Dependence on the Motif YIPP for the Physical Association of Jak2 Kinase with the Intracellular Carboxyl Tail of the Angiotensin II AT$_1$ Receptor*

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Angiotensin II is the effector molecule of the renin-angiotensin system. Virtually all of its biochemical actions are mediated through a single class of cell-surface receptors called AT$_1$. These receptors contain the structural features of the seven-transmembrane, G-protein-coupled receptor superfamily. Angiotensin II, acting through the AT$_1$ receptor, also stimulates the Jak/STAT pathway by inducing ligand-dependent Jak2 tyrosine phosphorylation and activation. Here, we show that a glutathione S-transferase fusion protein containing the carboxyl-terminal 54 amino acids of the rat AT$_1A$ receptor physically binds to Jak2 in an angiotensin II-dependent manner. Deletional analysis, using both in vitro protocols and cell transfection analysis, showed that this association is dependent on the AT$_1A$ receptor motif YIPP (amino acids 319–322). The wild-type AT$_1A$ receptor can induce Jak2 tyrosine phosphorylation. In contrast, an AT$_1A$ receptor lacking the YIPP motif is unable to induce ligand-dependent phosphorylation of Jak2. Competition experiments with synthetic peptides suggest that each of the YIPP amino acids, including tyrosine 319, is important in Jak2 binding to the AT$_1A$ receptor. The binding of the AT$_1A$ receptor to the intracellular tyrosine kinase Jak2 supports the concept that the seven-transmembrane superfamily of receptors can physically associate with enzymatically active intracellular proteins, creating a signaling complex mechanistically similar to that observed with growth factor and cytokine receptors.

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The analysis of cytokines and their receptors has implicated the intracellular Jak family of kinases as critically important for the intracellular signaling initiated in response to ligand (1–3). Cytokines induce receptor dimerization and the activation, via tyrosine phosphorylation, of the associated Jak kinases. The Jak kinases phosphorylate the cytokine receptors, leading to the binding and eventual activation of intermediate signaling molecules referred to as STAT (signal transducers and activators of transcription). The STAT proteins are a family of transcription factors that migrate to the nucleus and induce gene transcription (4). The Jak/STAT pathway was first elucidated through the study of interferon signaling, but it is now known that this pathway participates in the signaling initiated by a wide variety of cytokines and growth factors. Recently, the vasoactive peptide angiotensin II was also found to activate the Jak/STAT pathway (5).

Angiotensin II is the effector molecule of the renin-angiotensin system. It is an 8-amino acid peptide that induces several physiologic responses that act to raise blood pressure. Virtually all of its biochemical actions are mediated through a single class of cell-surface receptors called AT$_1$ (6). Whereas humans have a single AT$_1$ receptor gene, rodents possess two genes encoding highly homologous receptor isoforms termed AT$_{1A}$ and AT$_{1B}$. These proteins are 95% identical and appear to bind ligand and to signal in an identical fashion (7, 8). All AT$_1$ receptors contain the structural features of the seven-transmembrane, G-protein-coupled receptor superfamily and are structurally quite different from cytokine receptors. However, studies by our group (5) and by Baker and co-workers (9, 10) have independently demonstrated that angiotensin II, acting through the AT$_1$ receptor, also stimulates the Jak/STAT pathway. In rat aortic smooth muscle (RASM) cells, angiotensin II leads to the rapid tyrosine phosphorylation and activation of Jak2 (5). Angiotensin II also induces the physical association of Jak2 with the AT$_1$ receptor. The AT$_1$ receptor contains no intrinsic kinase activity. However, it is now known that ligand occupancy of this receptor stimulates several different intracellular signaling cascades in which tyrosine phosphorylation plays an important role (11). At present, the structural features of the AT$_1$ receptor necessary for intracellular tyrosine kinase activation are not understood. In this study, we show that the carboxyl-terminal 54 amino acids of the rat AT$_{1A}$ receptor physically bind to Jak2 in an angiotensin II-dependent manner. Both in vitro and in vivo analyses show that this association is dependent on the AT$_{1A}$ receptor motif YIPP (amino acids 319–322). The binding of the AT$_{1A}$ receptor to the intracellular tyrosine kinase Jak2 supports the concept that the seven-transmembrane superfamily of receptors can physically associate with enzymatically active intracellular proteins, creating a signaling complex mechanistically similar to that observed with growth factor and cytokine receptors.

