Jak2 Acts as Both a STAT1 Kinase and as a Molecular Bridge Linking STAT1 to the Angiotensin II AT1 Receptor

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Angiotensin II activates the Jak-STAT pathway via the AT1 receptor. We studied two mutant AT1 receptors, termed M5 and M6, that contain Y to F substitutions for the tyrosine residues naturally found in the third intracellular loop and the carboxyl terminus. After binding ligand, both the M5 and M6 AT1 receptors trigger STAT1 tyrosine phosphorylation equivalent to that observed with the wild type receptor, indicating that angiotensin II-mediated phosphorylation of STAT1 is independent of these receptor tyrosine residues. In response to angiotensin II, Jak2 autophosphorylates on tyrosine, and Jak2 and STAT1 physically associate, a process that depends on the SH2 domain of STAT1 in vitro. Evaluation of the wild type, M5, and M6 AT1 receptors showed that angiotensin II-dependent AT1 receptor-Jak2-STAT1 complex formation is dependent on catalytically active Jak2, not on the receptor tyrosine residues in the third intracellular loop and carboxyl tail. Immunodepletion of Jak2 virtually eliminated the ligand-dependent binding of STAT1 to the AT1 receptor. These data indicate that the association of STAT1 with the AT1 receptor is not strictly bimolecular; it requires Jak2 as both a STAT1 kinase and as a molecular bridge linking STAT1 to the AT1 receptor. 

Angiotensin II is a biologically active peptide that acts as both a hemodynamic regulator and as a growth factor. Growth factor properties of angiotensin II have been demonstrated in a variety of cell types. For example, in cultured vascular smooth muscle cells, angiotensin II induces hypertrophy, increases cell matrix formation and, under certain conditions, causes hyperplasia (1). These mitogenic effects have been linked to pathophysiological conditions such as hypertension, atherosclerosis, and restenosis (2). The cellular response to angiotensin II is directed by specific G protein-coupled receptors. There are two major classes of angiotensin II receptors that are now referred to as the AT1 and AT2 receptor subtypes (3). The use of subtype specific inhibitors has demonstrated that the hemodynamic and growth factor effects of angiotensin II are mediated almost exclusively, by the AT1 receptor (4).

AT1 receptors bear the structural features of the seven-transmembrane, G protein-coupled receptor superfamily and thus are structurally different from cytokine or growth factor receptors. Despite this, evidence suggests that the AT1 receptor can activate the Jak-STAT signaling pathway transducing cell surface signals into the cell cytoplasm and nucleus (5, 6). The Jak family of intracellular tyrosine kinases consists of Jak1, Jak2, Jak3, and Tyk2, which range in size from 130 to 135 kDa (7, 8). These proteins have a carboxyl-terminal kinase domain immediately preceded by a pseudokinase domain. The Jaks lack a transmembrane domain and have no SH2, SH3, PTB, or PH domains. With the exception of Jak3, Jaks are ubiquitously expressed.

The STAT (signal transducer and activators of transcription) proteins are latent cytoplasmic transcription factors originally identified as part of cytokine signal transduction pathways. Subsequently, a wide variety of ligands were found to activate STAT family members. STAT proteins are involved in various biological functions like cell transformation, development, differentiation, immunity, and apoptosis (9). Structurally, STATs possess a single highly conserved tyrosine phosphorylation site, an SH2 domain, a DNA-binding domain, and a transactivation domain.

In vascular smooth muscle cells, angiotensin II induces the rapid tyrosine phosphorylation and activation of Jak2 (5). This results in the tyrosine phosphorylation of STAT1, STAT2, STAT3, and STAT5. In cardiac myocytes, Charlene et al. (10) showed that angiotensin II, acting through the AT1 receptor, can induce STAT1 and STAT3 phosphorylation, translocation to the nucleus, and initiation of gene transcription. These data show that the AT1 receptor can signal to the nucleus via the tyrosine phosphorylation of STAT proteins, analogous to that of cytokine receptors.

