Phosphorylation of Tyrosine 319 of the Angiotensin II Type 1 Receptor Mediates Angiotensin II-induced Trans-activation of the Epidermal Growth Factor Receptor*

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Although tyrosine kinases are critically involved in the angiotensin II (Ang II) type 1 (AT1) receptor signaling, how AT1 receptors activate tyrosine kinases is not fully understood. We examined the structural requirements of the AT1 receptor for transactivation of the epidermal growth factor (EGF) receptor (EGFR). Studies using carboxyl terminal-truncated AT1 receptors indicated that the amino acid sequence between 312 and 337 is required for activation of EGFR. The role of the conserved YIPP motif in this sequence in transactivation of EGFR was investigated by mutating tyrosine 319. Ang II failed to activate EGFR in cells expressing AT1-Y319F, whereas EGFR was activated even without Ang II in cells expressing AT1-Y319E, which mimics the AT1 receptor phosphorylated at Tyr-319. Immunoblot analyses using anti-phospho Tyr-319-specific antibody showed that Ang II increased phosphorylation of Tyr-319. EGFR interacted with the AT1 receptor but not with AT1-Y319F in response to Ang II stimulation, whereas the EGFR-AT1 receptor interaction was inhibited in the presence of dominant negative SHP-2. The requirement of Tyr-319 seems specific for EGFR because Ang II-induced activation of other tyrosine kinases, including Src and Jak2, was preserved in cells expressing AT1-Y319F. Extracellular signal-regulated kinase activation was also maintained in AT1-Y319F through activation of Src. Overexpression of wild type AT1 receptor in cardiac fibroblasts enhanced Ang II-induced proliferation. By contrast, overexpression of AT1-Y319F failed to enhance cell proliferation. In summary, Tyr-319 of the AT1 receptor is phosphorylated in response to Ang II and plays a key role in mediating Ang II-induced transactivation of EGFR and cell proliferation, possibly through its interaction with SHP-2 and EGFR.

The signaling mechanism of the angiotensin II (Ang II) type 1 (AT1) receptor has traditionally been portrayed to be dependent on heterotrimeric G proteins, including Goq and Gox proteins and their downstream targets, primarily phospholipase C (1). This results in inositol triphosphate generation, which in turn causes an increase in intracellular calcium concentrations and diacylglycerol formation, leading to activation of protein kinase C. However, recent investigations revealed that tyrosine phosphorylation is also intimately involved in AT1 receptor signaling (2–6). Ang II-induced ERK1/2 activation, for example, requires tyrosine kinase activation, including Src family tyrosine kinases (7, 8) and epidermal growth factor receptor (EGFR) (9, 10). It is unclear, however, how AT1 receptors, which lack intrinsic tyrosine kinase activities, are able to stimulate tyrosine kinases.

We have recently shown that an AT1 receptor second intracellular loop mutant, lacking heterotrimeric G protein coupling, is able to activate Src tyrosine kinase (11). This suggests that heterotrimeric G protein-independent mechanisms are able to activate Src. Furthermore, increasing lines of evidence suggest that the carboxyl terminus (C-tail) of the AT1 receptor plays an important role in the AT1 receptor signaling (11, 12). For example, ligand binding to the AT1 receptor induces physical association of the C-tail of the AT1 receptor with Jak2, thereby causing phosphorylation and translocation of STAT to the nucleus (13). Other signaling molecules, including phospholipase Cγ and SHP-2, also have been shown to interact with the C-tail of the AT1 receptor (14, 15). These results suggest that direct interaction between the heterotrimeric G protein-coupled receptor and intracellular signaling molecules may play an important role in mediating activation of downstream-signaling mechanisms.

Accumulating data suggests that EGFR is involved in signal transduction of many G protein-coupled receptors, including the AT1 receptor (9, 10) (for review, see Ref. 16). Ang II induces tyrosine phosphorylation of EGFR and its association with Shc and Grb2, leading to subsequent activation of the Ras-Raf-MEK-ERK1/2 pathway (9). Although several signaling mechanisms are involved in Ang II-induced activation of EGFR (9, 17–20), whether or not direct interaction between the AT1 receptor and intracellular signaling molecules is required for EGFR activation and, if so, the amino acid sequence of the AT1 receptor mediating Ang II-induced EGFR activation has not been identified.

