To further study the role of EGF-R transactivation in Ang II–stimulated TGF-β mRNA expression, we determined TGF-β mRNA levels in HEGF-R 533del cells or the cells pretreated with the MEK inhibitor PD98059. Ang II–stimulated TGF-β mRNA accumulations were completely inhibited in HEGF-R 533del cells and by treatment with PD98059 (Figure 8A), indicating that ERK activated downstream of EGF-R is a major mediator to regulate TGF-β mRNA accumulation by Ang II.

We also examined the role of TGF-β in regulating fibronectin gene expression. TGF-β (1, 10, and 100 ng/mL) did not significantly increase the CAT activity of the longest chimeric construct (p1908-CAT) (data not shown). Therefore, we next tested the effect of TGF-β on fibronectin mRNA stability by inhibiting gene transcription with actinomycin D. In unstimulated control cells, the half-life of fibronectin mRNA was 14±0.1 hours. When cells were stimulated with Ang II or TGF-β, the half-life was significantly (P<0.01) increased to 23.6±0.1 and 23.2±0.1 hours, respectively, compared with that in controls (Figure 8B). Moreover, we tested the involvement of de novo protein synthesis by using cycloheximide.
synthesis in Ang II– or TGF-β–induced stabilization of fibronectin mRNA. Pretreatment of cells with cycloheximide completely reversed Ang II– or TGF-β–induced increases in mRNA stability to the control level (Figure 8B). These observations suggested that TGF-β secreted into the medium by Ang II contributes to the accumulation of fibronectin mRNA mainly by increasing its mRNA stability rather than affecting its gene transcription and that this posttranscriptional effect is exerted via de novo protein synthesis.

**Discussion**

The major new findings of this study were that Ang II–induced synthesis and secretion of fibronectin and TGF-β are mediated by downstream signaling of EGF-R transactivated by Ca²⁺/calmodulin-dependent tyrosine kinase, in which the ERK-mediated pathway plays an important role, and that Ang II–induced fibronectin mRNA expression is regulated by 2 different mechanisms, transcriptional control by binding of the c-fos/c-jun complex to the AP-1 site and posttranscriptional control by autocrine and/or paracrine effects of TGF-β, which is exerted by increasing the mRNA stability and requires de novo protein synthesis.

Fibronectin is important for cell adhesion and cell migration, events that occur in wound healing, organogenesis, and cardiac remodeling, and it is possible that this protein molecule plays an important role in the remodeling of cardiac interstitium secondary to myocardial hypertrophy. A direct role for Ang II in remodeling is supported by the observation that cultured rat cardiac fibroblasts demonstrate increased fibronectin and collagen types I and 3 transcript expression, as well as secretion of new collagen after stimulation with Ang II. However, little is known about the mechanism by which Ang II stimulates collagenous protein synthesis in cardiac fibroblasts. Recently, we and Eguchi et al. clearly showed that in cardiac fibroblasts Ang II–induced ERK activation and c-fos expression is mainly mediated through downstream signaling of EGF-R transactivated by Ang II in a Ca²⁺/calmodulin-dependent, PKC-independent manner, and that Ang II as well as the Ca²⁺ ionophore A23187 induced tyrosine phosphorylation of the EGF-R, which was sufficient to recruit the adaptor proteins that are involved in Ras activation. In this study, we further extended these previous studies and found for the first time that such a Ca²⁺/calmodulin-dependent transactivation mechanism of EGF-R is operating on both Ang II–induced fibronectin and TGF-β gene expression.

We also showed that the AP-1 sequence present in the promoter region plays an important role in Ang II responsiveness of the fibronectin gene and that AP-1 complex (containing c-fos/c-jun) is associated with this cognate recognition sequence. A serum response element (SRE) mediates c-fos induction by growth factors, cytokines, and other stimuli that activate ERK. The c-fos promoter contains SRE, and induction of c-fos expression occurs on formation of a ternary complex factor composed of p62TPF and the serum response factor at the SRE. ERK was shown to phosphorylate p62TPF (also known as Elk-1 or SAP-1), resulting in enhanced ternary complex formation. Recent studies have shown that Janus kinase/signal transducer(s) and activator(s) of transcription and c-jun N-terminal kinase (JNK) also induce c-fos gene expression. As Ang II–induced c-fos expression in cardiac fibroblasts or VSMCs is completely inhibited by specific inhibition of EGF-R function, it is likely that Ang II–stimulated c-fos expression is mainly regulated by phosphorylated p62TPF after EGF-R–mediated ERK activation. c-jun is 1 of the major components of the transcriptional factor AP-1, which regulates expression of many genes with an AP-1 binding site in their promoter regions. The transcriptional activating activity of c-jun is regulated at the posttranslational level by phosphorylation of c-jun. c-jun is phosphorylated at 2 serine residues within N-terminal transactivation domain by JNK. JNK was reported to be activated by downstream signaling of EGF via a phosphatidylinositol 3-kinase–dependent mechanism in HeLa cells. We also found that stimulation of EGF-R is able to stimulate JNK activity in cardiac fibroblasts (S.M., unpublished observation, 1998). In this study, we showed that Ang II–induced transcriptional activity of fibronectin gene and binding of c-fos/c-jun complex to the AP-1 site are completely abolished by specific inhibition of EGF-R function. This finding suggests that in cardiac fibroblasts the transcriptional activating activity of c-jun is enhanced by downstream signaling of EGF-R. Given that inhibition of ERK activity by MEK inhibitor partially inhibited the Ang II–stimulated transcriptional activity of fibronectin gene and binding of the c-fos/c-jun complex, it is likely that the JNK pathway activated downstream of EGF-R is also involved in the transcriptional regulation of fibronectin gene.

