The Angiotensin II-dependent Nuclear Translocation of Stat1 Is Mediated by the Jak2 Protein Motif \textsuperscript{231}YRFRR\textsuperscript{*}

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In response to angiotensin II, Jak2 autophosphorylates and binds the angiotensin II AT\textsubscript{1} receptor. By studying a variety of Jak2 deletion proteins, we now show that the Jak2 protein motif \textsuperscript{231}YRFRR is required for the co-association of this kinase with the AT\textsubscript{1} receptor. We also used a full-length Jak2 protein containing a \textsuperscript{231}FAAAA amino acid substitution. Although this protein still autophosphorylated in response to angiotensin II, it did not co-associate with the AT\textsubscript{1} receptor. This uncoupling indicates that AT\textsubscript{1}/Jak2 co-association is not necessary for angiotensin II-induced Jak2 autophosphorylation and that Jak2 autophosphorylation \textit{per se} is insufficient for AT\textsubscript{1} receptor co-association. In response to angiotensin II, the Jak2-231FAAAA mutant will tyrosine phosphorylate Stat1. However, in the absence of AT\textsubscript{1}/Jak2 co-association, Stat1 did not translocate into the cell nucleus and failed to mediate gene transcription. This notable result indicates that Stat1 tyrosine phosphorylation alone is insufficient for Stat1 nuclear translocation. In summary, we now show that, although Jak2-mediated tyrosine phosphorylation of Stat1 is independent of receptor co-association, Jak2-mediated recruitment of Stat1 to the AT\textsubscript{1} receptor is critical for Stat1 nuclear translocation and subsequent gene transcription.

Angiotensin II is the effector molecule of the renin-angiotensin system. It is vital for maintaining a wide variety of physiological responses, including salt and water balance, blood pressure, and vascular tone. These effects are transduced through a seven-transmembrane surface receptor called AT\textsubscript{1} (1). In addition to promoting the hydrolysis of heterotrimeric G proteins, activation of the AT\textsubscript{1} receptor by angiotensin II also results in the activation of several non-receptor tyrosine kinases, including Jak2 (2–5).

Jak2 is a member of the \textit{Janus} family of non-receptor tyrosine kinases that also includes Jak1, Jak3, and Tyk2. These proteins are \textasciitilde130 kDa in mass and contain seven conserved Jak homology domains. Typically, Jak activation by a cytokine receptor leads to STAT activation and thus transmission of a signal from the extracellular surface of the cell into the nucleus (6). Studies by our laboratory (2) and by Baker and co-workers (7–9) have shown that, similar to cytokines, activation of the AT\textsubscript{1} receptor by angiotensin II promotes 1) STAT tyrosine phosphorylation, 2) STAT nuclear translocation, 3) STAT DNA binding activity, and 4) STAT-dependent transcriptional activation.

Recently, our laboratory examined proximal signaling events that mediate Jak/STAT activation by the AT\textsubscript{1} receptor. Specifically, we demonstrated that the physical co-association of the AT\textsubscript{1} receptor with Jak2 is dependent on the AT\textsubscript{1} receptor motif \textsuperscript{319}YIPP found within the carboxyl terminus of the receptor protein (10). Subsequently, we found that Jak2 must be catalytically active to associate with the AT\textsubscript{1} receptor, as inhibition of Jak2 kinase activity either by pharmacological means or by a dominant-negative Jak2 protein greatly reduces AT\textsubscript{1}/Jak2 co-association (11).

The studies in this report examined whether, in addition to a functional kinase domain, there are any other requirements for Jak2 co-association with the AT\textsubscript{1} receptor. Here we demonstrate that the Jak2 protein motif \textsuperscript{231}YRFRR, located in the amino-terminal portion of the molecule, is also required for co-association with the AT\textsubscript{1} receptor. Conversion of the Jak2 protein motif from \textsuperscript{231}YRFRR to \textsuperscript{231}FAAAA functionally unouples Jak2 autophosphorylation from AT\textsubscript{1} receptor binding, demonstrating that Jak2 autophosphorylation occurs independently of receptor co-association. When Jak2 fails to co-associate with the AT\textsubscript{1} receptor, a functional consequence is that Stat1 fails to translocate into the nucleus and mediate gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 cells and BSC-40 cells were cultured and growth-arrested exactly as described (12, 13). Cell culture reagents were obtained from Life Technologies, Inc. All other reagents were purchased from Sigma.