To elucidate the molecular mechanism of Ang II-induced EGFR activation, we investigated the structural requirements of the AT1 receptor and the associating signaling mechanism.
leading to transactivation of EGFR. Our results indicate that tyrosine 319 in the conserved Y725 motif in the carboxyl terminus of the AT1 receptor plays an essential role in mediating Ang II-induced transactivation of EGFR and cell proliferation.

EXPERIMENTAL PROCEDURES

MATERIALS—Ang II was purchased from Peninsula. Anti-FLAG M2 affinity gel was from Sigma. Horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody (RC20H) and anti-EGF receptor monoclonal antibody were from Transduction Laboratories. Anti-v-Src monoclonal antibody was from Calbiochem. Anti-AT1 receptor monoclonal antibody was from BioSource. Rabbit anti-active ERK1/2 polyclonal antibody was from Promega. Anti-EGF receptor sheep polyclonal antibody were from Transduction Laboratories. Anti-v-Src monoclonal antibody was from Amersham Biosciences. Enolase was from Roche Molecular Biochemicals. AG1475 was from Biomol.

Plasmids—The full-length rat AT1a wild type receptor (AT1-WT) cDNA subcloned into pcDM8 was obtained from Dr. S. Sano (Mitsubishi Kasei Institute of Life Science, Tokyo, Japan). Plasmid encoding dominant negative SHP-2 (SHP-2-(1–225)) was generated by PCR, and the PCR product was subjected to TA cloning using pCR3.1 vector (Invitrogen). Plasmid encoding Myc-dominant negative SHP-2 (SHP-2-(1–225)) was generated by PCR, and the PCR product was subjected to TA cloning using pCR3.1 vector (Invitrogen). Plasmid encoding the dominant negative AT1 receptor carboxyl terminus peptide was generated by subcloning cDNA encoding AT1-(292–359) into a mammalian expression vector pHM6 (Roche Molecular Biochemicals), which has an HA tag in the amino terminus. Site-directed mutagenesis was performed to generate HA-AT1-(292–359)-Y319F using QuikChange. The DNA sequence of all constructs was confirmed by DNA sequence analyses. Expression plasmids for EGFR and JAK2 were provided by Dr. A. Yoshimura (Kyushu University, Fukuoka, Japan). Plasmid for Myc-SHP-2 was provided by Dr. Y. Tannish (Kawasaki Institute of Science, Tokyo, Japan). Plasmid encoding Myc-dominant negative SHP-2 (SHP-2-(1–225)) was generated by PCR, and the PCR product was subjected to TA cloning using pCR3.1 vector (Invitrogen). Plasmid encoding dominant negative Src (R296F/Y298F) and its control vector (pUSE) were purchased from Upstate Biotechnology.

Cell cultures, Transfection, and Receptor Binding Assays—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO2 atmosphere. Transfections were performed on 70% confluent monolayers in 60-mm dishes for immunoprecipitation and Src kinase assays or in 35-mm dishes for IPx assays. For transient transfection, 2.5 μl of Opti-MEM I (Invitrogen) containing 4 μg of DNA, 8 μl of LipofectAMINE Plus reagent, and 12 μl of LipofectAMINE was used for a 60-mm dish. One ml of Opti-MEM I containing 2 μg of DNA, 6 μl of LipofectAMINE Plus reagent, and 4 μl of LipofectAMINE was used for a 35-mm dish. Empty pcDNA 3.0 plasmid was added as needed to keep the total amount of DNA constant per transfection. Cells were incubated in serum-free Opti-MEM at 37 °C for 3 h. COS-7 cells were then incubated with 10% fetal bovine serum in DMEM and incubated overnight. Transfected cells were serum-starved in serum-free DMEM for 24–26 h before transfection. Assays were performed 48 h after transfection. Saturation binding curves were determined using a modification of the whole cell receptor binding assay (16) as described previously (11). The dissociation constant (Kd) for [3H]-AngII binding was determined using Scatchard analysis.