ATF-2, a member of the family of CRE binding proteins, is not activated by agents that increase cAMP. ATF-2 can form a complex not only with c-jun but also with itself and some other members of the ATF family, and the complex binds the ATF/CRE motif to enhance gene transcription.

Although the fibronectin gene has an ATF/CRE motif in the 5′-flanking region, the results from the promoter analyses (Figure 6A) and gel retardation assays (Figure 6B) indicated that the ATF/CRE motif is not involved in Ang II responsiveness of this gene. Expression of the atrial natriuretic peptide, endothelin-1, and collagenase genes containing AP-1 sites is regulated by AP-1 activity, whereas the c-jun promoter and adhesion molecule E-selectin are upregulated by binding of the ATF-2/c-jun complex to the ATF/CRE motif. This study demonstrated for the first time that fibronectin is included in the gene family regulated by the AP-1 complex. Given that collagenase activity is also stimulated by Ang II, it is interesting that both collagenase and fibronectin genes are transcriptionally regulated by AP-1 activity. Further studies are needed to define the interaction between Ang II–induced collagenous protein synthesis and degradation.

An important function of Ang II in the heart may be stimulation of cardiac fibroblasts to release growth factors. Ang II can directly modulate vascular smooth muscle growth by stimulating release of platelet-derived growth factors, TGF-β, and basic fibroblast growth factors. Primary cultures of neonatal cardiac fibroblasts secrete significant amounts of TGF-β when stimulated with Ang II, which was
shown in turn to cause fibrillar collagen synthesis.\textsuperscript{39} Although similar paracrine or autocrine action of TGF-\textbeta\ to induce synthesis of fibronectin and collagen proteins was reported in glomerular mesangial cells,\textsuperscript{43} it remains unclear how TGF-\textbeta\ regulates the expression of fibronectin. In the present study, we clearly demonstrated that Ang II increases the level of TGF-\beta\ mRNA and production of both latent and active TGF-\beta\ into medium and that this induction is mediated via ERK activated downstream of EGF-R transactivated by Ang II. We also found that TGF-\beta\ did not affect the promoter activity of the fibronectin gene but stimulated fibronectin mRNA accumulation by stabilizing its mRNA metabolism, and that this action requires de novo protein synthesis. TGF-\beta\ has been shown to induce promoter activity of the collagen type 1 gene through a nuclear factor 1 binding site in mouse NIH 3T3 and rat osteosarcoma cells,\textsuperscript{60} whereas posttranscriptional regulation of collagen type 1 gene has also been reported in lung fibroblasts.\textsuperscript{61} As there is no nuclear factor site in the 1.9 kb of promoter region of the fibronectin gene, it is plausible that the effect of TGF-\beta\ is exerted at the posttranscriptional level by stabilization of fibronectin mRNA. Eghbali et al\textsuperscript{59} reported that in cardiac fibroblasts the effect of TGF-\beta\ on collagen type 1 synthesis required de novo synthesis of proteins by examining the effect of cycloheximide and that TGF-\beta\ did not stimulate the induction of proto-oncogenes c-fos, c-jun, or Egr-1. We also confirmed these findings in our cells (Y.M., unpublished observation, 1998). Thus, this study demonstrated that induction of fibronectin by Ang II is regulated by both transcriptional and posttranscriptional mechanisms, and the latter is due to autocrine or paracrine effects of TGF-\beta\. Although our study also indicated that the effect of TGF-\beta\ on fibronectin mRNA stabilization required de novo protein synthesis, identification of the proteins produced and the mechanism of stabilization of fibronectin mRNA remain to be determined.

In summary, we demonstrated that the expression of fibronectin as well as TGF-\beta\, a strong mediator of collagenous protein synthesis, is increased through an AR\textsubscript{T}\,-mediated transactivation mechanism of EGF-R induced by a Ca\textsuperscript{2+}\,-dependent tyrosine kinase. We also showed that ERK activation via EGF-R transactivation is a major mediator to regulate the synthesis and release of fibronectin and TGF-\beta\, whereas a pathway(s) (probably JNK) other than ERK activated downstream of EGF-R is also partially involved in the transcriptional control of fibronectin gene. Recently, we have shown that a Ca\textsuperscript{2+}\,-dependent tyrosine kinase, Pyk2/CAK\beta\ /RAFTK, is partially involved in this transactivation mechanism by examining the effects of dominant negative Pyk2.\textsuperscript{38} However, the Pyk2-mediated action alone cannot sufficiently account for transactivation of EGF-R, and another mechanism is likely to be responsible for tyrosine phosphorylation of EGF-R. The direct involvement of the EGF-R in Ang II--induced fibronectin and TGF-\beta\ expression presents a novel paradigm for cross-talk between AT\textsubscript{T}\, and growth factor receptor signaling pathways. Therefore, it is important for us to interpret the effects of Ang II on extracellular matrix formation in association with the signaling cascade regulating cellular proliferation and/or differentiation by growth factors.

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References


Angiotensin II–Induced Transactivation of Epidermal Growth Factor Receptor Regulates Fibronectin and Transforming Growth Factor- β Synthesis via Transcriptional and Posttranscriptional Mechanisms

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