Plasmid Constructs—The HA-tagged AT\textsubscript{1} cDNA was kindly provided by Dr. R. J. Lefkowitz (14) and was cloned into pcDNA3 at the HindIII/NotI restriction sites. Construction of the pRC-WT, pRC-ATD, pRC-PKD, pRC-CTD, and pRC-AFL Jak2 expression vectors has been described (13, 15, 16). The following Jak2 deletion constructs were generated by polymerase chain reaction using Pfu DNA polymerase and cloned into pRC-AFL at the NotI/HindIII restriction sites. The top strand oligonucleotides were as follows: pRC-68, 5'-CATGATAATGCGGCCG-CAATGTTGATCTGCTCCATAGCGTG-3'; pRC-120, 5'-ATTGCTGCTCCCATAGCGTG-3'; pRC-168, 5'-CATGATAATGCGGCCGCAATGTTGATCTGCTCCATAGCGTG-3'.

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\‡ The abbreviations used are: STAT, signal transducer and activator of transcription; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; pAb, polyclonal antibody; mAb, monoclonal antibody.

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pRC-186, 5'-CATGATAATGGCCCGCAATGGAAGATGCTAGAAGG-3'; pRC-211, 5'-CATGATAATGGCCCGCAATGGAAGATGCTAGAAGG-3'; pRC-221, 5'-CATGATAATGGCCCGCAATGGAAGATGCTAGAAGG-3'; pRC-230, 5'-CATGATAATGGCCCGCAATGGAAGATGCTAGAAGG-3'; pRC-235, 5'-CATGATAATGGCCCGCAATGGAAGATGCTAGAAGG-3'; and pRC-240, 5'-CATGATAATGGCCCGCAATGGAAGATGCTAGAAGG-3'. The right strand oligonucleotide for all constructs was 5'-GATCATTGCAGATCTAAGGCAGTTCC-3' and utilized an internal A/II site. The pRC-240 plasmid was made by cutting pRC-ATD with A/II and closing with ligase. The pRC-FLAG-225, pRC-FLAG-235, and pRC-FLAG-240 constructs were made by placing the FLAG peptide sequence (DYKDDDDK) between the initiation methionine and the respective Jak2 sequence. Other than pRC-CTD, which lacks the Jak2 kinase domain, all deletion constructs were found to have readily detectable levels of tyrosine phosphorylation as measured by anti-phosphotyrosine Western blotting. The Jak2-Y231F and Jak2-231FAAAA mutants were generated using the QuikChange site-directed mutagenesis system (Stratagene). All constructs were confirmed by DNA sequence analysis.

Transient Cell Transfection—AT\textsubscript{1}/Jak2 co-association in BSC-40 cells was done using the vaccinia virus transfection/infection protocol (17, 18). Cells were seeded in 100-mm dishes and transfected at near-confluency with 10 \mu g of pHA-AT\textsubscript{1} and 10 \mu g of Jak2 expression vector in 20 \mu l of Lipofectin. 4 h later, vaccinia virus clone vTF7-3 was added at a multiplicity of infection of 1.0 and incubated for 4 h. The medium was aspirated, and cells were incubated overnight in serum-containing medium and lysed 18–20 h after infection. COS-7 cells were transiently transfected exactly as described (10).

Immunoprecipitation—To prepare lysates, cells were washed with 2 volumes of ice-cold phosphate-buffered saline containing 1 mm Na\textsubscript{3}VO\textsubscript{4} and lysed in 1.0 ml of ice-cold radioimmune precipitation assay buffer containing protease inhibitors (18). The samples were sonicated and incubated on ice for 1 h. Samples were spun at 12,000 \times g for 5 min at 4 °C, and supernatants were normalized using the Dc protein assay (Bio-Rad). Normalized lysates (400 \mu g/ml) were immunoprecipitated with 1 \mu g of antibody and 20 \mu l of Protein A/G-agarose beads (Santa Cruz Biotechnology). After centrifugation, protein complexes were washed three to five times with radioimmune precipitation assay buffer and resuspended in sample buffer. Bound proteins were boiled, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. The immunoprecipitating anti-Jak2 pAb (HR758) and anti-HA mAb (12CA5) were from Santa Cruz Biotechnology and Roche Molecular Biochemicals, respectively. The anti-Tyr(P) mAb (PY20) and anti-Stat1 mAb (S21120) were from Transduction Laboratories (Lexington, KY) and Roche Molecular Biochemicals, respectively. The anti-Tyr(P) mAb (PY20) and anti-Stat1 mAb (S21120) were from Transduction Laboratories.

