AT1 Receptor Mutant Lacking Heterotrimeric G Protein Coupling Activates the Src-Ras-ERK Pathway without Nuclear Translocation of ERKs*

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Angiotensin II (Ang II) type 1 receptors (AT1Rs) activate tyrosine kinases, including Src. Whether or not tyrosine kinase activation by AT1R occurs independently of heterotrimeric G protein coupling is unknown. To address these questions, we used an AT1aR intracellular second loop mutant, which lacks heterotrimeric G protein coupling (AT1a-i2m). Surprisingly, Ang II-induced Src activation was preserved in AT1a-i2m, which was not attenuated by inhibiting protein kinase C and Ca2+ or by inhibiting Goq or Gqi in CHO-K1 cells. By contrast, Ang II-induced Src activation was abolished in a C-terminally truncated AT1a-(1–309), where Ang II-induced inositol phosphate response was preserved. Ang II activates ERKs via a Src-Ras-dependent mechanism in AT1a-i2m. ERKs activated by AT1a-i2m phosphorylate their cytoplasmic targets, including p90RSK, but fail to translocate into the nucleus or to cause cell proliferation. Ang II-induced nuclear translocation of ERKs by wild type AT1aR was inhibited by overexpression of nuclear exportin Crm-1, while that by AT1a-i2m was restored by leptomycin B, an inhibitor of Crm-1. In summary, while Src and ERKs are activated by Ang II even without heterotrimeric G protein coupling, the carboxyl terminus of the AT1 receptor is required for activation of Src. Interestingly, ERKs activated by heterotrimeric G protein-independent mechanisms fail to phosphorylate nuclear targets due to lack of inhibition of Crm-1-induced nuclear export of ERKs. These results suggest that heterotrimeric G protein-dependent and -independent signaling mechanisms play distinct roles in Ang II-mediated cellular responses.

The signaling mechanism of the angiotensin II (Ang II)1 type 1 (AT1) receptor has traditionally been portrayed as being dependent on heterotrimeric G proteins (1). The AT1 receptor activates phospholipase Cγ (PLCγ) via Goq proteins. This causes generation of inositol trisphosphates as well as diacylglycerol, which in turn causes release of Ca2+ from the intracellular Ca2+ store sites and activation of protein kinase C (PKC), respectively. The AT1 receptor also couples to Gαs, thereby regulating adenylyl cyclase (2). Besides coupling with the heterotrimeric G proteins, activation of tyrosine kinases is also intimately involved in the AT1 receptor signaling (3, 4). Both nonreceptor type tyrosine kinases (Src, Fyn, Yes, Pyk2, focal adhesion kinase, and JAK2) and receptor type tyrosine kinases (EGF and platelet-derived growth factor receptors) are activated by the AT1 receptor (5–8). These tyrosine kinases regulate downstream signaling mechanisms, including PLCγ, Ras-Raf-MEK-ERK, and STAT (6, 9, 10), thereby playing a critical role in cell growth responses by Ang II.

Several mechanisms have been shown to mediate tyrosine kinase activation by heterotrimeric G protein-coupled receptors (GPCRs; reviewed in Refs. 11–13). First, the downstream effectors of Ga and Gβγ mediate tyrosine kinase activation. For example, Ca2+ and PKC activated through the Goq-PLCγ pathway directly or indirectly affect activities of tyrosine kinases, including Src, focal adhesion kinase, PYK2, and epidermal growth factor receptor (7, 14–17). Gβγ causes activation of tyrosine kinases through its effects upon downstream signaling molecules, such as phosphatidylinositol 3′-kinase γ (18). Second, Go directly interacts with tyrosine kinases and regulates their kinase activities. Goq and Gqi directly regulate Bruton’s tyrosine kinase (19) and Src family tyrosine kinases (20). Third, direct interaction between the GPCRs and signaling molecules seems to affect tyrosine kinase activities. Arrestins associate with the carboxyl terminus of the β-AR phosphorylated by GRK in a ligand binding-dependent manner. Arrestins work as scaffold proteins, thereby regulating downstream kinases, such as Src, ERKs, ASK1, JNK3, and EGF receptor (21, 22) (reviewed in Ref. 23). SHP-2 binds to the carboxyl terminus of the AT1 receptor, thereby mediating interaction between the AT1 receptor and JAK2 (24). Fourth, active Src has been shown

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** The abbreviations used are: Ang II, angiotensin II; AT1, Ang II type 1; AT1R, AT1 receptor; PKC, protein kinase C; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; STAT, signal transducers and activators of transcription; PLC, phospholipase C; GPCR, G protein-coupled receptor; AR, adrenergic receptor; BAPTA-AM, 1,2-bis(2-aminoethyl)-amine-N,N,N′,N′-tetraacetic acid acetyoxymethyl ester; GTPyS, guanosine 5′-O-(3-thiotriphosphate); AEBSF, 4-2-aminoethyl)-benzenesulfonyl fluoride; FITC, fluorescein isothiocyanate; IPx, inositol phosphate(s); IP3, inositol trisphosphate; PTX, pertussis toxin; WT, wild type; PMA, phorbol 12-myristate 13-acetate.

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to directly associate with the β2- and β2-ARs (25, 26). However, how interaction between the GPCR and Src affects the activity of Src remains unclear. Fifth, activation of epidermal growth factor receptor by GPCR is mediated by metalloproteinase-induced cleavage of pro-HB-EGF in PC3 cells (27) or by an N-acetylcysteine-dependent mechanism in cardiac fibroblasts (17) (reviewed in Ref. 12).

Some of the aforementioned cross-talks between GPCRs and tyrosine kinases are initiated by activation of heterotrimeric G proteins alone or via downstream signaling molecules (7, 17–20, 28, 29). By contrast, in some cases, GPCRs may stimulate tyrosine kinases through G protein-independent mechanisms (reviewed in Ref. 12). Some of the aforementioned cross-talks between GPCRs and Src affect the activity of Src by receptor binding assays. Experimental results were verified with multiple independent stable cell lines for each construct.