EXPERIMENTAL PROCEDURES

Cell Culture—RASM cells were cultured to near confluence at 37 °C under 5% CO$_2$ in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal serum and supplemented with antibiotics. The cells were

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1 The abbreviations used are: RASM, rat aortic smooth muscle; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase.
growth-arrested by incubation in serum-free DMEM for 36–48 h prior to angiotensin II stimulation.

Preparation of RASM Cell Lysates—Growth-arrested RASM cells were stimulated with 100 μM angiotensin II for varying times, washed two times with phosphate-buffered saline containing 1 mM Na2VO4, and lysed at 4°C with a solution containing 100 μM aprotinin and 10 μM leupeptin. The cell lysate was collected by scraping the monolayer into a microcentrifuge tube, and the tube was centrifuged to sediment any remaining debris. The supernatant was then used for the GST pull-down experiments.

GST Fusion Protein Construction—A 166-base pair fragment of the Ca18b cDNA encoding the AT1A receptor was amplified by polymerase chain reaction and cloned into the pGEX-KG vector via XhoI and BamHI restriction sites (13, 14). Point mutations were created using the Bio-Rad Muta-Gene phagemid kit. Other deletion constructs were made using polymerase chain reaction. All constructs were verified by DNA sequence analysis.

Jak2 Binding Assay—GST fusion proteins were expressed in Escherichia coli DH5α cells and purified by affinity chromatography using immobilized glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.). Glutathione-Sepharose beads containing 5 μg of fusion protein or GST were incubated with 1.0 ml of RASM cell lysate (0.9–1.0 mg of protein) for 2 h at 4°C. The beads were washed three to four times with icecold lysis buffer containing 1 M NaCl (deletion construct D-85 (see Fig. 2, lower panel, lanes 10 and 11) was washed in 1.5 M NaCl), and the bound proteins were eluted with SDS sample buffer. Eluted GST fusion proteins were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with Jak2 polyclonal antisera (Upstate Biotechnology, Inc.).

Physical Association of Jak2 Kinase with the AT1A Receptor

RESULTS AND DISCUSSION

A GST fusion protein containing the carboxyl-terminal 54 amino acids of the AT1A receptor was covalently linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. The column was then blocked with 1 μl of glycine. The Jak2 binding assay was similar to that described above except that binding was assessed in the presence of increasing concentrations of competitor protein as indicated in Fig. 1B.

Peptide Synthesis—The synthetic peptides were synthesized using an Applied Biosystems Model 420 synthesizer. The peptides were purified by high-performance liquid chromatography, and the identity of each peptide was verified by mass spectrometry using a Jedu SX-102 instrument. The amino acid sequences of the synthetic peptides are as follows: peptide P1, 311YIPPKRT222; peptide P2, IKKPPKYP219 (scrambled version of peptide P1); and peptide P3, 311KYFLQKL315. In addition, we synthesized mutant versions of peptide P1 designated peptide P4 (KYAPPKKA), peptide P5 (KYYAPPKA), peptide P6 (KYIAPPKA), peptide P7 (KYAAAKKA), and peptide P8 (KFIPPKKA). The change in the amino acid sequence of peptide P1 is underlined.

Cell Transfection—A rat AT1A receptor containing a mutation of the 311YIPP motif to FAAA was created using the Bio-Rad Mutagenesis Phagemid kit. This construct, as well as the wild-type AT1A receptor, was cloned into the XhoI site of the mammalian expression vector pCMVneo (Invitrogen, San Diego, CA).