Western Blotting—Proteins were detected using enhanced chemiluminescence exactly as described (19). Blotting antibodies were anti-Jak2 pAb (Upstate Biotechnology, Inc.), anti-Tyr(P) mAb (Upstate Biotechnology, Inc.), anti-HA mAb (Roche Molecular Biochemicals), anti-Stat1 mAb (Transduction Laboratories), anti-FLAG mAb (Sigma), anti-Eps15 pAb (Santa Cruz Biotechnology), anti-phospho-Stat1 pAb (Oncogene Research Products), and anti-SV40 large T antigen mAb (Santa Cruz Biotechnology).

Preparation of Nuclear Extracts—After treatment with angiotensin II, nuclear extracts were prepared using the high salt method of extraction (20–22). Nuclear proteins were then dialyzed for 2 h at 4 °C against 4 \textmu l of solution containing 20 m\textmu M HEPES (pH 7.9 at 4 °C), 20% glycerol, 100 m\textmu M KCl, 0.2 m\textmu M EDTA, 0.5 m\textmu M dithiothreitol, and 4 °C, and supernatants were normalized using the Dc protein assay (Bio-Rad). Nuclear extracts (20–22) were utilized in this assay. Western blotting aliquots from these same samples with anti-Jak2 pAb (Fig. 1A), anti-HA mAb and blotting with anti-Jak2 pAb.

RESULTS

The Jak2 Protein Motif \textsuperscript{231}YRFRR Is Required for AT\textsubscript{1} Receptor Binding in Vivo—To determine regions of Jak2 that are required for co-association with the AT\textsubscript{1} receptor, we utilized an in vivo vaccinia virus-based expression system that exploits two aspects of Jak2 protein expression. First, we previously demonstrated that overexpression of wild-type Jak2 in COS-7 cells results in AT\textsubscript{1}/Jak2 co-association independent of angiotensin II treatment (11). The expressed Jak2 is activated not by angiotensin II, but rather by oligomerization and autophosphorylation of the highly expressed protein. Although the COS-7 expression system worked well for wild-type Jak2, we (12, 13, 15, 16) and others (23) observed that some Jak2 deletion proteins are poorly expressed. To overcome this, BSC-40 cells (a vaccinia virus permissive cell line) are transfected with an expression vector containing the Jak2 cDNA under the control of the T7 promoter. The cells are then infected with a vaccinia virus that produces T7 polymerase. This system greatly enhances Jak2 protein expression and has been used to examine various aspects of Jak2 signal transduction (12, 13, 15, 16, 23). This system was used to overexpress various Jak2 deletion constructs along with an HA-tagged AT\textsubscript{1} receptor cDNA (pHA-AT\textsubscript{1}). As with the COS-7 cell system, Jak2 is activated by oligomerization/autophosphorylation of the highly expressed protein, which in turn binds the AT\textsubscript{1} receptor. In vivo AT\textsubscript{1}/Jak2 co-association is measured by immunoprecipitating with anti-HA mAb and blotting with anti-Jak2 pAb.

Prior to assessing co-immunoprecipitation of Jak2 with the AT\textsubscript{1} receptor, several controls were performed. First, we demonstrated that overexpression of Jak2 required the presence of both the transfected cDNA and infection of the recombinant vaccinia virus, as omission of either one resulted in the loss of overexpression (data not shown). Second, we confirmed that the vaccinia-derived Jak2 protein was post-translationally modified by tyrosine phosphorylation and was catalytically active (data not shown).

To demonstrate specificity of the co-immunoprecipitation system, BSC-40 cells were transfected with plasmids encoding wild-type Jak2 (pRC-WT) and the HA-tagged AT\textsubscript{1} receptor under the control of the T7 promoter. The cells were then infected with vaccinia virus clone vTF7-3, which produces T7 polymerase. Lysates were prepared 18–20 h after infection. The lysates were then immunoprecipitated with anti-HA mAb and blotted with anti-Jak2 pAb (Fig. 1A). AT\textsubscript{1}/Jak2 co-association was seen only when the Jak2 plasmid was present (lane 3 versus lanes 1 and 2). Furthermore, omission of either the HA-tagged AT\textsubscript{1} receptor plasmid (lane 4) or the immunoprecipitating anti-HA mAb (lane 5) resulted in specific loss of Jak2 co-association, indicating that neither was Jak2 immunoprecipitated nonspecifically by anti-HA mAb nor was Jak2 binding the Protein A/G-agarose nonspecifically.