The paradigm of STAT activation by a cytokine receptor is that STATs are transiently recruited to the receptor complex via binding to specific phosphotyrosine residues through their SH2 domain (8, 9). The receptor bound STAT proteins are then tyrosine phosphorylated by Jaks associated with the membrane proximal portion of the receptor. The tyrosine phosphorylation of STAT proteins is obligatory for STAT dimerization, nuclear translocation, and subsequent DNA binding. In addition, selective recruitment of STAT proteins to phosphotyrosine residues of the receptor is a mechanism that may impart selectivity of STAT activation to different cytokines (11, 12). Although it is established that the AT1 receptor activates the Jak-STAT pathway, the biochemical sequence of events leading to association of the AT1 receptor and STAT proteins has not been defined. The problem in applying the paradigm of cytokine receptors for STAT activation by the AT1 receptor is that phosphotyrosine-dependent protein-protein interaction has never been established for a G protein-coupled receptor. In analyzing this problem, there are two logical possibilities. One mechanism involves a direct interaction of STATs with the AT1?
receptor. A second potential mechanism is that an ancillary molecule may interact with STATs, which in turn form a complex with the receptor.

In this report, we studied the mechanism of AT1 receptor-mediated STAT1 phosphorylation in cultured COS-7 and CHO1AT1 cells. We demonstrate that angiotensin II stimulates STAT1 phosphorylation through the specific activation of Jak2 tyrosine kinase activity. The AT1 receptor contains one tyrosine residue in the third intracellular loop, one tyrosine in the seventh transmembrane domain, and four tyrosines in the carboxyl-terminal cytoplasmic domain. We mutated these residues to phenylalanine and examined the ability of the mutant receptors to induce angiotensin II-dependent STAT1 signaling. Although STAT1 phosphorylation is regulated by the AT1 receptor, it is independent of the phosphorylation of these six AT1 receptor tyrosine residues. Rather, phosphoryrosine residues in Jak2 appear critical for recruiting STAT1 to the AT1 receptor complex. These findings offer important advances in understanding the biochemical mechanism of STAT1 activation by seven transmembrane receptors.

MATERIALS AND METHODS

Antibodies—The polyclonal antibody used for immunoprecipitation of Jak2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody used for Western blotting of Jak2 was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies directed against STAT1 and the HA tag (clone 12CA5) were purchased from Transduction Laboratories (Lexington, KY) and Roche Molecular Biochemicals, respectively. Antiphosphoryrosine PY99 and 4G10 monoclonal antibodies were obtained from Santa Cruz and Upstate Biotechnology.

Plasmids—A full-length cDNA encoding the rat AT1a receptor was cloned into the pGEX vector containing a SV-40 promoter and a Zeocin resistance cassette. Mutants of the AT1 receptor were constructed by site-directed mutagenesis using dUtGung Escherichia coli according the protocols in the Bio-Rad mutagen kit. The M5 mutant contains phenylalanine in place of tyrosine at amino acids 292, 302, 312, 319, and 339. The M6 AT1 receptor contains a Y226F mutation in addition to those of the M5 AT1 construct. The GST-STAT1 fusion proteins were generous gifts of Dr. Christian Schindler of Columbia University. We obtained the HA-tagged AT1 receptor construct from Dr. R. Lefkowitz at Duke University (14). Subsequently, we made the HA-tagged pZeoM5/AT1, pZeoM6/AT1, and pZeoFAAA/AT1 receptor constructs. For each, the HA sequence YPYDVPDYA was expressed at the amino terminus of the receptor protein. pBOSWTJak2 (wild type Jak2) and pBODJK22VIII (dominant-negative Jak2) were generous gifts from Dr. D. M. Wojchowski and were previously described (15).