COS-7 cells were transfected at 60% confluence in 100-mm tissue culture dishes using Lipofectin (Life Technologies Inc.). Each dish was washed once and layered with 3 ml of DMEM containing 2.5 μg of a plasmid encoding murine Jak2 (pBOSwtJk2) (16), 10 μg of the appropriate AT1A receptor expression plasmid, and 20 μl of Lipofectin. After 5 h at 37°C, the Lipofectin-containing medium was aspirated, replaced with serum-containing DMEM, and incubated for 24–28 h. Plates were then washed and incubated for 16–20 h in 5 ml of serum-free DMEM supplemented with 0.5% (w/v) bovine serum albumin. Cells were stimulated with 100 ng/mL angiotensin II for the indicated times, and lysates were prepared as described above. To assess association of Jak2 with the AT1A receptor, the receptor was immunoprecipitated using a polyclonal antibody directed against the carboxyl terminal of the AT1A receptor (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were then probed with Jak2 polyclonal antisera. To measure the tyrosine phosphorylation of Jak2, the protein was immunoprecipitated using a polyclonal anti-Jak2 antibody (Santa Cruz Biotechnology). Immunoprecipitates were collected by the addition of 20 μl of Protein A/G Plus (Santa Cruz Biotechnology, Inc.). Precipitates were washed three times with radioimmune precipitation assay lysis buffer (RIPA), and proteins were eluted by boiling in SDS sample buffer. Proteins were separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted as described above.

Binding Assays—COS-7 cells were transfected as described above. Approximately 30–32 h after transfection, cells were trypsinized and seeded at a density of 1.5 × 105 cells/well using 24-well tissue culture plates. The following day, transfected cells were tested for their ability to bind [125I-Sar1,Ile8]angiotensin II (DuPont). 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 0.05% (w/v) bovine serum albumin) 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. Plates were set at 1 nm, and serial dilutions were made 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 icecold binding buffer without bovine serum albumin. Cells were lysed with 250 μl of 2% NaOH for 30 min at 25°C, and 150 μl was counted on a Beckman γ-counter. Protein concentrations were determined using the Bio-Rad D protein assay with the remaining lysates. Nonspecific binding was defined as binding in the presence of 1.0 μM angiotensin II.

Effect of Peptide P1 on Jak2 Binding to the GST-AT1A Fusion Protein

10 separate experiments. Maximal Jak2 binding was observed 3 min after the addition of angiotensin II (lane 1). Jak2 did not bind to the fusion protein, an observation that was confirmed in >10 separate experiments. Maximal Jak2 binding was observed 3 min after the addition of angiotensin II (lane 4). Treatment of RASM cells with angiotensin II for longer periods of time (lanes 5 and 6) resulted in less Jak2 binding to the GST-AT1A fusion protein. An equivalent experiment was performed using GST (lanes 7 and 8); no Jak2 binding to GST was detected.

We have also used an identical protocol to investigate the binding of Jak1 and Tyk2. In contrast to Jak2, angiotensin II induced no detectable complex formation of Jak1 or Tyk2 with the GST-AT1A fusion protein (data not shown).

To investigate the specificity of the binding assay, we covalently linked the GST-AT1A fusion protein to an agarose matrix. This preparation allowed us to compete for the binding of Jak2 to the GST-AT1A fusion protein matrix with increasing concentrations of free GST-AT1A fusion protein, GST, or an unrelated protein such as albumin (Fig. 1B). Lane 1 shows that in the absence of a competitor, there was a substantial binding of Jak2 to the GST-AT1A fusion protein matrix. Increasing concentrations of free GST-AT1A fusion protein competed with this binding (lanes 2–4). In contrast, free GST or bovine serum albumin showed no competition with the binding of Jak2 to the fusion protein matrix (lanes 5–8).
lanes 2–8

fusion protein. In

fusion protein was covalently attached to agarose. RASM cells were

buffer containing 1M NaCl, and 130-kDa Jak2 was detected by Western

bound to glutathione-Sepharose. Protein complexes were washed in

a RASM cell lysate was prepared from cells treated with angiotensin II

receptor fusion protein (lanes 5–8). (Fig. 2,

binding (lanes 5–8). For each of these proteins

sequence.