Using this system, we then coexpressed the HA-tagged AT\textsubscript{1} receptor with several Jak2 deletion constructs. A schematic representation of these constructs is shown as Fig. 1B. Initially, expression vectors encoding wild-type Jak2, an amino-terminal deletion (ATD), an internal deletion (PKD), or a C-terminal deletion (CTD) were utilized. As shown in Fig. 2A (upper panel), both wild-type Jak2 and the pseudo-kinase deletion (PKD; Δ523–746) co-associated with the receptor. However, the amino-terminal deletion (ATD; Δ1–240) and the C-terminal deletion (CTD; Δ1000–1129) did not. We confirmed that all Jak2 proteins were expressed by immunoprecipitating and Western blotting aliquots from these same samples with anti-Jak2 pAb (Fig. 2A, lower panel). We believe the inability of the CTD mutant to bind the receptor is due to the loss of the kinase domain since previous work demonstrated that inhibi-
The specificity of vaccinia virus-mediated AT1/Jak2 co-association was examined as follows: 

**A**. BSC-40 cells were transfected with the indicated plasmids and infected with vaccinia virus clone vTF7-3. Lysates were prepared and immunoprecipitated with anti-HA mAb (lanes 1-4) or control IgG (lane 5) and then Western-blotted with anti-Jak2 pAb to assess AT1/Jak2 co-association. Shown is one of three independent experiments. B. Shown is a summary of Jak2 deletion analysis studies and co-association with the HA-tagged AT1 receptor. The schematic represents full-length Jak2 and the seven respective Jak homology domains (1-7). Also shown are the deletions generated and a table summarizing whether each was able to co-associate with the HA-tagged AT1 receptor in vivo. Co-IP, co-immunoprecipitate.

**Fig. 1. Specificity of vaccinia virus-mediated AT1/Jak2 co-association.** A, BSC-40 cells were transfected with the indicated plasmids and infected with vaccinia virus clone vTF7-3. Lysates were prepared and immunoprecipitated with anti-HA mAb (lanes 1-4) or control IgG (lane 5) and then Western-blotted with anti-Jak2 pAb to assess AT1/Jak2 co-association. Shown is one of three independent experiments. B. Shown is a summary of Jak2 deletion analysis studies and co-association with the HA-tagged AT1 receptor. The schematic represents full-length Jak2 and the seven respective Jak homology domains (1-7). Also shown are the deletions generated and a table summarizing whether each was able to co-associate with the HA-tagged AT1 receptor in vivo. Co-IP, co-immunoprecipitate.

| Jak2 Construct | Co-IP with AT1 |
|----------------|----------------|
| pRC-AMY         | +              |
| pRC-ATD (Δ1-240) | -              |
| pRC-PCD (Δ231-764) | +              |
| pRC-CTD (Δ1000-1129) | -              |
| pRC-AFL (Δ251-473) | +              |
| pRC-68 (Δ1-103 & Δ251-473) | -              |
| pRC-120 (Δ1-120 & Δ231-473) | -              |
| pRC-186 (Δ1-186 & Δ231-473) | -              |
| pRC-211 (Δ1-211 & Δ231-473) | -              |
| pRC-221 (Δ1-221 & Δ231-473) | -              |
| pRC-225 (Δ1-225 & Δ231-473) | -              |
| pRC-230 (Δ1-230 & Δ231-473) | -              |
| pRC-235 (Δ1-235 & Δ231-473) | -              |