**Experimental Procedures**

**Plasmids**—Mammalian expression plasmid encoding Goα was obtained from ATCC. Expression plasmid encoding RGS4 was obtained from the Guthrie cDNA Resource Center. Expression plasmid encoding Crm-1 was from Dr. M. Yoshida (33).

**Materials**—Ang II was purchased from Peninsula. Anti-v-Src monoclonal antibody was from Calbiochem. Rabbit anti-ERK1/2 polyclonal antibody was from Zymed Laboratories Inc., and rabbit anti-active ERK1/2 polyclonal antibody was from Promega. Anti-phospho-Erk1/2 monoclonal antibody, anti-phospho-p44/42 MAP kinase E10 monoclonal antibody, horseradish peroxidase-conjugated anti-rabbit IgG, and anti-mouse F(ab′)2 fragments were from Cell Signaling Technology. Dowex AG1-X8 formate resin was from Bio-Rad. *3-Iodotyrosyl* 

**AT1a Receptor Constructs**—The full-length rat AT1a receptor cDNA subcloned into pcDNA mammalian expression vector was obtained from Dr. J. Harrison. In addition, two kinds of AT1a receptor mutants were used. One mutant has a series of point mutations in the conserved DRYXXVIXLXX sequence at the amino-terminal portion of the second intracellular (2) loop, where Asp126Val574, Tyr127Cys71, and Met7124 were converted to Gly126, Gly127, Ala127, and Ala127, respectively (designated as AT1-2m) (32). In another mutant, Asp93 was replaced with Asn (designated as AT1-D74N) (34). Combination of a carboxy-terminally truncated AT1a receptor, AT1a(1–309) was generated by PCR. Generation of these mutants has been described previously (35).

**Stable Transfection**—The plasmid encoding either wild type AT1a (pcDNA-AT1a-WT), AT1a-12m (pcDNA-AT1a-12m), AT1a-D74N (pcDNA-AT1a-D74N), or the control plasmid (pcDNA) was transfected into CHO-K1 cells by electroporation with series of freeze/thaw and centrifuged at 21,000 g for 1 h at 4 °C. The pellet was washed twice with ice-cold phosphate-buffered saline, and then solubilized with 1 ml of 5% trichloroacetic acid. Nonspecific binding (less than 5% of total binding) was determined by adding 1 μl unlabelled Ang II. The Bmax and the dissociation constant (Kd) for [3-Iodotyrosyl]* *were determined. Suspended membranes were incubated with 0.1 nM 125I-Ang II (36). AT1a-expressing CHO-K1 cells were grown in Ham’s F-12 medium containing 10% fetal bovine serum in 24-well plates. After 24 h of serum starvation, each well was aspirated and rinsed with assay buffer (OPTI-MEM (Life Technologies, Inc.) medium with 0.1% fetal bovine albumin). Assay buffer containing 30 μM [3-Iodotyrosyl]* *was added to cells and incubated for a volume of 1 ml for 1 h at 37 °C. The cells were immediately placed on ice, washed three times with ice-cold phosphate-buffered saline, and then solubilized with 1 ml of 5% trichloroacetic acid. Nonspecific binding (less than 5% of total binding) was determined by adding 1 μl unlabelled Ang II. The Bmax and the dissociation constant (Kd) for [3-Iodotyrosyl]* *were determined by using Prism 3.0 (GraphPad). Protein assay was performed on each sample using the Bio-Rad protein assay kit.

**Effect of GTP**S on Ang II Binding**—**Cells were plated in 10-cm culture dishes and grown in Ham’s F-12 medium containing 10% fetal bovine serum. After an overnight incubation, cells were washed three times with phosphate-buffered saline, scraped in 5 ml Tris buffer (pH 7.5), containing 0.01% soybean trypsin inhibitor, and 0.5 mM AEBSF. After 10 min of incubation on ice, the cells were subjected to two cycles of 10,000 × g for 10 min at 4 °C. Pellets were washed and centrifuged once, and the radioactivity was counted.

**Phosphoinositide Production**—Measurement of inositol phosphates (IPx) was based upon the method of Berridge et al. (37), as described by Thompson et al. (38). Cells were incubated with [3H]myoinositol (10 μCi/ml) in F-12 for 24 h at 37 °C. Labeling was terminated by aspirating medium, washing cells with 3 ml of ice-cold phosphate-buffered saline, adding 8 ml of 0.5 M NaCl, 30 mM Hepes buffer, pH 7.4, 0.5 mM KCl, 3.6 mM NaHCO3, 2.2 mM CaCl2, 1.0 mM MgCl2, and 1 mg/ml aprotinin, and harvesting cells with phosphate-buffered saline, 0.02% EDTA. Cells were centrifuged twice (300 × g, 5 min) in reaction buffer, and the pellet was resuspended in an equal volume of reaction buffer containing 60 mM LiCl. The 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 incubation). The mixture was incubated for 30 min at 23 °C, and then 0.5 ml of ice-cold 20% trichloroacetic acid was added. Precipitates were pelleted (4000 × g, 20 min), and the trichloroacetic acid-insoluble fraction was transferred to new tubes, washed with water-saturated diethyl ether, and neutralized with NaHCO3. IPx were isolated by adsorption to 0.5 ml of Dowex AG1-X8 formate resin slurry and rinsed five times with 3 ml of unlabeled myoinositol, followed by elution with 1 ml of 1.2 M HCl in formate buffer (pH 3.0). IPx was identified by liquid scintillation counter in 5 ml of Scinti-verse. To normalize released IPx for lipid content, the trichloroacetic acid-insoluble fraction of each sample was processed for lipid analysis. After resuspending the pellet in 0.5 ml of H2O and 1.5 ml of chloroform/methanol (1:2), a 200-μl aliquot of the organic phase was counted by liquid scintillation spectrophotometry in 5 ml of Scinti-verse.