in vitro

AT1A fusion protein (Fig. 4A). Peptide P1 demonstrated a dose-dependent inhibition of

JAK2 binding to the AT1A receptor fusion protein (lanes 2–4). No inhibition was observed using either the scrambled peptide

(P2) or the peptide encompassing amino acids 311–318 (P3) (lanes 5–10). Even when 50 μg of competitor peptide P2 or P3 was used, no competition was observed (data not shown). On a molar basis, peptide P1 is a less effective inhibitor than the entire GST-AT1A fusion protein (Fig. 1B), a finding probably related to the small size of the peptide.

The specificity of individual amino acids within the YIPP motif was tested by synthesizing peptides P4–P8 and testing the ability of each peptide to inhibit Jak2 binding to the GST-AT1A fusion protein (Fig. 4B). Competition assays were performed using 10 μg of competitor peptide. These data demonstrate that peptides containing a single change in the IPP portion of peptide P1 were no longer able to compete (Peptides P4–P6). Peptide P7, containing a 3-amino acid substitution, was also unable to compete. Peptide P8, containing FIPP in place of YIPP, showed a partial ability to inhibit Jak2 binding to the GST-AT1A fusion protein. These results are consistent with data in Fig. 3, showing that a GST-AT1A fusion protein with a Y319F mutation bound Jak2, but with reduced efficiency.

To investigate the behavior of the AT1A receptor carboxyl tail in vivo, RASM cells were electroporated with the GST-AT1A fusion protein; deletion construct D-35, D-55, or D-85; or GST (Fig. 5). We have previously shown that electroporation is an effective method of inserting proteins into RASM cells (15). After electroporation, the cells were incubated for 30 min at 37 °C and then washed extensively. Angiotensin II was added for 3 min, and the cells were lysed and incubated with glutathione-Sepharose beads at 4 °C. The beads were washed with buffer containing 1 M NaCl and eluted with SDS sample buffer. Jak2 was then assessed by Western blot analysis. In the absence of angiotensin II, no Jak2 associated with the GST-AT1A fusion protein (data not shown). However, after angiotensin II addition, both the GST-AT1A fusion protein and deletion construct D-35 bound Jak2 (Fig. 5, lanes 1 and 2). In contrast, deletion constructs D-55 and D-85, both of which lack the YIPP motif, bound very much less Jak2 (lanes 3 and 4). No binding of Jak2 was observed with GST (lane 5). These data recapitulate the in vitro studies and indicate the importance of the YIPP sequence.

We have also used a cell transfection approach to evaluate the importance of the YIPP motif. These experiments used the mammalian expression vector pZeo containing either a full-length, wild-type AT1A receptor (called pZeo/WT) or a construct in which the YIPP motif was converted to FAA (called pZeo/FAAA). Transient transfection was performed using COS-7 cells, which contain little endogenous AT1 receptor or Jak2 (data not shown). Initial experiments used standard procedures to measure the binding of [125I]-Sar1,Ile8]angiotensin II to the wild-type receptor or the FAA mutant expressed in these cells. Scatchard analysis indicated Kd values of 0.172 nM for the wild-type receptor and 0.225 nM for the FAA mutant. Thus, the conversion of the YIPP motif to FAA does not markedly

![Figure 1: Jak2 binds to the GST-AT1A receptor fusion protein](image)