Cardiac fibroblast cultures were prepared as described previously (17). In brief, hearts were removed from 1-day-old Crl:Wistar rats (Charles River Laboratories) and minced in small pieces. The tissue was minced with scissors and incubated in CHAPS buffer (150 mM NaCl, 15 mM HEPES, pH 7.0, 1% deoxycholic acid, 1% Igepal, 0.1% SDS, 0.1 mM Na3VO4, 1 mM NaN3, 0.5 mM 4-2-aminoethylbenzenesulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin) for immunoprecipitation or hypotonic lysis buffer (25 mM NaCl, 25 mM Tris, pH 7.4, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM Na3VO4, 10 mM NaF, 0.5 mM 4-2-aminoethylbenzenesulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin) for ERK1/2 kinase assays. Cell lysates were incubated on ice for 10 min and subject to centrifugation for 30 min. Protein concentrations of the supernatants were adjusted to be 1 mg/ml with the lysis buffer. For immunoprecipitation of EGFR-FLAG, the cell lysates (500 μg) were incubated with 40 μl of anti-FLAG M2 affinity gel at 4 °C for 2 h. For immunoprecipitation of endogenous EGFR, the cell lysates were incubated with 4 μg of anti-EGF receptor monoclonal antibody for 1 h followed by protein G-agarose (30 μl of slurry) for 45 min. The immune complexes were washed 3 times with lysis buffer and denatured in Laemmli sample buffer. After SDS-PAGE, samples were transferred onto polyvinylidene fluoride microporous membranes (Millipore). Immuno-blots were performed as described previously. Phosphorylated levels of the EGFR, the AT1 receptor, or ERK1/2 were analyzed by immunoblotting with anti-phospho-specific antibody and scanning densitometry, and the results were expressed as fold increase compared with the control.

Ang II Binding Assays—Polycyclic analogues of angiotensin II were synthesized in our laboratory or purchased from supplier. Binding assays were performed as described previously (17). Cells were incubated with [125I]-AngII (1.6 nM) for 1 h followed by protein G-agarose (30 μl of slurry) for 45 min. The immune complexes were washed 3 times with lysis buffer and denatured in Laemmli sample buffer. After SDS-PAGE, samples were transferred onto polyvinylidene fluoride microporous membranes (Millipore). Immunoblots were performed as described previously. Phosphorylated levels of the EGFR, the AT1 receptor, or ERK1/2 were analyzed by immunoblotting with anti-phospho-specific antibody and scanning densitometry, and the results were expressed as fold increase compared with the control.

Phosphoinositide Production—Measurement of inositol phosphates (IPX) was based upon the method of Berriege et al. (19) as described previously (11). Cells were incubated with [3H]-inositol (10 μCi/ml) in DMEM for 24 h at 37 °C. Labeling was terminated by aspirating the medium, rinsing cells with oxygenated reaction buffer (142 mM NaCl, 30 mM Hepes buffer, pH 7.4, 5.6 mM KCl, 3.6 mM NaHCO3, 2.2 mM CaCl2, 1.0 mM MgCl2, and 1 mg/ml p-glucose), and harvesting cells with phosphate-buffered saline, 0.02%EDTA. Cells were centrifuged twice (300 g for 5 min) in reaction buffer, and the pellet was resuspended in an equal volume of reaction buffer containing 60 mM LiCl. Stimulation of IPx production was initiated by mixing 0.25 ml of cell suspension with 0.25 ml of 0–100 nM Ang II in reaction buffer (without LiCl). The mixture was incubated for 30 min at 37 °C, then 0.5 ml of ice-cold 20% trichloroacetic acid was added. Precipitates were pelleted (4100 × g, 20 min), and the trichloroacetic acid-soluble fraction was transferred to new tubes, washed with saturated diethyl ether, and neutralized with NaHCO3. IPXs were isolated by adsorption to 0.5 ml of Dowex AG-1X-8 formate resin slurry and rinsed 5 times with 3 ml of unlabeled 5 mM myoinositol followed by elution with 1 ml of 1.2 M ammonium formate, 0.1 M formic acid. The elutes were counted by liquid scintillation counter in 5 ml of ScintiVerse.