Cell Culture—Cell culture reagents were purchased from Life Technologies. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, glutamine, sodium pyruvate, and antibiotics. CHO-K1 cells expressing pZeoAT1 were grown in Ham’s F-12 medium containing 150 mg/ml of Zeocin. All cells were maintained at 37 °C and 5% CO2. Cells at 80% confluence were serum starved for 16–20 h prior to stimulation with angiotensin II.

Transient and Stable Transfection—COS-7 cells were transiently transfected using Lipofectin (Life Technologies Inc.) as described previously (16). Stable transfectants of CHO/AT1 cells were generated by Lipofectin using 10–20 μg of plasmid DNA. After an overnight incubation, cells were transferred to medium containing 400 μg/ml Zeocin. The medium was replaced after every 3–4 days. Zeocin resistant colonies were isolated after 2–3 weeks.

Angiotensin II Binding Assay—Cells were seeded at a density of 1.5 × 105 cells/well using 24-well tissue culture plates and tested for their ability to bind 125I-Sar1-Ile8-angiotensin II (Du Pont). (4) Cells were incubated for 1 h at 25 °C in binding buffer (10 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 10 mM NaHCO3, 1.2 mM KH2PO4, 1 mM CaCl2, and 0.25% bovine serum albumin (w/v)) to remove endogenous ligand. After aspiration, 200 μl of fresh binding buffer was added to each well, and binding was initiated by the addition of 125I-Sar1-Ile8-angiotensin II. A saturating concentration of 125I-Sar1-Ile8-angiotensin II was set at 1 nM, and serial dilutions are from this point. All samples were done in duplicate. Cells were incubated for 1 h at 25 °C, and reactions were terminated by placing the plates on ice. Cells were washed four times with ice-cold binding buffer without bovine serum albumin. Cells were lysed with 250 μl of 2 N NaOH for 30 min at 25 °C, and 150 μl were added to 500 μl of H2O. Protein concentrations were determined using the Bio-Rad D6 Protein Assay with the remaining lysates. Nonspecific binding is defined as binding in the presence of 1.0 μM angiotensin II.

Immunoprecipitation and Immunoblotting—Subconfluent cells were serum starved for 16–20 h following 100 nM angiotensin II stimulation. Cells were washed twice with phosphate-buffered saline containing 1 mM Na3VO4. The cells were then lysed either in RIPA buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 2.5 mM EDTA) or gentle lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, and 10% glycerol) containing phosphatase and protease inhibitors. Cell lysates were cleared by centrifugation and then incubated with appropriate antibodies for 6–18 h. The immunocomplexes were collected with protein A/G-agarose. The beads were washed extensively with either lysis buffer or wash buffer (50 mM Tris-HCl, pH 7.4, 0.1% triton X-100 and 150 mM NaCl), and proteins were eluted in SDS sample buffer. Proteins were separated on 8% SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were visualized with horseradish peroxidase conjugated to specific antibodies (Biositech Pharm) following enhanced chemiluminescence (NEL Life Science Products). To measure the tyrosine phosphorylation of Jak2 or STAT1, proteins were immunoprecipitated from transfected COS-7 cells lysates using either polyclonal anti-Jak2 antibody or monoclonal anti-STAT1 antibody and then immunoblotted with anti-phosphotyrosine monoclonal antibodies.

Jak2 and STAT1 Binding Assays—For in vitro binding studies, we used GST (GST)-SH2 and a mutant form of GST (GST)-SH2 containing an R602K mutation. After expressing these proteins in DH5α, we were purified to homogeneity using gluthathione Sepharose affinity chromatography as described by Gupta et al. (13). To study angiotensin II–dependent Jak2 interaction with the STAT1 fusion proteins, COS-7 cells were transfected with plasmids encoding the wild type AT1 receptor and wild type Jak2. The cells were washed twice with phosphate-buffered saline, cell lysates were prepared and incubated with 2 μg of GST (GST) fusion protein bound to glutathione-Sepharose for 2 h at 4 °C. Protein complexes were washed extensively with wash buffer and analyzed by SDS-PAGE. Jak2 in the GST-STAT1 fusion protein complexes was visualized by immunoblotting with Jak2 antibody.