**FIG. 1.** Specificity of vaccinia virus-mediated AT1/Jak2 co-association. A, BSC-40 cells were transfected with the indicated plasmids and infected with vaccinia virus clone vTF7-3. Lysates were prepared and immunoprecipitated with anti-HA mAb (lanes 1-4) or control IgG (lane 5) and then Western-blotted with anti-Jak2 pAb to assess AT1/Jak2 co-association. Shown is one of three independent experiments. B, shown is a summary of Jak2 deletion analysis studies and co-association with the HA-tagged AT1 receptor. The schematic represents full-length Jak2 and the seven respective Jak homology domains (1-7). Also shown are the deletions generated and a table summarizing whether each was able to co-associate with the HA-tagged AT1 receptor in vivo. Co-IP, co-immunoprecipitate.
rylates in Response to Angiotensin II—The vaccinia virus deletion analysis studies presented in Figs. 1 and 2 were performed without angiotensin II treatment. To examine the role of the Jak2231YRFRR motif in ligand-mediated binding to the AT1 receptor, we generated two additional Jak2 molecules. The first was a full-length Jak2 in which the231YRFRR motif was converted to 231FAAAA (Jak2-231FAAAA). The second was a full-length Jak2 containing a single Y231F point mutation (Jak2-Y231F). To evaluate the Jak2-231FAAAA construct, COS-7 cells were transiently cotransfected with pHA-AT1 and this plasmid. The cells were then treated with angiotensin II, and ligand-dependent AT1/Jak2 co-association was measured. We found that the Jak2-231FAAAA mutant did not co-associate with the AT1 receptor in response to angiotensin II (Fig. 3A, upper panel). We confirmed equal expression of both wild-type and Jak2-231FAAAA mutant proteins by immunoprecipitating and Western blotting equal aliquots from these samples with anti-Jak2 pAb (Fig. 3A, lower panel). In contrast to this result, the Y231F mutant bound the AT1 receptor in a manner that was virtually identical to wild-type Jak2, indicating that in the context of the 231YRFRR motif, tyrosine 231 appears to provide a structural contribution rather than a phosphotyrosine binding residue (data not shown).

To determine whether the Jak2-231FAAAA mutant auto-phosphorylates in response to angiotensin II, COS-7 cells were transfected as described for Fig. 3A, but the lysates were then

![Image](http://www.jbc.org/Downloaded/PDFFigure.png)
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**A** Jak2 Molecule Containing a $^{231}$FAAAA Mutation in Place of $^{231}$YRFRR Mediates the Angiotensin II-dependent Tyrosine Phosphorylation of Stat1—We then measured the angiotensin II-dependent tyrosine phosphorylation of Stat1. This protein is a substrate of Jak2 and is tyrosine-phosphorylated in response to angiotensin II (2, 6). COS-7 cells were transfected with plasmids encoding the AT$_1$ receptor and an empty vector control, wild-type Jak2, or Jak2-$^{231}$FAAAA. 2 days later, the cells were treated with angiotensin II, and the resulting lysates were immunoprecipitated with anti-Tyr(P) mAb and blotted with anti-Stat1 mAb (Fig. 4A). In the absence of transfected Jak2 expression vector, no Stat1 phosphorylation was observed in this system. In contrast, addition of wild-type Jak2 plasmid resulted in the angiotensin II-dependent tyrosine phosphorylation of Stat1. When the Jak2-$^{231}$FAAAA mutant protein was transfected into cells, we found that it phosphorylated Stat1 in a manner that was identical to that of wild-type Jak2.

To better establish a time course for angiotensin II-mediated Stat1 tyrosine phosphorylation and to measure Stat1 tyrosine phosphorylation by an alternate protocol, transfected COS-7 cells were treated with angiotensin II for periods of 0–30 min. A reciprocal protocol was then performed whereby the lysates were immunoprecipitated with anti-Stat1 mAb and Western-blotted with anti-phospho-Stat1 pAb (Fig. 4B, *upper panel*). This blotting antibody is specific for phosphorylated Stat1 protein at tyrosine 701, a site of phosphorylation that correlates with Stat1 activation (24, 25). These data showed that Jak2-$^{231}$FAAAA phosphorylated Stat1 over a time course that was nearly identical to that of wild-type Jak2, with peak Stat1 tyrosine phosphorylation occurring between 3 and 6 min after angiotensin II treatment for both groups. Furthermore, the alternate protocol produced a result that was similar to that in Fig. 4A. Collectively, these results indicate that Jak2-$^{231}$FAAAA is not only capable of autophosphorylation, but is also able to phosphorylate a substrate in response to angiotensin II.