**Src Kinase Assay**—The tyrosine kinase activity of Src was determined by the immune complex kinase assay using enolase as a substrate as described previously (39). Cell lysates were prepared in lysis buffer (150 mM NaCl, 15 mM HEPES, pH 7.0, 1% deoxycholic acid, 1% IGEPAL, 0.1% SDS, 0.1 mM Na2VO4, 1 mM NaF, 0.5 mM AEBSF, 0.5 mM μg/mL aprotinin, 0.5 μg/mL leupeptin). The cell lysates containing equal amount of protein (750 μg) were incubated with anti-v-Src monoclonal antibody (30 μg of 12C1) overnight at 4 °C. The protein-G-Sepharose was then added. The immunoprecipitates 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% Brij 35). Pellets were incubated for 15 min at 37 °C in the kinase buffer in the presence of 1 μCi of [γ-32P]ATP and 0.25 μg of enolase as substrates. The reaction was terminated by the addition of Laemmli sample buffer on ice. Reaction products were resolved by electrophoresis at 12% SDS-PAGE, and proteins were followed by autoradiography. Results were analyzed by densitometry.

**Dominant Negative Ras Adenovirus Vector**—Adenovirus-mediated transduction was performed as described previously (39). Cells grown in 60-mm dishes were transduced with an adenovirus vector harboring dominant-negative Ras (Ad5.N17Ras) (courtesy of Dr. M. D. Schneider, Baylor College of Medicine, Houston, TX) at a multiplicity of infection of 100. For a control study, Ad5/E141sp1B (courtesy of Dr. B. French, University of Texas Southwestern Medical Center, Dallas, TX) was used.
Baylor College of Medicine) was used. All experiments were performed 48 h after transduction.

**Immunoblotting**—To determine the phosphorylation of ERKs, cells were scraped in a hypotonic lysis buffer (25 mM NaCl, 25 mM Tris, pH 7.5, 0.5 mM EGTA, 10 mM sodium pyrophosphate, 1 mM Na3VO4, 10 mM NaF, 0.5 mM AEBSF, 0.5 mM leupeptin) and incubated on ice for 30 min. After centrifugation, supernatants were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad). Immunoblot analyses were performed using anti-phosphotyrosine antibody (RC20H; Transduction Laboratories), anti-active mitogen-activated protein kinase polyclonal antibody (Promega), or anti-phosphospecific p80(GS) polyclonal antibody (Cell Signaling Technology). The membranes were stripped and reprobed with antibodies against ERKs (Zymed Laboratories Inc.) to determine the total amount of ERKs.

**Transient Transfection and Reporter Gene Assay**—Cells were plated in 24-well plates. Transfections were carried out using 1 μl/ml of LipofectAMINE (Invitrogen) with 0.4 μg of DNA in 250 μl/well OPTI-MEM1 medium (Invitrogen). To determine whether Elk1 is activated by a given stimulus, the PathDetect™ in vivo signal transduction pathway trans-reporting system (Stratagene) was used, in which a plasmid (0.15 μg/ml) consisting of the activation domain of Elk1 fused with the DNA binding domain of the yeast GAL4, was co-transfected. Total amounts of DNA were adjusted to 0.4 μg/ml by adding an empty vector. 12 h after the transfection, the culture medium was changed to serum-free. Cells were further cultured in the presence or absence of Ang II (10−9 M) for an additional 12 h. Cells were then lysed with the Lysis buffer (Promega), and luciferase activities were measured. An SV40 promoter-driven β-galactosidase construct (SV40 β-gal; 0.01 μg/ml) was co-transfected, and the β-galactosidase activity was determined by using Lumi-Gal 530 (Lumigen). The luciferase values were divided by the β-galactosidase values to correct for differences in the transfection efficiency.

**Immunostaining**—Cells were grown on glass coverslips. After stimulation, cells were fixed with methanol at −20 °C for 10 min followed by a brief dip in acetone at −20 °C. For staining of phospho-Elk-1 and phospho-ERK, anti-phospho-Elk-1 antibody (Cell Signaling Technology; 1 μg/ml) and phospho-p44/42 MAP kinase E10 monoclonal antibody (Cell Signaling Technology; 1 μg/ml) were used, respectively. FITC-conjugated anti-mouse IgG antibody (Jackson Immunoresearch) was used as secondary antibody at 1:200 dilution in phosphate-buffered saline. Microscopic analyses were performed by using confocal microscopy. Detailed methods of immunostaining have been described (39).

**Preparation of Nuclear Extracts**—Nuclear proteins were extracted as described (40). Cells were washed and scraped with Tris-buffered saline. After centrifugation, cells were resuspended in 200 μl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM Na3VO4, 0.5 mM AEBSF) by gentle pipetting and allowed to swell on ice for 15 min. Cells were lysed by adding 12.5 μl of 10% Nonidet P-40 and vortexing vigorously for 10 s, followed by centrifugation (12,000 rpm) for 30 s. Supernatant was saved as the cytosolic fraction. Pellets were resuspended in 50 μl of ice-cold buffer B (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.1 mM Na3VO4, 0.5 mM AEBSF) and rocked for 15 min. The samples were centrifuged at 14,000 rpm for 5 min. The supernatant was saved as the nuclear fraction. The samples were stored at −80 °C.

**Cell Proliferation Experiments**—Cells were plated at a density of 0.3 × 105 per well in six-well plates. 12 h after plating, cells were serum-starved for 12 h and incubated with Ang II (10−8 M) for 36 h. Ang II was added every 12 h. After stimulation, cells were washed twice with phosphate-buffered saline and treated with 0.1 ml of 0.05% trypsin for 5 min, followed by the addition of 0.4 ml of Dulbecco’s modified Eagle’s medium to neutralize the trypsin. The cells were suspended thoroughly by pipetting. Cell numbers were counted using a hemocytometer.