To assess the in vivo association of Jak2 with STAT1, COS-7 cells were stimulated with 100 nM angiotensin II for various time periods, and lysates were prepared. STAT1 was precipitated using monoclonal anti-STAT1 antibody. Western blots were then probed with anti-Jak2 antibody. To determine the association of STAT1 and Jak2 with the AT1 receptor, immunoprecipitation was performed using anti-HA antibody (12CA5), followed by Western blotting with STAT1 or Jak2 antibodies. In those experiments comparing transfection of wild type Jak2 versus dominant-negative Jak2, cell culture dishes were transfected with somewhat greater quantities of the Jak2 dominant-negative construct to achieve equal protein expression of both the wild type and dominant-negative proteins (see Fig. 3, A and B).

RESULTS

Angiotensin II–dependent STAT1 Phosphorylation—We analyzed the activation of STAT1 in cultured COS-7 cells. These cells express high levels of STAT1, low levels of Jak2, and no endogenous AT1 receptor. Therefore, we transiently transfected COS-7 cells with plasmids encoding the wild type AT1 receptor and wild type Jak2. Cell lysates were prepared after stimulation with angiotensin II for 0, 1, 3, 6, and 15 min and were immunoprecipitated with STAT1 monoclonal antibody. Precipitated proteins were analyzed by Western blot using anti-phosphotyrosine antibody 4G10 (Fig. 1A, upper panel). The onset of STAT1 phosphorylation is rapid and transient, with peak phosphorylation occurring at about 6 min after stimulation with angiotensin II. Identical results were obtained when we inverted the order of antibody addition (Fig. 1B, upper panel). Further, we confirmed previous published work showing that Jak2 is heavily autophosphorylated on tyrosine in response to angiotensin II (Fig. 1B, lower panel). The time course of this phosphorylation is similar to that of STAT1. To
verify that the tyrosine phosphorylation of STAT1 in our system is dependent on the AT1 receptor, transfected COS-7 cells were treated with losartan, a highly specific AT1 receptor antagonist. Losartan completely blocked angiotensin II-dependent STAT1 phosphorylation irrespective of the order of antibody addition (data not shown). Similarly, when COS-7 cells were transiently transfected with wild type Jak2 and an empty vector in place of the AT1 receptor, no angiotensin II-dependent STAT1 phosphorylation was observed (data not shown).

Both genetic and biochemical studies suggest that cytokine-dependent activation of Jak kinases are associated with the phosphorylation and activation of STATs. We therefore wanted to investigate whether a catalytically active Jak2 is a requirement for STAT1 phosphorylation. To address this question, COS-7 cells were co-transfected with the wild type AT1 receptor construct and either wild type Jak2 (Jak2 WT) or a dominant-negative Jak2 construct (Fig. 1C). The dominant-negative Jak2 construct contained two point mutations that rendered the Jak2 protein catalytically inactive (15). Cell lysates were immunoprecipitated with anti-STAT1 antibody and immunoblotted with anti-phosphotyrosine antibody. There was a marked angiotensin II-dependent tyrosine phosphorylation of STAT1 in cells transfected with wild type Jak2. In contrast, no such phosphorylation was observed in cells transfected with dominant-negative Jak2. To confirm equal immunoprecipitation of STAT1 in each lane, the blot was stripped and reprobed with anti-STAT1 antibody (Fig. 1C, lower panels). Western blot analysis was also used to confirm equal protein expression of both the wild type and dominant-negative protein (data not shown).