**B** IP: αHA-mAb
IB: αJak2-pAb

**A** IP: αHA-mAb
IB: αJak2-pAb

**B** IP: αSta1-mAb
IB: αJak2-pAb

**A** Jak2 WT
Jak2-$^{231}$FAAAA

**B** Jak2 WT
Jak2-$^{231}$FAAAA

**FIG. 3.** Conversion of $^{231}$YRFRR to $^{231}$FAAAA inhibits the angiotensin II-dependent co-association of Jak2 with the AT$_1$ receptor, but not Jak2 tyrosine autophosphorylation. COS-7 cells were transfected with 10 μg of HA-tagged AT$_1$ receptor and either 5 μg of wild-type Jak2 (Jak2 WT) or 12.5 μg of Jak2-$^{231}$YRFRR. The cells were treated with 10$^{-7}$ M angiotensin II (Ang II) for the indicated times, and lysates were prepared. A, lysates were immunoprecipitated (IP) with anti-HA mAb and immunoblotted (IB) with anti-Jak2 pAb to assess AT$_1$/Jak2 co-association (*upper panel*). Equal aliquots from these samples were immunoprecipitated and blotted with anti-Jak2 pAb to assess Jak2 expression (*lower panel*). B, lysates were immunoprecipitated with anti–Jak2 pAb and then blotted with either anti-Tyr(P) mAb to assess Jak2 tyrosine autophosphorylation (*upper panel*) or anti-Jak2 pAb to measure Jak2 expression (*lower panel*). Shown is one of three independent experiments for each.

**FIG. 4.** Conversion of $^{231}$YRFRR to $^{231}$FAAAA does not inhibit the angiotensin II-dependent tyrosine phosphorylation of Stat1. COS-7 cells were transfected with 10 μg of HA-tagged AT$_1$ receptor and either 2.5 μg of wild-type Jak2 (Jak2 WT) or 12.5 μg of Jak2-$^{231}$YRFRR. The cells were treated with 10$^{-7}$ M angiotensin II (Ang II) for the indicated times, and lysates were prepared to assess Stat1 tyrosine phosphorylation. A, lysates were immunoprecipitated with anti-Tyr(P) mAb and Western-blotted (immunoblotted) (IB) with anti-Stat1 mAb. B, lysates were immunoprecipitated with anti-Stat1 mAb and Western-blotted either with anti-phospho-Stat1 pAb to assess Stat1 tyrosine phosphorylation (*upper panel*) or with anti-Stat1 mAb to assess equal precipitation of Stat1 protein (*lower panel*). Shown is one of three independent experiments for each.
This result is reminiscent of the growth hormone-mediated activation of Stat3; recruitment of Stat3 to the growth hormone receptor is critically dependent on Jak2 kinase (30).

A Jak2 Molecule Containing a 231YRFRR Mutation in Place of 231FAAA Fails to Mediate Stat1-dependent Gene Transcription—To assess the functional impact of the Jak2-231FAAA mutation, we chose to investigate angiotensin II-dependent, Stat1-mediated gene transcription. For this assay, COS-7 cells were transfected as described for Fig. 5, but we also included a luciferase reporter plasmid containing a Stat1-binding, sis-inducible element. This plasmid contains a tandem repeat of a minimal DNA enhancer element, the thymidine kinase TATA-containing promoter, and the firefly luciferase cDNA (31). Each copy of the DNA enhancer contains a sis-inducible element, a serum response element, and an AP-1-binding site. We have previously demonstrated that this plasmid is a good indicator of Jak/STAT-mediated gene transcription (12, 16). The transfected cells were then treated with angiotensin II for varying times, and luciferase activity was measured (Fig. 6). Transfection of the Jak2-231FAAA mutant resulted in substantially reduced angiotensin II-mediated luciferase activity with respect to wild-type Jak2 controls. The greatest difference in luciferase values between the two groups occurred 24 h after angiotensin II addition ($p < 0.0001$; $n = 6$ for each point). These data indicate that the failure of Jak2-231FAAA to facilitate Stat1 nuclear translocation also impairs Stat1-dependent transcriptional activation.

**DISCUSSION**

This work is significant for three fundamental reasons. First, we have identified a minimal region of Jak2 (231YRFRR) that is required for Jak2 co-association with a cell-surface receptor. Second, the generation of the Jak2-231FAAA mutant functionally uncoupled ligand-mediated Jak2 autophosphorylation from AT1 receptor co-association. Third, the work suggests that when Jak2 fails to bind the AT1 receptor, one functional consequence is that Stat1 does not translocate into the nucleus and therefore fails to mediate gene transcription.