**Statistics**—Data are given as 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 the p < 0.05 level.

**RESULTS**

**Stable Transfection of CHO-K1 Cells with Wild Type AT1a Receptor and AT1a-i2m**—To examine the role of the heterotrimeric G proteins in tyrosine kinase activation by AT1a receptors, we used AT1a-i2m and AT1a-D74N, which have been shown to lack or have impaired coupling with heterotrimeric G proteins in COS7 cells (32, 34, 35). We stably transfected either the wild type AT1a receptor (AT1a-WT), AT1a-i2m, or AT1a-D74N into CHO-K1 cells, which have no endogenous Ang II receptors. We isolated 20–25 clones with various levels of AT1a receptor expression for each receptor. We determined Kd and Bmax in each clone using 125I-Ang II. Cell lines expressing each receptor with similar Kd (1.78 ± 0.58, 1.82 ± 0.32, 0.77 ± 0.32 nM for AT1a-WT, AT1a-i2m, and AT1a-D74N, respectively) and Bmax (64.5 ± 1.7, 61.7 ± 2.1, and 73.3 ± 5.4 fmol/mg for AT1a-WT, AT1a-i2m, and AT1a-D74N, respectively) were used for the further experiments (cell line WT-#25, i2m-#12, and D74N-#7, respectively). We designated these cell lines as CHO-WT, CHO-i2m, and CHO-D74N. The results presented in this study were reproducible in other cell lines expressing either AT1a-WT, AT1a-i2m, or AT1a-D74N with similar expression levels (cell line WT-#20, i2m-#7, and D74N-#9, respectively).

**GTP·S Does Not Affect Ang II Binding to AT1a-i2m**—Receptor interaction with heterotrimeric G proteins was evaluated by measuring 125I-Ang II binding to the receptors in the presence or the absence of the nonhydrolyzable GTP analogue GTP·S. GTP·S shifts the G protein-coupled receptor from a high affinity state to a low affinity state. Since this shift is considered as an indicator for effective interaction of the receptor with heterotrimeric G proteins (41), we examined the effect of GTP·S on 125I-Ang II binding to AT1a-WT, AT1a-i2m, and AT1a-D74N. As shown in Fig. 1, GTP·S decreased Ang II binding to the AT1a-WT and AT1a-D74N in a dose-dependent manner. By contrast, Ang II binding to AT1a-i2m was not affected by GTP·S even at 10−4 M, suggesting that AT1a-i2m lacks heterotrimeric G protein coupling in CHO-K1 cells. Thus, we further characterized the signaling mechanism of AT1-i2m in comparison with AT1a-WT.

**Functional Goα and Gqα Coupling Is Missing in CHO-i2m**—Binding of Ang II to the AT1a receptor activates Goα protein, followed by activation of PLC and release of inositol triphosphates (IP3). To confirm that AT1a-i2m lacks functional coupling with the Goα PLC pathway, we examined accumulation of the total IPx by Ang II in CHO-WT and CHO-i2m. Ang II did not induce IPx accumulation in untransfected CHO-K1 cells, while Ang II dose-dependently increased intracellular accumulation of IPx in CHO-WT. On the other hand, no detectable IPx accumulation was observed in CHO-i2m even at 10−6 M Ang II (Fig. 2A).

To further confirm that AT1a-i2m lacks functional coupling...
with the Goq,PLC pathway, we examined the effect of Goq overexpression on Ang II-induced IPx accumulation. We used transient co-transfection to induce co-expression of AT1a receptors and Goq, in each cell transfected. Transfection of control plasmid alone did not induce IPx accumulation by Ang II (data not shown). Transient expression of AT1a-WT induced significant IPx accumulation by Ang II, while co-transfection with AT1a-WT and Goq produced an even greater increase (Fig. 2B). By contrast, transient expression of AT1a-i2m failed to induce Ang II-induced accumulation of IPx, and co-transfection with AT1a-i2m and Goq failed to increase it (Fig. 2B). The result confirms that the lack of Goq,PLC coupling in AT1a-i2m is not due to the shortage of Goq, and that AT1-i2m lacks functional Goq,PLC coupling in CHO-K1 cells.

Ligand binding to the AT1a receptor activates Goq protein, which negatively regulates adenylyl cyclase. In CHO-WT, Ang II stimulation reduced forskolin-induced cAMP accumulation to 72% of the control values obtained in the absence of Ang II. Treatment of cells with pertussis toxin (PTX, 250 ng/ml for 24 h) abolished this reduction, indicating that the AT1a-WT inhibits adenylyl cyclase activity via Goq (Fig. 2C). In CHO-i2m, by contrast, Ang II failed to affect forskolin-induced cAMP accumulation, and PTX treatment did not affect it (Fig. 2C).

Ang II also did not affect forskolin-induced cAMP production in CHO-K1 cells transiently transfected with AT1a-i2m and Goq (data not shown). These results suggest that AT1a-i2m lacks coupling with Goq, in CHO-K1 cells.

Ang II Activates Src in CHO-i2m—Ligand binding to the AT1 receptor activates tyrosine kinases, including Src family tyrosine kinases (5, 6, 42-44), which in turn mediate important downstream cellular responses. To examine if Src is activated by Ang II independently of G protein coupling in CHO-K1 cells, we performed immune complex Src kinase assays using enolase as a substrate. Src-dependent phosphorylation of enolase is shown.

To further confirm that Ang II-induced Src activation is Goq,PLC-independent in CHO-i2m, we examined the role of PKC and Ca2+ as important mediators of the Goq,PLC pathway, in Ang II-induced Src activation. GF109203X, a specific PKC inhibitor, failed to affect Ang II-induced Src activation in CHO-WT and CHO-i2m, indicating that Ang II can activate Src even in the absence of PKC in CHO-K1 cells (Fig. 4A). BAPTA-AM, a chelator of Ca2+, also failed to affect Ang II-induced Src
activation in CHO-WT and CHO-i2m (Fig. 4B). We confirmed that GF109203X and BAPTA-AM effectively blocked PKC and increases in the intracellular Ca\(^{2+}\) levels, respectively, in CHO-K1 cells by using the Path Detect reporter gene assay system (data not shown) (see also Fig. 7B and Ref. 43). These results suggest that both PKC and Ca\(^{2+}\) are dispensable for Ang II-induced Src activation in CHO-WT and CHO-i2m.