Receptor Phosphotyrosine-independent Activation of STAT1—The AT1 receptor is a member of the seven transmembrane receptor superfamily. A structural feature that distinguishes the AT1 receptor is the long intracellular carboxyl terminus of the protein, a region of about 60 amino acids containing four tyrosine residues. This portion of the receptor is important for angiotensin II-mediated activation of intracellular tyrosine kinases, including Jak2 (16). To investigate the role of tyrosine residues in STAT1 activation, we used site-directed mutagenesis to create two mutant receptors called M5 AT1 and M6 AT1. To make the M5 AT1 construct, we mutated the carboxy-terminal five tyrosine residues to phenylalanine. One of these residues, Tyr292, is within the seventh transmembrane domain (17). The other four tyrosines are found within the intracellular carboxyl terminus. To create the M6 AT1 receptor, the M5 AT1 construct was further modified by a Y226F mutation to the only tyrosine within the third intracellular loop. Ligand binding affinity was studied in CHO cells stably transfected for either the M5 AT1 or M6 AT1 receptors. The measured Kd values for M5 AT1 and M6 AT1 were 0.13 ± 0.02 and 0.14 ± 0.03 nM, values not significantly different from that of the wild type AT1 receptor (0.11 ± 0.02 nM).

To analyze the ability of the M5 AT1 and M6 AT1 receptors to activate STAT1 phosphorylation, two separate protocols were used. In the first protocol, COS-7 cells were transiently transfected with each of the mutant receptors, as well as a plasmid encoding Jak2 WT. After the addition of angiotensin II, STAT1 phosphorylation was analyzed by immunoprecipitation with anti-phosphotyrosine antibody followed by Western blotting with anti-STAT1 antibody (Fig. 2A, lanes 1–6). In response to angiotensin II, both the M5 AT1 and M6 AT1 receptors induced STAT1 tyrosine phosphorylation in a fashion indistinguishable from that of wild type receptor. This is in contrast to another AT1 receptor mutant termed FAAA AT1, in which the carboxy-terminal motif YIPP (amino acids 319–322) was mutated to FAA. Previously, we reported that FAAA AT1 was unable to stimulate Jak2 activation (16). When the FAAA AT1 receptor was transiently transfected into COS-7 cells, no ligand-dependent activation of STAT1 was observed (Fig. 2A, lanes 7 and 8).

The ability of the M5 AT1 and M6 AT1 receptors to stimulate the phosphorylation of STAT1 was also measured in CHO cell clones stably transfected with these receptors. Clones were selected with a total ligand binding capacity similar to a CHO cell clone stably transfected with the wild type AT1 receptor. Nontransfected, native CHO cells do not phosphorylate STAT1 in response to angiotensin II (data not shown). However, as shown in Fig. 2B, clones expressing the wild type AT1 receptor, the M5 receptor, and the M6 receptor responded to ligand with a marked increase in the tyrosine phosphorylation of STAT1. In the stably transfected CHO cells, STAT1 tyrosine phospho-
The role of AT1 receptor tyrosine residues in angiotensin II-induced STAT1 phosphorylation. A, COS-7 cells were transiently transfected with cDNA encoding Jak2 WT and either the wild type AT1 receptor (lanes 1 and 2), M5 AT1 (lanes 3 and 4), M6 AT1 (lanes 5 and 6), or FAAA AT1 (lanes 7 and 8). After serum starvation, cells were left untreated (−) or stimulated with angiotensin II (Ang II) for 6 min (+). Cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine antibody and immunoblotted (IB) with anti-STAT1 antibody. Only the FAAA AT1 receptor failed to stimulate STAT1 tyrosine phosphorylation. B, CHO-K1 cells were stably transfected with the wild type AT1 receptor, M5 AT1, or M6 AT1. The cells were stimulated with angiotensin II for 0, 10, 20, 30, and 60 min. Cell lysates were immunoprecipitated with anti-STAT1 antibody and immunoblotted with anti-phosphotyrosine antibody. All three cell lines stimulated STAT1 tyrosine phosphorylation in response to angiotensin II. Equal immunoprecipitation was verified by stripping the blot and reprobing with anti-STAT1 antibody (data not shown). The data represent those observed in twelve independent experiments.