**Figure 1: Jak2 binds to the GST-AT1A receptor fusion protein.**

A, a RASM cell lysate was prepared from cells treated with angiotensin II for 0, 0.5, 1, 3, 6, and 10 min (lanes 1–6, respectively). 1 mg of cell protein was incubated for 2 h with 5 μg of GST-AT1A fusion protein bound to glutathione-Sepharose. Protein complexes were washed in buffer containing 1 M NaCl and 130-kDa Jak2 was detected by Western blot analysis. In the absence of ligand (lane 1), Jak2 did not bind to the fusion protein. In response to angiotensin II, Jak2 bound to the fusion protein in a time-dependent fashion, with maximal binding present 3 min after ligand addition (lane 4). Using a similar protocol, Jak2 showed no binding to GST (lanes 7 and 8). B, the GST-AT1A receptor fusion protein was covalently attached to agarose. RASM cells were treated with angiotensin II for 3 min, and a cell lysate was prepared. Lane 1 shows that Jak2 binds to the immobilized GST-AT1A receptor fusion protein. In lanes 2–8, increasing concentrations of soluble competitor proteins were added to the cell lysate as indicated. Free AT1A receptor fusion protein showed a dose-dependent inhibition of Jak2 binding (lanes 2–4). In contrast, free GST or bovine serum albumin (BSA) had no effect on the binding of Jak2 to the immobilized GST-AT1A receptor fusion protein (lanes 5–8).

To identify specific regions within the AT1A receptor carboxyl tail important for the association with Jak2, we created a series of GST fusion proteins containing overlapping portions of the AT1A receptor tail (Fig. 2, upper panel). Each of these proteins was purified and individually tested for the ability to bind to Jak2 kinase. As before, Jak2 association was quantitated by Western blot analysis. We observed that fusion proteins containing the motif YIPP (Tyr-Ile-Pro-Pro, amino acids 319–322) bound Jak2 in a fashion similar to the full-length construct (Fig. 2, lower panel, lanes 1–4, 7, and 10). Fusion protein lacking this motif showed either markedly reduced or no binding of Jak2 (lanes 5, 6, 8, 9, and 11). The carboxyl-terminal 54 amino acids of the AT1A receptor contain tyrosine residues at positions 319 and 339. While Jak2 association with cytokine receptors is not thought to be dependent on tyrosine residues, the presence of a tyrosine in the 319YIPP motif convinced us to make GST-AT1A fusion proteins in which these individual tyrosine residues were converted to phenylalanine. When the conversion at position 319 was made, the GST fusion protein bound less Jak2 than the parent protein (Fig. 3, lower panel, lane 2). This was consistently observed in five separate experiments. In contrast, the Y339F change had no effect on Jak2 binding (lane 3). To further investigate the specificity of the YIPP motif, we created an 8-amino acid peptide (KYIPPKAK) corresponding to positions 318–325 of the AT1A receptor. As controls, we also synthesized this peptide in a scrambled configuration (IKKPAPYK) and a peptide corresponding to positions 311–318 of the AT1A receptor (KYFQLLK). Increasing amounts of the synthetic peptides were used to compete for the angiotensin II-dependent binding of Jak2 to a GST-AT1A fusion protein containing the carboxyl-terminal 54 amino acids of the receptor (Fig. 4A). Peptide P1 demonstrated a dose-dependent inhibition of Jak2 binding to the AT1A receptor fusion protein (lanes 2–4). No inhibition was observed using either the scrambled peptide (P2) or the peptide encompassing amino acids 311–318 (P3) (lanes 5–10). Even when 50 μg of competitor peptide P2 or P3 was used, no competition was observed (data not shown). On a molar basis, peptide P1 is a less effective inhibitor than the entire GST-AT1A fusion protein (Fig. 1B), a finding probably related to the small size of the peptide.

The specificity of individual amino acids within the YIPP motif was tested by synthesizing peptides P4–P8 and testing the ability of each peptide to inhibit Jak2 binding to the GST-AT1A fusion protein (Fig. 4B). Competition assays were performed using 10 μg of competitor peptide. These data demonstrate that peptides containing a single change in the IPP portion of peptide P1 were no longer able to compete (Peptides P4–P6). Peptide P7, containing a 3-amino acid substitution, was also unable to compete. Peptide P8, containing FIPP in place of YIPP, showed a partial ability to inhibit Jak2 binding to the GST-AT1A fusion protein. These results are consistent with data in Fig. 3, showing that a GST-AT1A fusion protein with a Y319F mutation bound Jak2, but with reduced efficiency.

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affect ligand affinity.