Ang II activates Src through \(G_{\alpha}\) protein in some cell types (45). To determine the role of \(G_{\alpha}\) in Ang II-induced Src activation in CHO-i2m, we used PTX, which uncouples receptors from \(G_{\alpha}/G_{\alpha}\) (46). Since PTX blocked Ang II-induced decreases in forskolin-induced cAMP accumulation in CHO-WT (Fig. 2C), our PTX treatment effectively uncouples AT1a receptors from \(G_{\alpha}\). The PTX treatment, however, failed to inhibit Src activation by Ang II in both CHO-WT and CHO-i2m, suggesting that Src is activated independently of \(G_{\alpha}\) (Fig. 4C). These results are consistent with a notion that Src is activated by Ang II independently of heterotrimeric G protein coupling in CHO-K1 cells.

Co-expression of RGS4 Does Not Affect Ang II-induced Src Activation in CHO-i2m—To further evaluate the involvement of the heterotrimeric G proteins in Ang II-induced activation of Src, we examined the effect of RGS4, which is known to inhibit the cellular responses mediated by \(G_{\alpha}\) and \(G_{\alpha}\) (47). Co-expression with RGS4 and AT1a-WT significantly inhibited Ang II-induced IP\(_x\) accumulation in CHO-K1 cells, suggesting that RGS4 significantly inhibits \(G_{\alpha}\)-mediated responses in our experimental conditions (Fig. 5A). As expected, expression of RGS4 did not affect Ang II-induced IP\(_x\) accumulation in CHO-i2m. Interestingly, RGS4 failed to inhibit Ang II-induced activation of Src when it is co-expressed with either AT1a-WT or AT1a-i2m (Fig. 5B). These results suggest that neither \(G_{\alpha}\) nor \(G_{\alpha}\) coupling plays an essential role in ligand-dependent activation of Src in AT1a-WT and AT1a-i2m.

Ang II-induced Src Activation Was Abolished in AT1a-(1–309)—It has been shown that the carboxyl terminus of the AT1 receptor directly interacts with several signaling molecules (24, 48–51). To examine the mechanism of heterotrimeric G protein-independent activation of Src by the AT1 receptor, we examined if the carboxyl terminus of AT1 receptor mediates Ang II-induced Src activation. Transient expression of a carboxyl terminus truncation mutant AT1a-(1–309) showed equal levels of cell surface Ang II binding sites as AT1a-WT in CHO-K1 cells (data not shown). Ang II caused comparable levels of increases in IP\(_x\) production in CHO-K1 cells transfected with AT1a-(1–309) and those with AT1a-WT, suggesting that AT1a-(1–309) is able to activate heterotrimeric G protein-dependent cell signaling (Fig. 6A). By contrast, Ang II failed to activate Src in CHO-K1 cells transfected with AT1a-(1–309), suggesting that the carboxyl terminus of the AT1 receptor plays an important role in Ang II-induced Src activation (Fig. 6B).

Ang II Activates ERK1/2 in CHO-i2m—We next examined if ERKs are activated by Ang II in CHO-i2m and, if so, whether or not Ang II-induced Src activation is involved in subsequent activation of ERK1/2 in CHO-i2m. Ang II activated ERK1 and ERK2 in both CHO-WT and CHO-i2m, suggesting that ERKs are activated even in the absence of heterotrimeric G protein coupling in CHO-K1 cells (Fig. 7A). To determine the role of Src in Ang II-induced ERK activation in CHO-WT and CHO-i2m, we used PP1, a specific inhibitor of the Src family tyrosine kinases (52). Although PP1 partially inhibited Ang II-induced ERK activation in CHO-WT, PP1 pretreatment completely inhibited Ang II-induced ERK activation in CHO-i2m (Fig. 7B). To determine whether Ras is involved in ERK activation by Ang II in CHO-i2m, we used adenovirus-mediated transduction of dominant negative Ras. Although activation of ERKs by Ang II was not inhibited by dominant negative Ras in CHO-WT, dominant negative Ras completely abolished Ang II-induced ERK activation in CHO-i2m (Fig. 7C). These results suggest that Ang II-induced ERK activation in CHO-i2m is mediated by...
A were transiently transfected with expression vector encoding either AT1a-WT or AT1a (1–309). A, cells were then labeled with [3H]myo-inositol and incubated with Ang II (100 nM) for 30 min. n = 3. Data are expressed by -fold increases in IPx production compared with basal values obtained in AT1a-WT transfected CHO-K1 cells in the absence of Ang II. B, the cells were stimulated with Ang II (100 nM) for 30 s. The activity of Src was determined by the immune complex kinase assay. Src-dependent phosphorylation of enolase is shown. n = 3.

Fig. 6. Ang II fails to activate Src in AT1a-(1–309). CHO-K1 cells were transiently transfected with expression vector encoding either AT1a-WT or AT1a (1–309). A, cells were then labeled with [3H]myo-inositol and incubated with Ang II (100 nM) for 30 min. n = 3. Data are expressed by -fold increases in IPx production compared with basal values obtained in AT1a-WT transfected CHO-K1 cells in the absence of Ang II. B, the cells were stimulated with Ang II (100 nM) for 30 s. The activity of Src was determined by the immune complex kinase assay. Src-dependent phosphorylation of enolase is shown. n = 3.