In regard to 231YRFRR-mediated receptor co-association, exactly how 231YRFRR mediates the binding of Jak2 with the AT1 receptor is not fully understood. Our data suggest that it is probably a structural motif rather than a simple phosphotyrosine interaction in that the Jak2-Y231F mutant bound the AT1 receptor, whereas the Jak2-231FAAA mutant did not. Consistent with our observation is a report describing a naturally occurring Jak3 mutation from a patient with autosomal severe combined immunodeficiency; a single Y100C amino acid substitution prevents co-association of Jak3 with the common $\gamma$-chain of the interleukin-2 receptor (32). Similar to our results, the authors of this report concluded that Tyr100 does not mediate a phosphotyrosine-dependent interaction, but rather contributes to a larger structural motif, as the Y100C mutation disrupts receptor co-association, whereas a Y100F mutation does not. The amino acid sequence immediately downstream of Tyr100 is 100YRLRF, and this bears some homology to the 231YRFRR motif described in this report.

With respect to the second point, when COS-7 cells are transfected with Jak2-231FAAA and then treated with angiotensin II, Jak2-231FAAA autophosphorylates, but does not co-associate with the AT1 receptor. This observation suggests that ligand-mediated Jak2 autophosphorylation has therefore been uncoupled from AT1 receptor co-association. Since the Jak2-231FAAA mutant can be activated independently of receptor co-association, one interpretation is that Jak2 autophosphorylation temporally precedes receptor co-association. The current findings are important in that they strongly suggest that AT1/Jak2 co-association is not necessary for angiotensin...
II-induced Jak2 activation and that Jak2 activation, although necessary, is not sufficient for its interaction with the AT1 receptor (11).

Perhaps most significant is the observation that when Jak2 fails to bind the AT1 receptor, Stat1 does not translocate into the nucleus and therefore fails to mediate gene transcription. The data in Fig. 4 demonstrate that Stat1 tyrosine phosphorylation alone is insufficient for Stat1 nuclear translocation, and the data in Fig. 5 indicate that failure of Jak2 to bind the AT1 receptor correlates with markedly decreased Stat1 nuclear translocation.

Our finding that Stat1 tyrosine phosphorylation alone is insufficient for Stat1 nuclear localization has precedent in other published studies. Herrington et al. (33) reported that a Stat5b protein with a small mutation in the DNA-binding region was defective in nuclear location despite its ability to be tyrosine-phosphorylated and to dimerize in response to ligand. Similarly, Strehlow and Schindler (34) reported a modified Stat1 protein that was phosphorylated on tyrosine and that dimerized, but was unable to be transported to the nucleus. Although these reports described a phenotype similar to what we observed with Jak2 Motif 231YRFRR, in both studies, the phenotype was a direct result of a STAT structural mutation. Nonetheless, these studies support our observation by providing other examples of STAT proteins that are phosphorylated on tyrosine and that dimerize, but are unable to transport to the nucleus.

We recently reported that, in response to angiotensin II, Jak2 not only serves as a Stat1 kinase, but also acts as a molecular bridge in recruiting Stat1 to the AT1 receptor (35). One interpretation of the Jak2 Motif 231YRFRR data presented here is that recruitment of Stat1 to the AT1 receptor by wild-type Jak2 further modifies Stat1 in a manner that promotes its nuclear translocation. The exact modification(s) that occur at the AT1 receptor and whether this phenomenon is applicable to other STAT family members is under intense investigation. The concept that Jak2 acts as a molecular bridge in recruiting STAT proteins to a cell-surface receptor is not without precedent. In the case of growth hormone, Jak2 acts as a scaffold in recruiting Stat3 to the growth hormone receptor (30). The present data are significant in that they extend this observation by demonstrating that when Jak2 fails to act as a scaffold in recruiting Stat1 to the AT1 receptor, one consequence is that Stat1-mediated gene transcription is severely compromised.

In summary, this work provides novel insight into the mechanism of Jak2 activation and receptor co-association. Specifically, it defines a region of Jak2 that is required for receptor co-association and advances our understanding of the temporal sequences that occur when the Jak/STAT signaling pathway is activated. Most importantly, this work establishes a correlation between the loss of a Jak2 signaling complex at the level of a cell-surface receptor with the loss of transcriptional regulation within the nucleus.

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