To measure the physical association of the AT$_{1A}$ receptor with Jak2, constructs encoding both these proteins were transfected into COS-7 cells (Fig. 6). Two days after transfection, the cells were treated with angiotensin II for 0, 3, 6, or 15 min. Cells were lysed, and proteins were immunoprecipitated using a rabbit polyclonal anti-rat AT$_{1A}$ receptor antibody and Protein A/G. The precipitated proteins were washed, and associated Jak2 was measured by Western blot analysis using polyclonal anti-Jak2 antibody. Controls for this protocol included transfection of the vector pZeo lacking any angiotensin II receptor insert (lanes 1–4, 7, and 10); fusion proteins lacking this motif showed either no or very much reduced binding of Jak2 (lanes 5, 6, 8, 9, and 11). W.T., wild type.

In smooth muscle cells, angiotensin II stimulates the tyrosine phosphorylation of Jak2 (5). We have analyzed this using cotransfection of the AT$_{1A}$ receptor and Jak2 into COS-7 cells. Two days after transfection, cells were treated with angiotensin II, lysed, and immunoprecipitated with a rabbit polyclonal anti-rat AT$_{1A}$ receptor antibody and Protein A/G. The precipitated proteins were washed, and associated Jak2 was measured by Western blot analysis using polyclonal anti-Jak2 antibody. Controls for this protocol included transfection of the vector pZeo lacking any angiotensin II receptor insert (lanes 1–4, 7, and 10); fusion proteins lacking this motif showed either no or very much reduced binding of Jak2 (lanes 5, 6, 8, 9, and 11). W.T., wild type.

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A GST fusion protein containing the carboxyl-terminal portion of the rat AT1A receptor. This association appears to be dependent on the receptor motif YIPP and is stable to washing in 1.5 M NaCl (Fig. 2, lower panel, lane 10). Transient cellular expression of either the wild-type AT1A receptor or the receptor containing a mutation of the YIPP motif confirmed the importance of this sequence in the association of Jak2 with the seven-transmembrane AT1A receptor (lanes 2–4). Inhibiting the binding of Jak2 to a dose-dependent fashion. Peptide P2 (IKKAPPKAK) contains the same amino acids as peptide P1, but in a scrambled configuration. This peptide showed no inhibition of Jak2 binding (lanes 5–7). Peptide P3 (KYFLQLK), containing amino acids 311–318 of the AT1A receptor, also did not inhibit the binding of Jak2 (lanes 8–10). All experiments were performed with a RASM cell lysate prepared from cells treated with angiotensin II for 3 min. B, inhibition studies similar to those described in A were performed with 10 μg of peptides P1 and P4–P8. Peptides P4–P6 contain single alanine substitutions for the sequence IPP (amino acids 320–322), but were unable to inhibit the binding of Jak2 to the GST-AT1A receptor fusion protein. Peptide P8, containing phenylalanine in place of tyrosine 319, has a reduced capacity to inhibit the binding of Jak2.

Changes induced by angiotensin II are not known. Presumably, ligand binding to the AT1A receptors within cells changes the chemistry of these cells such that the Jak2 present in a cell lysate now binds to the GST-receptor fusion proteins. Whether our protocol is stripping activated Jak2 from endogenous receptors or whether activated Jak2 is naturally shuttling onto and off of AT1 receptors is not known. Indeed, it is not known whether the association of Jak2 with the GST-AT1A fusion protein is a bimolecular event or whether additional linker molecules participate. Finally, we are not certain if the Jak2-AT1A association that occurs within cells is a precedent or a consequence of Jak2 activation.