FIG. 6. Ang II Fails to Stimulate Elk1 in CHO-i2m—ERKs phosphorylate nuclear transcription factors, such as Elk1, which in turn mediate cell growth responses in many cell types. We therefore examined if Ang II-induced activation of ERKs leads to activation of Elk1 in CHO-K1 cells. In this experiment, Elk1-GAL4 activates the co-transfected reporter gene (pFR-Luc) when Ser383 in the activation domain of Elk1 is phosphorylated. In CHO-WT, Ang II significantly activated Elk1-GAL4 (Fig. 8A). Ang II-induced Elk1-GAL4 activation in CHO-WT is mediated by ERKs, since co-transfection with dominant negative MEK1 abolished Ang II-induced Elk1-GAL4 activation (data not shown). Interestingly, Ang II failed to activate Elk1-GAL4 in CHO-i2m despite the fact that ERKs are activated in CHO-i2m (Fig. 8A). By contrast, PMA significantly increased nuclear staining of phospho-Elk1 antibody. Ang II significantly increased nuclear staining of phospho-Elk1 in CHO-K1 cells. In this experiment, phospho-Elk1 staining was determined by immunostaining with anti-phospho-Elk1 antibody. Ang II significantly increased nuclear staining of phospho-Elk1 in CHO-K1 cells. The status of Elk1 Ser383 phosphorylation was examined by immunostaining in CHO-i2m cells expressing Elk1-GAL4 in CHO-i2m (Fig. 8C). These results suggest that Ang II-induced activation of ERK is not sufficient for phosphorylation and activation of the nuclear transcription factor Elk1 in CHO-i2m.

To examine the role of Src and PKC in Ang II-induced Elk1 activation in CHO-WT, we examined the effect of PP1 and GF109203X on Ang II-induced Elk1-GAL4 activation. Although PP1 treatment failed to block Ang II-induced Elk1 activation, GF109203X completely blocked it (Fig. 8B). Since Ang II-induced activation of ERKs was preserved in the presence of GF109203X in CHO-WT (Fig. 6D), these results suggest that, while activation of ERKs alone is not sufficient, activation of PKC is required for Ang II-induced Elk1 activation in CHO-K1 cells.

The status of Elk1 Ser383 phosphorylation was examined by immunostaining with anti-phospho-Elk1 antibody. Ang II significantly increased nuclear staining of phospho-Elk1 in CHO-WT but not in CHO-i2m (Fig. 8C). These results suggest that Ang II-induced activation of ERK is not sufficient for phosphorylation and activation of the nuclear transcription factor Elk1 in CHO-i2m.

Nuclear Translocation of ERKs Is Missing in CHO-i2m—To elucidate the mechanism by which Ang II fails to stimulate Elk-1 in CHO-i2m despite the fact that Ang II stimulates ERKs, we examined the subcellular localization of activated forms of ERKs by immunostaining with anti-phosphospecific ERK antibody. To show equal loading, membranes were stripped and reprobed with antibody against ERKs. A, CHO-WT and CHO-i2m were stimulated with indicated concentrations of Ang II for 5 min. n = 5. B, CHO-WT or CHO-i2m were pretreated with or without PP1 (10 nM) for 30 min, and then stimulated with Ang II (100 nM) for 5 min. n = 3. C, CHO-WT and CHO-i2m were transduced with control adenovirus or adenovirus harboring dominant negative Ras (DN-Ras), at 100 multiplicity of infection. 48 h after transduction, cells were stimulated with Ang II (100 nM) for 5 min. n = 3. D, CHO-WT transduced with dominant negative Ras and/or pretreated with GF109203X (10 nM) were stimulated with Ang II (100 nM) for 5 min. n = 3.

Fig. 7. Ang II activates ERKs in both CHO-WT and CHO-i2m. Activation of ERKs was evaluated by immunoblot analyses using anti-phosphospecific ERK antibody. To show equal loading, membranes were stripped and reprobed with antibody against ERKs. A, CHO-WT and CHO-i2m were stimulated with indicated concentrations of Ang II for 5 min. n = 5. B, CHO-WT or CHO-i2m were pretreated with or without PP1 (10 nM) for 30 min, and then stimulated with Ang II (100 nM) for 5 min. n = 3. C, CHO-WT and CHO-i2m were transduced with control adenovirus or adenovirus harboring dominant negative Ras (DN-Ras), at 100 multiplicity of infection. 48 h after transduction, cells were stimulated with Ang II (100 nM) for 5 min. n = 3. D, CHO-WT transduced with dominant negative Ras and/or pretreated with GF109203X (10 nM) were stimulated with Ang II (100 nM) for 5 min. n = 3.

GF109203X on Ang II-induced Elk1-GAL4 activation. Although PP1 treatment failed to block Ang II-induced Elk1 activation, GF109203X completely blocked it (Fig. 8B). Since Ang II-induced activation of ERKs was preserved in the presence of GF109203X in CHO-WT (Fig. 6D), these results suggest that, while activation of ERKs alone is not sufficient, activation of PKC is required for Ang II-induced Elk1 activation in CHO-WT but not in CHO-i2m (Fig. 8C). These results suggest that Ang II-induced activation of ERK is not sufficient for phosphorylation and activation of the nuclear transcription factor Elk1 in CHO-i2m.
examined by using anti-phospho-p90RSK-specific antibody. Ang II significantly increased p90RSK phosphorylation in both CHO-WT and CHO-i2m, consistent with our hypothesis (Fig. 9C).

Ang II-induced Nuclear Translocation of ERKs Is Regulated by Nuclear Exportin Crm-1—Recent evidence suggests that nuclear localization of ERKs is regulated in part by a nuclear exportin Crm-1 and its interaction with PEA-15 nuclear exportin complex (53, 54). To examine the effect of the nuclear exportin on nuclear translocation of ERK by Ang II, we transiently co-expressed AT1a-WT and Crm-1 in CHO-K1 cells. Overexpression of Crm-1 inhibited Ang II-induced Elk-1 activation and nuclear localization of phospho-ERKs in AT1a-WT, suggesting that nuclear translocation of ERKs is negatively regulated by nuclear exportin Crm-1 (Fig. 10, A and B). To test if endogenous Crm-1 is involved in nuclear translocation of ERKs by Ang II, we examined the effect of leptomycin B, a specific inhibitor of Crm-1 (33) in CHO-WT. Leptomycin B reversed the inhibition of Ang II-induced ERK translocation by the PKC inhibitor in CHO-WT (Fig. 10C, see also Fig. 8B). These results suggest that Crm-1 plays an important role in regulation of nuclear localization of ERKs by Ang II.