Src Kinase Assay—The tyrosine kinase activity of Src was determined by the immunecomplex kinase assay using enolase as a substrate as described previously (7, 11). Cell lysates were prepared in a lysis buffer (150 mM NaCl, 15 mM HEPES, pH 7.0, 1% deoxycholic acid, 1% Igepal, 0.1% SDS, 0.1 mM Na3VO4, 1 mM NaF, 0.5 mM 4-2-aminoethylbenzenesulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin). The cell lysates were resuspended on ice in a total reaction buffer (10 μl) consisting of 20 μl of 5% NP-40, 0.25 μl of anti-v-Src monoclonal antibody at 4 °C for 1 h. Protein G-Sepharose was then added. The immunoprecipitated were washed twice with lysis buffer without SDS or deoxycholic acid and then washed once with kinase buffer (50 mM HEPES, pH 7.6, 0.1 mM EDTA, 10 mM MnCl2, 0.015% Brig 35). Pellets were incubated for 15 min at 37 °C in the kinase buffer with 1 μM of [γ-32P]ATP and 0.25 μg of enolase as a substrate. The reaction was terminated by precipitation of Laemmli sample buffer on ice. Reaction mixtures were boiled and subjected to 12% SDS-PAGE followed by autoradiography. Results were analyzed by densitometry.

Adenovirus Vectors—Adenovirus-mediated transduction was performed as described previously (20). Cells grown in 60-mm dishes were transfected with an adenovirus vector harboring the wild-type Ras (Ad5/N17Ras) (courtesy of Dr. M. Schneider, Baylor College of Medicine, Houston, TX) at a multiplicity of infection of 100. For a control study, Ad5/E1sp1B (courtesy of Dr. B. French, University of Virginia, Charlottesville, VA) was used. Adenovirus vectors harboring the wild type AT1 receptor or AT1-Y319F were generated by using the
of the experiments. In

with AT1R and EGFR-FLAG were treated with Ang II (10⁻⁷ M) for the indicated time. Cell lysates were subjected to immunoprecipitation (IP) with antibody against FLAG and immunoblotted with antibodies against phosphotyrosine (PY) or EGFR. A, shown is the representative of the experiments. B, fold increase of phosphorylation level of EGFR is shown. n = 7.

AdEasy system (21). All experiments were performed 48 h after transduction.

Cell Proliferation Experiments—Cells were plated at a density of 0.3 × 10⁵/well in six-well plates. Twelve hours after plating, cells were serum-starved for 12 h and then stimulated with Ang II (10⁻⁷ M) in the presence or absence of AG1478 (250 nM) for 36 h. Ang II or AG1478 was added every 12 h. After stimulation, cells were washed twice with phosphate-buffered saline. The cell layer was scraped with 1 ml of standard sodium citrate containing 0.25% SDS and vortexed extensively. Total DNA content was determined by the Hoechst dye method as described previously (17).

Statistics—Data are given as the mean ± S.E. Statistical analyses were performed using the analysis of variance. The post-test comparison was performed by the method of Tukey. Significance was accepted at p < 0.05 level.

RESULTS

Ang II Activates EGFR in COS-7 Cells Expressing the Wild Type AT1 Receptor—Ang II stimulation of COS-7 cells without transfection of the AT1 receptor did not activate either endogenous or transfected EGFRs (not shown). By contrast, in COS-7 cells transfected with the AT1 receptor, Ang II, caused time-dependent increases in tyrosine phosphorylation of either endogenous (not shown) or transfected EGFRs (Fig. 1A). Tyrosine phosphorylation of EGFR by Ang II was observed within 3 min, reached a peak around 5 min, and lasted for more than 60 min (Fig. 1B (n = 7) and 2.8 ± 0.1-fold at 60 min (n = 3)). This suggests that stimulation of the AT1 receptor causes transactivation of EGFR in COS-7 cells.