We have considered the possibility that the interaction of Jak2 with the GST-AT1A fusion protein is the result of the YIPP sequence acting as a substrate-binding site for Jak2. This seems unlikely given that Jak2 still shows affinity for a receptor fusion protein lacking Tyr319 (Fig. 3). A peptide lacking this same tyrosine also showed some ability to inhibit the interaction of Jak2 with the GST-AT1A fusion protein (Fig. 4B). To
The wild-type AT1A receptor induced increased Jak2 tyrosine phosphorylation. Cell lysates (lanes 1–4) were preincubated with or without anti-Jak2 antibody, followed by Western blot analysis of the precipitated proteins with anti-phosphotyrosine antibody. The wild-type AT1A receptor induced increased Jak2 tyrosine phosphorylation in response to angiotensin II (lanes 5–8). After the addition of angiotensin II, the level of Jak2 tyrosine phosphorylation was measured by immunoprecipitation of proteins using a Jak2 antibody. No increase was observed with the AT1A receptor lacking the YIPP motif (lanes 9–12). In the absence of transfected Jak2 (lane 13), no Jak2 was identified using this protocol.

Previous studies of Jak2 binding to cytokine receptors have demonstrated the importance of the SH2 domain that interacts with the indicated SH2 target sequences when the tyrosine is phosphorylated. For instance, phospholipase C-γ1 contains an SH2 domain that interacts with the indicated SH2 target sequences of the platelet-derived growth factor and epidermal growth factor receptors. In contrast to phospholipase C-γ, the study of cytokine receptors has suggested that Jak2 binding is not dependent on tyrosines and is consistent with the lack of an SH2 domain in these kinases. Whether our data, showing an effect of Tyr319 on Jak2 binding, can be explained by a nonspecific steric effect or whether these data indicate that tyrosine (and perhaps a linker protein) may play a specific role in Jak2-AT1A receptor association is an issue that still must be addressed.

The study of growth factor receptors has also demonstrated that ligand binding induces receptor dimerization and the assembly of a signaling complex. This complex consists of the receptor, linker proteins, and enzymatically active molecules (such as Jak2) capable of transducing the binding of ligand into an intracellular signaling cascade. Whether this sequence acts alone to define the Jak2-binding site or acts in combination with other regions of the AT1A receptor is an issue that still must be addressed.

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12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
13. Murphy, T. J., Alexander, R. W., Griendling, K. K., Runge, M. S., and Bernstein, K. E. (1991) Nature 351, 233–236
14. Griendling, K. K., Alexander, R. W., Murphy, T. J., Runge, M. S., and Bernstein, K. E. (1991) J. Biol. Chem. 266, 15744–15748
15. Zhuang, H., Patel, S. V., He, T., Sonstebey, S. K., Niu, Z., and Wojchowski, D. M. (1994) J. Biol. Chem. 269, 11349–11353
16. Lebrun, J.-J., Ali, S., Ullrich, A., and Kelly, P. A. (1995) J. Biol. Chem. 270, 10664–10670
17. Colamonici, O., Yan, H., Domanski, P., Handa, R., Smalley, D., Mullersman, J., Witte, M., Krishnan, K., and Kroler, J. (1994) Mol. Cell. Biol. 14, 8133–8142
18. Dinerstein, H., Lago, F., Goujon, L., Ferrag, F., Esposito, N., Finidori, J., Kelly, P. A., and Poatel-Vinay, M.-C. (1995) Mol. Endocrinol. 9, 1751–1757
19. Tanner, J. W., Chen, W., Young, R. L., Longmore, G. D., and Shaw, A. S. (1995) J. Biol. Chem. 270, 6523–6530
20. Marrero, M. B., Schieffer, B., Paxton, W. G., Duff, J. L., Berk, B. C., and Bernstein, K. E. (1995) Cardiovasc. Res. 30, 530–536
21. Berk, B. C., and Corson, M. A. (1997) Circ. Res. 80, 607–616
22. Pascal, S. M., Singer, A. U., Gish, G., Yamazaki, T., Shoelson, S. E., Pawson, T., Kay, L. E., and Forman, K.-J. D. (1994) Cell 77, 461–472
23. Ohyama, R., Yamano, Y., Chaki, S., Kondo, T., and Inagami, T. (1992) Biochem. Biophys. Res. Commun. 189, 677–683
24. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) Annu. Rev. Biochem. 62, 455–481
25. Malarkey, K., Belham, C. M., Paul, A., Graham, A., McLees, A., Scott, P. H., and Plevin, R. (1995) Biochem. J. 309, 361–375