Angiotensin II-mediated Jak2-STAT1 Association—For many cytokine receptors, the consensus sequence YDKPH may act as a specific motif for STAT1 recruitment to the cytoplasmic domain of the receptor (11, 12). Analysis of the AT1 receptor sequence revealed that the receptor lacks the putative binding motif for STAT1. However, the consensus-binding motif for STAT1 is present in Jak2 (19). Previous studies, as well as data presented in Fig. 1B, provide compelling evidence that Jak2 autophosphorylates following angiotensin II stimulation (5). In addition, published reports have demonstrated a physical association of Jak2 with the AT1 receptor in response to angiotensin II (16). To investigate whether Jak2 acts as a bridge molecule for STAT1 association with the AT1 receptor, we first studied angiotensin II-mediated association of Jak2 and STAT1 by co-immunoprecipitation. For these experiments, COS-7 cells were co-transfected with the AT1 wild type receptor and the Jak2 WT constructs. Cells were stimulated with angiotensin II for 0, 3, 6, or 15 min. A cell lysate was then immunoprecipitated with anti-STAT1 antibody followed by Western blot analysis using anti-Jak2 (Fig. 3A, panel a). This experiment showed that, in response to angiotensin II, Jak2 and STAT1 associate in a time-dependent fashion with maximal binding observed at 6 min (lane 3). Substitution of pooled rabbit IgG as the immunoprecipitating antibody led to no detectable Jak2 association (Fig. 3A, lane 5). When the blot shown in Fig. 3A was reprobed with anti-phosphotyrosine antibody and when Jak2 identified by comparison with panel a, we observed that the Jak2 that associated with STAT1 was heavily phosphorylated on tyrosine (Fig. 3A, panel b). The experiments shown in Fig. 3A were controlled by verifying that individual groups of transfected cells expressed equal amounts of STAT1 and Jak2 (Fig. 3A, panels c and d). When the protocol used in Fig. 3A was repeated using COS-7 transfected with the dominant-negative Jak2 construct in place of wild type Jak2, we observed no ligand-dependent association of STAT1 and Jak2 (Fig. 3B, panel a) and no tyrosine phosphorylation of Jak2 (Fig. 3B, panel b).

The observation that STAT1 associates with catalytically active Jak2 but not with the dominant-negative construct, suggesting that autophosphorylation of Jak2 on tyrosine may play an important role in this bimolecular interaction. All STAT family members possess SH2 domains that interact with phosphorylated tyrosine residues. To investigate the role of the STAT1 SH2 domain, we used two GST fusion proteins. The fusion protein termed GST-STAT1-SH2 contains 111 amino acids encoding the SH2 domain of human STAT1. The second fusion protein,
GST-STAT1-SH2mut, was similar except that Arg<sup>602</sup> was changed to Lys<sup>602</sup>. Previously, it was reported that the mutation of Arg<sup>602</sup> disabled the interaction of this SH2 domain fusion protein with the phosphotyrosine residues of Jak1 (13). COS-7 cells were cotransfected with wild type Jak2 and the wild type AT<sub>1</sub> receptor. After stimulation with angiotensin II for 0, 3, 6, or 15 min, cell lysates were incubated with the GST-STAT1 fusion proteins bound to glutathione-Sepharose beads. After incubation and extensive washing, bound Jak2 was assessed by Western blot analysis (Fig. 3C). In response to angiotensin II, increasing amounts of Jak2 associated with the GST-STAT1-SH2 fusion protein, with maximal association observed at 6 min (lane 3). In contrast, no such ligand-dependent association was observed with the GST-STAT1-SH2mut fusion protein. To verify that each sample received an equal amount of fusion protein, the membrane was stripped and reprobed with anti-GST antibody (lower panel). Results were observed in six separate experiments.