Tyrosine 319 in the Carboxyl Terminus of the AT1 Receptor Plays an Important Role in Ang II-induced Activation of EGFR—Because it has been suggested that the C-tail of the AT1 receptor plays an important role in mediating cell-signaling mechanisms of the AT1 receptor (11), we examined the role of the AT1 receptor C-tail in Ang II-induced transactivation of EGFR. We co-transfected EGFR-FLAG and carboxyl-terminal-truncated AT1 receptors into COS-7 cells, and EGFR was immunoprecipitated with anti-FLAG antibody. Although Ang II caused significant increases in tyrosine phosphorylation of EGFR in cells transfected with AT1-(1–338), it failed to do so in cells transfected with AT1-(1–311) (Fig. 2A). These results suggest that the amino acid sequence located between amino acid 312 and 337 of the AT1 receptor is required for activation of EGFR by Ang II stimulation.

Between amino acids 312 and 337 of the AT1 receptor, the YIPP motif (319–322) motif is evolutionarily conserved in AT1 receptors cloned from many species (Fig. 2B). It has been shown that several signaling molecules directly or indirectly associate with the YIPP motif in the AT1 receptor (13, 14). To test the role of this conserved motif in Ang II-induced EGFR activation, we made a mutant where a tyrosine residue at position 319 in this motif was mutated to phenylalanine (AT1-Y319F). Interestingly, Ang II-induced activation of EGFR was abolished in COS-7 cells transfected with AT1-Y319F (Fig. 2C).

AT1 Receptor Y319F Mutant Increases Basal EGFR Activities in COS-7 Cells—To test if phosphorylation of tyrosine 319 is involved in activation of EGFR, we mutated tyrosine 319 to glutamate (Y319E) to mimic the status of phosphorylation. Expression of AT1-Y319E in COS-7 cells significantly increased phosphorylation of EGFR in basal conditions, and Ang
Tyrosine 319 in AT1 Receptor Mediates EGFR Activation

II stimulation failed to increase phosphorylation of EGFR (Fig. 3A). These results suggest that phosphorylation of tyrosine 319 may be involved in Ang II-induced activation of EGFR.

Tyrosine 319 Is Phosphorylated in Response to Ang II Stimulation—To test if tyrosine 319 of the AT1 receptor is phosphorylated in vivo in response to Ang II stimulation, we generated phosphoserine 319-specific anti-AT1 receptor antibody (anti-phosphoserine 319 antibody). Either wild type AT1 receptor (AT1-WT) or AT1-Y319F was transfected in COS-7 cells. The AT1 receptor phosphorylated at tyrosine 319 was immunoprecipitated by the anti-phosphotyrosine 319 antibody and immunoblotted with the same antibody. Although AT1-WT was not detected by anti-phosphotyrosine 319 antibody in unstimulated cells, it was detected after the cells were stimulated with Ang II for 3 min (Fig. 3B). Phosphorylation of tyrosine 319 was transient and returned to the basal level at 5 min. No apparent signals of phosphotyrosine 319 were detected in samples obtained from cells expressing AT1-Y319F (Fig. 3B), suggesting that the signal found in AT1-WT was most likely from phosphorylated Tyr-319. Duplicate samples were subjected to immunoprecipitation using anti-(total) AT1 receptor (AT1R) antibody and immunoblotted with the same antibody. n = 3.

II-induced Activation of EGFR in AT1-WT Is Inhibited in the Presence of AT1 Carboxyl Terminus Minigene—To confirm that tyrosine 319 of the AT1 receptor is important for Ang II-induced transactivation of EGFR, we examined the effect of overexpression of a mini-gene-containing AT1 receptor carboxyl terminus peptide (HA-AT1-C) upon Ang II-induced EGFR activation in COS-7 cells. Expression of HA-AT1-C inhibited Ang II-induced activation of EGFR in COS-7 cells (Fig. 4A). Overexpression of HA-AT1-C alone did not inhibit activation of EGFR by EGF treatment (Fig. 4B), suggesting that the effect of HA-AT1-C is stimulus-specific. Furthermore, a minigene-containing HA-AT1-C, whose tyrosine 319 is mutated to phenylalanine (HA-AT1-C-Y319F), failed to inhibit Ang II-induced EGFR activation in COS-7 cells (Fig. 4A). These results further support the notion that tyrosine 319 in the AT1 receptor plays an essential role in mediating EGFR activation by the AT1 receptor.