Furthermore, inhibition of Crm-1, possibly by PKC-dependent mechanisms, may be required for Ang II-induced nuclear translocation of ERKs. To test if inhibition of Crm-1 allows ERKs, which are activated by heterotrimeric G protein-independent mechanism, to go into the nucleus, we tested the effect of leptomycin B upon nuclear translocation of ERKs in CHO-i2m. Leptomycin B treatment caused significant increases in nuclear phospho-ERKs as well as phospho-Elk-1 by Ang II in CHO-i2m (Fig. 10D; see also Figs. 8C and 9A). These results suggest that the lack of Crm-1 inactivation may at least in part cause the failure of Ang II-induced nuclear translocation of ERKs in CHO-i2m.

Ang II Fails to Stimulate Cell Proliferation in CHO-i2m—We finally examined if Gox-q, PLC independent coupling of AT1a-i2m with Src and ERKs is sufficient to mediate Ang II-induced cell proliferation. CHO-K1 cells were serum-starved for 12 h and then incubated with Ang II for 36 h. Although Ang II failed to increase the cell number in parental CHO-K1 cells, which do not express AT1 receptors, Ang II treatment significantly increased the cell number in CHO-WT. This suggests that wild type AT1 receptors mediate cell proliferation in CHO-K1 cells. The Ang II-induced increases in cell number were attenuated in the presence of a specific inhibitor of MEK1, PD98059, suggesting that ERKs play an essential role in Ang II-induced cell proliferation in CHO-i2m, consistent with previous studies (55). By contrast, Ang II-induced cell proliferation was significantly attenuated in CHO-i2m, suggesting that activation of the Src-ERK pathway is not sufficient to mediate Ang II-induced cell proliferation in CHO-i2m (Fig. 11).
Most of the previously described functions of GPCRs are mediated by heterotrimeric G proteins. Recent evidence suggests, however, that GPCRs physically interact with intracellular signaling molecules, which potentially initiate heterotrimeric G protein-independent signaling (51) (reviewed in Refs. 4, 31, 56, and 57). Many lines of evidence indicate that GPCRs are able to initiate cross-talk with tyrosine kinases. However, whether or not GPCRs can activate tyrosine kinases even without heterotrimeric G protein-dependent mechanisms has not been clearly demonstrated in mammalian cells. Furthermore, even if such a mechanism exists, the functional significance of such mechanism remains unknown. In this study, we have addressed these issues by using AT1a-i2m, which lacks heterotrimeric G protein coupling (32). Our results indicate that AT1a-i2m is able to activate Src in the absence of the heterotrimeric G protein coupling in CHO-K1 cells. Interestingly, however, heterotrimeric G protein-independent activation of the Src-Ras-ERK pathway is not sufficient to induce nuclear translocation of ERKs, and the activated ERKs phosphorylate only their cytoplasmic targets (Fig. 12). These results suggest that heterotrimeric G protein-dependent and -independent signaling mechanisms play distinct roles in Ang II-mediated cellular responses.

i2m Lacks Functional Coupling with Heterotrimeric G Proteins in CHO-K1 Cells—In this study, we confirmed that the AT1a-i2m lacks functional coupling with heterotrimeric G proteins in CHO-K1 cells. Most importantly, we tested the effect of GTPγS on the high affinity receptor binding to the ligand, since reduction in GPCR binding to the ligand in the presence of GTPγS is attributable to the dissociation of Go and Gβγ subunits from the receptor and reflects its functional coupling with heterotrimeric G proteins (58). The absence of the effect of GTPγS on ligand binding to AT1a-i2m indicates that no G protein (e.g. Goq or Gai) is able to form a high affinity ternary complex with this mutant. Although several AT1a receptor mutants lacking Goq-PLC coupling have been reported (32, 34, 59), AT1a-i2m is unique among the AT1a receptor mutants for the fact that its ligand binding is not affected by GTPγS (Fig. 1). Thus, AT1a-i2m can be a very useful tool to determine the role of the heterotrimeric G protein coupling in the AT1a receptor-mediated cell signaling.

Ang II-induced Src Activation Occurs Independently of Heterotrimeric G Protein Coupling—Previous studies have suggested that Src is activated by stimulation of the GPCRs through multiple mechanisms. For example, Ca2+ and PKC-dependent activation of Pyk2 mediates Src activation by GPCRs (60, 61). Src activation by lysophosphatidic acid, α2-adrenergic, m2 muscarinic, or β2-adrenergic receptors is mediated by Gβγ-dependent mechanisms and subsequent recruitment of the β-arrestin-Src complex to the proximity of the receptor (22, 62). Src is directly activated by interaction with
Heterotrimeric G Protein-independent Activation of Src

Fig. 12. The mechanism of ERK activation and its subcellular localization in CHO-WT and CHO-i2m. In CHO-K1 cells expressing the wild type AT1a receptor (WT), ligand binding causes activation of ERKs through two distinct signaling pathways. One is via the Goq-PKC-dependent mechanism, and the other is via the Src-Ras-dependent mechanism. Although ERKs activated through the Goq-PKC pathway are translocated into the nucleus, ERKs activated through the Src-Ras pathway alone are not translocated into the nucleus due to interaction with the nuclear exportin, Crm-1. ERKs activated through the Src-Ras pathway may be translocated into the nucleus in the presence of the signal from the Goq pathway, which negatively regulates Crm-1, possibly through PEA-15. In CHO-K1 cells expressing AT1a-i2m (i2m), ligand binding activates ERKs via the Src-Ras-dependent mechanism alone in a heterotrimeric G protein-independent manner. In this case, ERKs stay in the cytoplasm through interaction with Crm-1 and fail to phosphorylate their nuclear targets.

GTP-bound forms of Goq and Goa (20). These results are all consistent with the notion that coupling with (and/or activation of) heterotrimeric G proteins is involved in activation of Src by GPCRs. Recently, Src has been shown to directly associate with both b-adrenergic receptors (25, 26). Even in this case, however, Src activation by b4-ARs is thought to be Goa-dependent (25). Thus, it has not been clearly shown that Src can be activated in the absence of activation of (coupling with) heterotrimeric G proteins.