**Fig. 3. In vivo association of STAT1 with Jak2.** A, COS-7 cells transfected with the wild type AT<sub>1</sub> receptor and Jak2 WT were stimulated with angiotensin II for 0, 3, 6, and 15 min. In panels a–c, cell lysates were immunoprecipitated with anti-STAT1 antibody and immunoblotted (IB) as indicated. Panel a shows the ligand-dependent association of STAT1 and Jak2. Maximal association was observed at 6 min (lane 3). In panel b, the blot was stripped and reprobed with anti-phosphotyrosine antibody, and Jak2 was identified by comparison with panel a. This shows that Jak2 is heavily phosphorylated on tyrosine when it associates with STAT1. Panel c verifies equal immunoprecipitation of STAT1. No STAT1/Jak2 association was observed when pooled rabbit IgG was substituted for the immunoprecipitating anti-STAT1 antibody (lane 5). To ensure that equivalent amounts of Jak2 were expressed, 10% of each cell lysate was immunoprecipitated in parallel with anti-Jak2 followed by immunoblotting with this same antibody (panel d). The results shown are representative of five separate experiments. B, COS-7 cells were transfected with cDNA encoding the wild type AT<sub>1</sub> receptor and a dominant-negative (DN) Jak2 construct. The protocols for the four panels of B are identical to those of the corresponding panels in A. These results are representative of three separate experiments. C, COS-7 cells, transfected with the wild type AT<sub>1</sub> receptor and Jak2 WT, were stimulated with angiotensin II for the indicated times. Cell lysates were then incubated with 2 μg of either wild type GST-STAT1-SH2<sup>wt</sup> or GST-STAT1-SH2mut fusion protein bound to glutathione Sepharose beads. The GST-STAT1-SH2mut contains an R602K mutation in the FLVR motif of the SH2 domain, which is critical for the function of the SH2 domain. After extensive washing, associated Jak2 was measured by immunoblotting with anti-Jak2 antibody (upper panel). Only the wild type SH2 domain fusion protein showed ligand-dependent association of Jak2. To verify that each sample received an equal amount of fusion protein, the membrane was stripped and reprobed with anti-GST antibody (lower panel). Results were observed in six separate experiments.
result in the detection of either Jak2 or STAT1. To investigate the role of Jak2 catalytic activity, the experiment was repeated, but we now used the Jak2 dominant-negative construct in place of wild type Jak2. As shown in Fig. 4C, catalytically inactive Jak2 resulted in substantially decreased levels of Jak2-AT1 receptor association. Further, the catalytically inactive Jak2 resulted in virtually no STAT1 binding to the receptor complex (Fig. 4C, bottom panel). These results provide direct evidence that the angiotensin II-dependent association of STAT1 with the AT1 receptor requires a catalytically active Jak2. Similarly, the AT1 receptor mutants M6 and FAAA were also epitope-tagged with HA to study the interaction of these receptors with STAT1 and Jak2. In response to angiotensin II, the M6 mutant bound both Jak2 and STAT1 (Fig. 4D). In contrast, the AT1 receptor mutant FAAA AT1 demonstrated no association with either Jak2 or STAT1 (Fig. 4D).

If Jak2 acts as an ancillary molecule to bring STAT1 to the AT1 receptor, we would predict that, in response to angiotensin II, there would be a physical co-association of these three proteins. To test this, COS-7 cells were transfected with cDNA encoding the HA-tagged wild type AT1 receptor and wild type Jak2. Cells were treated with angiotensin II for 0 or 6 min (Fig. 5A, – or +), and cell lysates were then immunoprecipitated with anti-Jak2 antibody. After separation by SDS-PAGE, the precipitated proteins were sequentially probed with anti-HA mAb, anti-STAT1 mAb, and anti-Jak2 antibody (Fig. 5A). This verified that in response to angiotensin II, there is a physical co-association of the Jak2 with both the AT1 receptor and STAT1.