Ang II Stimulates Interaction between AT1 Receptors and EGFR, Which Was Inhibited in the Presence of Dominant Negative SHP-2—We next examined if the AT1 receptor and EGFR physically associate with each other. We co-transfected AT1 receptors and EGFR into COS-7 cells and stimulated the cells with Ang II. EGFR immunoprecipitates were immunoblotted with antibodies against EGFR. Interestingly, AT1 receptors were co-immunoprecipitated with EGFR in samples stimulated with Ang II for 3 min (2.7 ± 0.2-fold versus 0 min, n = 4, Fig. 5A). This interaction between the AT1 receptor and EGFR was transient, since it was not observed at 5 min. Equal amounts of EGFRs were immunoprecipitated in each sample, and the immunoprecipitated EGFR was tyrosine-phosphorylated in response to Ang II stimulation, consistent with the results shown.
cells were treated with Ang II (10^{-7} M) for the indicated durations. Cell lysates were subjected to immunoprecipitation (IP) with antibodies against FLAG and immunoblotted with antibodies against phosphotyrosine (PY), EGFR, or AT1 receptor (AT1R). n = 4. B, COS-7 cells were co-transfected with EGFR-FLAG and AT1-Y319F. In the control cells (lane 4), no plasmid was transfected. Cell lysates were subjected to immunoprecipitation and immunoblotting as described in A. n = 4.

Because tyrosine 319 plays an essential role in mediating Ang II-induced activation of EGFR, we attempted to determine if activation of other tyrosine kinases is also affected in AT1-Y319F. Ang II caused significant increases in Src activities in cells transfected with either AT1-WT or AT1-Y319F (2.9 ± 0.1-fold in WT, 2.7 ± 0.2-fold in Y319F at 5 min) (Fig. 6B). Ang II also caused similar levels of increases in tyrosine phosphorylation of transfected JAK2-FLAG in cells expressing either AT1-WT or AT1-Y319F (3.5 ± 0.1-fold in WT, 3.4 ± 0.2-fold in Y319F at 5 min) (Fig. 6C). These results suggest that Ang II-induced activation of some tyrosine kinases, including Src and JAK2, is preserved in cells expressing AT1-Y319F.

**EGFR Plays an Important Role in Ang II-induced ERKs Activation by AT1-WT, Whereas Src Mediates Ang II-induced ERKs Activation by AT1-Y319F**—Ang II activated ERKs (3.5 ± 0.1-fold, p < 0.05 versus control) in COS-7 cells transfected with AT1-WT (Fig. 7A). AG1478, a specific inhibitor for EGFR, abolished Ang II-induced ERK activation in COS-7 cells transfected with AT1-WT (Fig. 7A), suggesting that EGFR plays an essential role in ERK activation by AT1-WT in COS-7 cells. Surprisingly, however, Ang II was still able to activate ERKs in AT1-Y319F (3.6 ± 0.2-fold, p < 0.05 versus control), where Ang II-induced EGFR activation is abolished. Because AG1478 did not affect Ang II-induced ERK activation in cells transfected
hours after transduction, cells were stimulated with or without Ang II (10^{-7} M) for 5 min. Cell lysates were subjected to immunoprecipitation (IP) with or without antibody against EGFR and immunoblotted with antibodies against phosphotyrosine (PY) or EGFR. For the control (C), antibody against EGFR was not added to the immunoprecipitation.

B. Ang II-induced cell proliferation was enhanced in cells overexpressing WT, whereas it was abolished in AT1-Y319F. Neonatal rat cardiac fibroblasts were transduced with either control adenovirus or adenovirus harboring WT or Y319F. Forty-eight hours after transduction, cells were stimulated with Ang II (10^{-7} M) for an additional 36 h. Cell numbers were estimated by total DNA content and normalized to those in control virus-transduced cells without Ang II treatment. NC, negative control; NS, not significant (n = 6).