In the present study, we have demonstrated that Ang II activates Src in CHO-K1 cells expressing AT1a-i2m, in which heterotrimeric G protein coupling is missing. The G protein-independent mechanism of Ang II-induced Src activation was also available in AT1a-WT (Fig. 5). This suggests that G protein-independent activation of Src is not limited to the property of the mutant. In Dictostyllum, cytosolic phosphorylation of STAT is increased by binding of the extracellular ligand cAMP to the cAR1, a seven-membrane spanning GPCR, even in the mutant where Gβγ, the only heterotrimeric G protein subunit in Dictostyllum, has been deleted (30) (reviewed in Ref. 63). Our results suggest that such a heterotrimeric G protein-independent mechanism of tyrosine kinase activation is evolutionally conserved.

What is the mechanism of heterotrimeric G protein-independent activation of Src? One mechanism may be that the AT1a receptor activates Src through direct interaction with intracellular signaling molecules. Such mechanisms have been proposed for activation of signaling molecules by GPCRs (56, 57). For the AT1a receptor, Jak2, PTP1D, PLCγ1, a novel 18-kDa protein, and RhoA have been shown to physically associate with the carboxyl terminus of the AT1a receptor (24, 48–51). Here, we have provided evidence that the carboxyl terminus of the AT1a receptor is required for Ang II-induced Src activation. Whether or not the molecules, which interact with the carboxyl terminus, regulate the activity of Src remains to be demonstrated. Recently, GPCRs, including the AT1a receptors, have been shown to homo- or hetero-oligomerize in a ligand-dependent manner (56, 64–68). Ligand-dependent oligomerization of GPCRs may induce interaction of intracellular signaling molecules, thereby changing their activities like in cytokine receptors. Further investigation will be required to elucidate the mechanism by which Ang II activates Src in CHO-i2m.

Nuclear Translocation of ERKs, Subsequent Activation of Elk1, and Cell Proliferation Are Missing in CHO-i2m—Another important finding in the present study is that although the AT1a receptor is able to activate ERKs even without heterotrimeric G protein coupling, heterotrimeric G protein-independent activation of ERKs alone is not sufficient for Ang II-induced cell proliferation. Since activation of ERKs is essential for Ang II-induced cell proliferation in CHO-WT (Fig. 9), the failure of cell proliferation by heterotrimeric G protein-independent activation of ERKs in CHO-i2m may be explained by the signaling mechanism downstream of ERKs. Our results indicate that nuclear translocation of activated ERKs is missing in CHO-i2m. Nuclear translocation of ERKs is essential for several biological phenomena (69, 70). In CHO-i2m, ERKs activated by Ang II were able to phosphorylate p90RSK in the cytoplasm, while they failed to phosphorylate Elk-1 in the nucleus. Thus, although Ang II activates ERKs in CHO-i2m, activated ERKs are retained in the cytoplasm and fail to activate nuclear transcription factors, including Elk1, which may play an essential role in cell proliferation.

Nuclear translocation of ERKs is mediated by phosphorylation and subsequent dimerization of ERKs (71, 72). Although dimerization itself does not affect kinase activities of ERKs, dimerization is required for nuclear translocation (71, 72). Since nuclear translocation of ERKs is inhibited by the PKC inhibitor in CHO-WT, despite the fact that ERK activation is preserved (Figs. 6D and 7B), activation of the Goq-PLC pathway and its downstream signaling mechanisms, including activation of PKC, may be required for nuclear translocation of ERKs by GPCRs. It has been recently shown that nuclear localization of ERKs is a part regulated by a nuclear export mechanism (53, 54, 72). Consistent with this observation, overexpression of a nuclear exportin Crm-1 inhibited both Elk-1 activation and nuclear translocation of phospho-ERK caused by stimulation of AT1a-WT with Ang II. Interestingly, a specific inhibitor of Crm-1, leptomycin B (33), restored Ang II-induced nuclear translocation of ERKs in both AT1a-WT in the presence of the PKC inhibitor and AT1a-i2m, suggesting that Crm-1 mediated nuclear export may play an important role in regulating subcellular localization of ERKs. A heterotrimeric G protein-dependent mechanism, perhaps PKC, may negatively regulate Crm-1-mediated nuclear export, thereby causing nuclear accumulation of ERKs. Consistent with this hypothesis, it has been recently shown that PEA15 negatively regulates nuclear localization of ERKs by stimulating nuclear export of ERKs in CHO-K1 cells (54). Since PEA15 is heavily phosphorylated by PKC (54), it will be interesting to determine if PKC-mediated phosphorylation of PEA15 regulates nuclear translocation of ERKs.

Alternatively, ERKs activated by a G protein-independent mechanism 1) may be compartmentalized (73, 2) may be trapped by cytoplasmic anchors, or 3) may not have an access to the machinery, which allows nuclear translocation (72). It has been recently shown that ERKs activated through an endocytosis machinery-dependent mechanism are localized at intracellular endosomal vesicles (73, 74). Our preliminary results suggest, however, that heterotrimeric G protein-independent activation of ERKs by AT1a-i2m was not affected by dominant negative arrestins (data not shown). Furthermore, phospho-ERKs were localized diffusely in the cytoplasm and did not show dramatic redistribution to the endosomal vesicle com-
partment (Fig. 8A) (74). Thus, if ERKs activated by heterotrimeric G protein-independent mechanism are compartmentalized, their specific subcellular distribution and their downstream targets remain to be elucidated.

In summary, we have provided evidence that the AT1 receptor is able to initiate cross-talk with Src tyrosine kinase independently of heterotrimeric G protein coupling. Although this heterotrimeric G protein-independent signaling mechanism runs in parallel with the heterotrimeric G protein-dependent one and both activate ERKs, the subcellular distribution of ERKs and their downstream targets may differ, depending upon the upstream signaling mechanisms.

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AT1 Receptor Mutant Lacking Heterotrimeric G Protein Coupling Activates the Src-Ras-ERK Pathway without Nuclear Translocation of ERKs
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