A second prediction of our model is that if, in response to angiotensin II, Jak2 acts as an ancillary molecule to bring STAT1 to the AT1 receptor, then depletion of Jak2 would prevent AT1-STAT1 association. However, if the ligand-dependent association of the AT1 receptor with STAT1 is bimolecular, then the AT1-STAT1 complex should persist in the absence of Jak2. To investigate this, COS-7 cells were again transfected with cDNA encoding the HA-tagged wild type AT1 receptor and wild type Jak2. Cells were treated with angiotensin II for 6 min to permit maximal AT1 receptor-STAT1 association (indicated in Fig. 5B and C with +). Cell lysates were prepared and Jak2 was immunodepleted by the addition of polyclonal anti-Jak2 antibody and protein A/G-agarose. Control samples received only protein A/G-agarose. Control samples received only protein A/G-agarose. All samples were maintained at 4 °C for 18 h during the immunodepletion protocol. The resulting supernatants were then immunoprecipitated with anti-HA monoclonal antibody and blotted to access receptor association of STAT1 (Fig. 5B) and Jak2 (Fig. 5C). Immunodepletion of Jak2 removed the vast bulk of both STAT1 and Jak2 binding to the AT1 receptor. These data strongly suggest that the ligand-mediated association of STAT1 with the AT1
Previous studies from our laboratory indicated that the association of Jak2 with the AT1 receptor was initiated by the binding of angiotensin II to the AT1 receptor (5,16). Using a HA-tagged AT1 receptor, this was confirmed in data presented here in Fig. 4A. Thus, in response to ligand, Jak2 autophosphorylates and forms a high affinity bond with the AT1 receptor, an interaction that is dependent on the AT1 receptor motif YIPP (16). Here, we examined the hypothesis that, in addition to being a STAT kinase, Jak2 also facilitates STAT1 binding to the AT1 receptor.

Murine Jak2 contains 49 tyrosine residues (19). Previous work showed that 15 of these can be autophosphorylated in vivo (23). In addition, Jak2 contains the protein motif YDKPH, which is thought capable of binding the SH2 domain of STAT1 (24,25). Data presented in Fig. 3 showed that association of Jak2 with STAT1 is markedly increased by the ligand angiotensin II. Further, this association was not observed with a dominant-negative, noncatalytic form of Jak2. Evidence presented in Fig. 3C implicates the SH2 domain of STAT1 as critical for the physical association of STAT1 with Jak2. Additionally, the data in Fig. 4 demonstrated the co-association of Jak2 and STAT1 with the AT1 receptor, an association once again absolutely dependent on a catalytically active Jak2 capable of autophosphorylation. Finally, the immunodepletion study presented in Fig. 5 (B and C) showed the intrinsic dependence of STAT1-AT1 receptor association on the presence of Jak2; even when cells were treated with ligand for 6 min, a period of maximum association of STAT1 with the AT1 receptor and a time period sufficient for any modification of the AT1 receptor, STAT1-AT1 receptor association was critically dependent on Jak2.

In summary, these studies give insight into the mechanism of STAT1 activation by a G protein-coupled receptor. Unlike the typical paradigm for cytokine receptors, the binding of Jak2 to the AT1 receptor appears to be ligand-dependent. In this system, a catalytically active Jak2 appears critical for ligand-mediated STAT1 tyrosine phosphorylation and its association with the AT1 receptor. Once autophosphorylated, we believe that it is the Jak2 phosphoryrosine that binds the SH2 domain of STAT1, thereby recruiting STAT1 to the receptor complex. Our analysis of the AT1 receptor is highly reminiscent of previous work showing that receptors for erythropoietin, growth hormone, epidermal growth factor, granulocyte-macrophage colony-stimulating factor, and prolactin can interact with STATs independent of receptor phosphoryrosine residues (26–30). Similar to our results, these studies suggested that signaling proteins such as SHC, IRS-1, or Jak enzymes were critical for receptor-STAT interaction. Recently Fujitani et al. (31) reported that Jak1 and Jak2 specifically recruit and phosphorylate STAT1 and STAT5, respectively. These experiments, together with our findings, support the concept that STATs associate with tyrosine phosphorylated Jaks through their SH2 domains.

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