Overexpression of the AT1-Y319F Mutant Abolishes Ang II-induced Cell Proliferation in Cardiac Fibroblasts—Our results presented thus far indicated that AT1-Y319F selectively lacks Ang II-induced transactivation of EGFR, whereas it maintains activation of some, if not all, signaling molecules, including IPx, Src, JAK2, and ERKs. To test if AT1-Y319F mutant has cellular functions different from AT1-WT, we expressed either AT1-WT or AT1-Y319F in primary cultured cardiac fibroblasts by adenovirus-mediated gene delivery. An adenovirus harboring an irrelevant sequence was used as a control vector. Cell cultures with comparable levels of expression of either AT1-WT or AT1-Y319F were used for these experiments. Overexpression of AT1-WT in cardiac fibroblasts increased Ang II-induced EGFR activation. By contrast, overexpression of AT1-Y319F failed to enhance Ang II-induced activation of EGFR, suggesting that Tyr-319 plays an essential role in mediating Ang II-induced EGFR activation in cardiac fibroblasts (Fig. 8A). Under these conditions, overexpression of the AT1-WT in cardiac fibroblasts significantly enhanced Ang II-induced cell proliferation, which was determined by the total DNA content, compared with control virus-transduced cells (Fig. 8B). By contrast, overexpression of AT1-Y319F abolished small increases in cell proliferation found in control virus-infected cardiac fibroblasts (Fig. 8B). To determine the role of EGFR activation in Ang II-induced cardiac fibroblast proliferation, we treated the cells with AG1478. Treatment with AG1478 completely abolished the Ang II-induced cell proliferation in both control virus-transduced and AT1-WT-transduced cardiac fi-
broblasts (Fig. 5B). These results suggest that the cell-signaling mechanism mediated by Tyr-319 in the AT1 receptor, including activation of EGFR, is required for Ang II-induced cell proliferation in cardiac fibroblasts.

**DISCUSSION**

**Tyrosine 319 Is Specifically Required for Ang II-induced EGF Receptor Activation**—The YIPP motif found in the AT1 receptor is conserved in all members of the AT1 receptor family so far cloned, suggesting that this motif is involved in important cellular functions of the AT1 receptor. This motif is also found in the platelet-derived growth factor α and β receptors and is involved in ligand-dependent activation of phospholipase Cγ (23). Although it has been shown that several signaling molecules associate with the motif in the AT1 receptor (13–15, 24, 25), the specific requirement of tyrosine 319 for activation of downstream protein kinases has not been clearly demonstrated *in vivo*. Because Ang II-induced activation of Src and JAK2 was not affected in AT1-Y319F, the requirement of tyrosine 319 in the AT1 receptor seems specific for activation of EGFR among the activation of major tyrosine kinases. The structural requirements of the AT1 receptor in Ang II-induced EGFR have not been previously determined.

It has been shown that the AT1 receptor is tyrosine-phosphorylated by ligand binding (14, 26). However, the tyrosine residue phosphorylated by ligand binding has not been identified *in vivo*. Because signaling molecules containing the SH2 domain interact with the YIPP motif of the AT1 receptor, it has been speculated that tyrosine 319 is phosphorylated (13–15). By using anti-phosphotyrosine 319-specific AT1 receptor antibody, we demonstrated that tyrosine 319 is phosphorylated in response to Ang II.

Although it has been speculated that ligand-dependent phosphorylation of the AT1 receptor may modulate the activities of downstream signaling molecules besides internalization of the receptor, this has not been clearly demonstrated. In fact, it has been recently shown that phosphorylation of the AT1 receptor by G protein-coupled receptor kinase does not play an essential role in Ang II-induced cell signaling (27). In our study, because expression of AT1-Y319E increased basal levels of EGFR phosphorylation and because Ang II failed to show additive effects on EGFR activation by Y319E, phosphorylation of tyrosine 319 seems to mediate Ang II-induced activation of the EGFR. At present we do not know which tyrosine kinase is responsible for phosphorylation of tyrosine 319.

**The Mechanism of EGFR Activation by the AT1 Receptor**—Ca²⁺-dependent mechanisms (9), other tyrosine kinases, such as Src and Pyk2 (28–30), metalloproteases (for review, see Ref. 31), and reactive oxygen species (32) have been proposed as mechanisms of Ang II-induced activation of EGFR in various cell types. The requirement of tyrosine 319 in the AT1 receptor for EGFR activation found in the present investigation may not contradict these previous observations but may represent another requirement for EGFR activation by AT1 receptors. Because production of IPX and activation of other kinases such as Src and JAK2 by Ang II are preserved in AT1-Y319F, activation of these molecules alone is not sufficient for Ang II-induced EGFR activation.

Growing lines of evidence suggest that heterotrimeric G protein-coupled receptors directly interact with intracellular signaling molecules (33, 34). The mini-gene containing AT1-C, but not AT1-C-Y319F, effectively blocked Ang II-induced activation of EGFR. Thus, protein-protein interaction at the AT1-C containing tyrosine 319 may mediate Ang II-induced EGFR activation. It has been shown *in vitro* that the SH2 domain of SHP2 interacts with the YIPP motif, where Tyr-319 is located, once tyrosine is phosphorylated (13, 15). Our results suggest that the AT1 receptor and EGFR transiently interact with each other in a ligand binding-dependent manner, and the timing of their interaction coincides with that of Tyr-319 phosphorylation. Therefore, we speculate that SHP2 binds to Tyr-319 when Tyr-319 is phosphorylated, thereby acting as a scaffold protein. Because dominant negative SHP-2 (22) was able to inhibit both AT1 receptor-EGFR interaction and Ang II-induced transactivation of EGFR, it is likely that SHP-2 mediates AT1 receptor-EGFR interaction. It has been recently shown that EGFR associates with β-adrenergic receptor in a ligand binding-dependent manner, possibly through a scaffold protein, β-arrestin (35). Thus, both SHP-2 and β-arrestin work as scaffold proteins to induce interaction between G protein-coupled receptors and EGFR for transactivation of EGFR. This hypothesis is consistent with a recent observation that assembling signaling molecules, including the AT1 receptor and EGFR, by scaffolding proteins such as caveolin is required for Ang II-induced transactivation of EGFR (36). It should be noted that phosphorylation of EGFR persists even after interaction between the AT1R and EGFR is lost. We speculate that once phosphorylation of EGFR is initiated, the activity of EGFR may be maintained through autophosphorylation until other mechanisms of inactivation are activated.

**Hypothesis about the Back-up Mechanism**—Although Ang II binding to the AT1-WT activates ERKs through an EGFR-dependent mechanism, binding to AT1-Y319F still activates ERKs through an Src-dependent mechanism despite the fact that AT1-Y319F failed to activate EGFR. This cellular mechanism, which is the Src-dependent mechanism, mediates the loss of EGFR activation to activate ERKs only in cells expressing AT1-Y319F is unclear at present. One possible explanation would be that EGFR may sequester molecules leading to Ras activation, such as Grb2, when tyrosine 319 of the AT1 receptor is available. It should be noted that it has been previously shown that tyrosine kinases are able to mediate Ang II-induced ERK activation only when a protein kinase C-dependent mechanism of ERK activation is blocked (37). Thus, tyrosine kinases may in general work as a back-up mechanism for the AT1 receptor to maintain the activity of ERKs.

**The Role of Tyrosine 319 in Cell Proliferation in Cardiac Fibroblasts**—Our results show that tyrosine 319 of the AT1 receptor plays an essential role in mediating Ang II-induced cell proliferation in cardiac fibroblasts. Although we have shown in this work that tyrosine 319 of the AT1 receptor plays a key role in Ang II-induced EGFR activation in cardiac fibroblasts, this does not exclude the possibility that activation of other molecules may also depend upon tyrosine 319. However, considering the fact that the EGFR-specific inhibitor AG1478 completely abolished Ang II-induced cell proliferation, it is likely that the effect of the mutation at tyrosine 319 is primarily mediated by its effect upon Ang II-induced EGFR activation. The signaling mechanisms of Ang II-induced cellular responses have been primarily studied by using either specific chemical inhibitors or inhibitor molecules such as dominant negatives (10). Our results suggest that the AT1 receptor mutant, which has a selective defect in the signaling mechanism, can be used to elucidate the cellular function of the signaling mechanisms activated by the AT1 receptors.

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