Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2

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1Vascular Biology Center, 2Department of Pharmacology and Toxicology, and 3Department of Pediatrics, Medical College of Georgia, Augusta 30912; and 4Center for Cell and Molecular Signaling, Emory University School of Medicine, Atlanta, Georgia 30322

Marrero, Mario B., Virginia J. Venema, Hong J u, Douglas C. Eaton, and Richard C. Venema. Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. Am. J. Physiol. 275 (Cell Physiol. 44): C1216–C1223, 1998.—Angiotensin II (ANG II) exerts its effects on vascular smooth muscle cells through G protein-coupled AT1 receptors. ANG II stimulation activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway by inducing tyrosine phosphorylation, activation, and association of JAK2 with the receptor. Association appears to be required for JAK2 phosphorylation. In the present study, electroporation experiments with neutralizing anti-Src homology phosphatase-1 (SHP-1) and anti-SHP-2 antibodies and time course determinations of SHP-1 and SHP-2 activation and complexation with JAK2 suggest that the tyrosine phosphatases, SHP-1 and SHP-2, have opposite roles in ANG II-induced JAK2 phosphorylation. SHP-1 appears responsible for JAK2 dephosphorylation and termination of the ANG II-induced JAK/STAT cascade. SHP-2 appears to have an essential role in JAK2 phosphorylation and initiation of the ANG II-induced JAK/STAT cascade leading to cell proliferation. The motif in the AT1 receptor that is required for association with JAK2 is also required for association with SHP-2. Furthermore, SHP-2 is required for JAK2-receptor association. SHP-2 may thus play a role as an adaptor protein for JAK2 association with the receptor, thereby facilitating JAK2 phosphorylation and activation.

vascular smooth muscle cells; tyrosine phosphatases; adaptor protein

THE JANUS KINASE (JAK)/signal transducers and activators of transcription (STAT) pathway is an important link between activation of cell surface receptors and nuclear transcriptional events leading to cell growth (8, 28). Signaling through this pathway was first elucidated for the cytokine family of receptors, including those for the interleukins and interferons. Recently, however, we and others have shown that the JAK/STAT cascade can also be activated by G protein-coupled receptors (6, 7, 11, 21, 26). For example, ligand binding to the ANG II AT1 receptor in vascular smooth muscle cells (VSMC) induces the rapid tyrosine phosphorylation and activation of JAK2 (21). The JAK family members, STAT1 and STAT2, are also tyrosine phosphorylated in response to ANG II, accompanied by a translocation of STAT1 to the nucleus. Nuclear translocation very likely activates transcription of early growth response genes. Consistent with this hypothesis are our recent observations that the pharmacological JAK2 inhibitor, AG490, or electroporation of blocking antibodies against STAT1 and STAT3 inhibits ANG II-induced VSMC proliferation and DNA synthesis (20). JAK2 appears to physically associate with the AT1 receptor because it can be communoprecipitated from VSMC lysates with anti-AT1 receptor antibodies (21). Association is mediated by ANG II-dependent binding of JAK2 to a YIPP motif in the COOH-terminal tail of the receptor. Furthermore, binding of the enzyme to the receptor appears to be required for JAK2 phosphorylation (3).

The phosphorylation states and activities of enzymes that are regulated by tyrosine phosphorylation are controlled by the combined actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPases) (29). Two cytoplasmicSrc homology 2 (SH2) domain-containing PTPases, termed SHP-1 and SHP-2, have been identified that interact with and regulate JAK2 in response to cytokine receptor activation. SHP-1 [formerly termed PTP1c, SH-PTP1, SHP, and HCP (1)] terminates proliferative signaling from hematopoietic cell cytokine receptors such as the interleukin-3 and erythropoietin receptors (16, 37). Downregulation of the erythropoietin-induced JAK/STAT cascade occurs through direct dephosphorylation of the JAK2 kinase by SHP-1 (14). The SHP-2 phosphatase [formerly termed SHPTP-2, SHPTP-3, Syp, PTP2C, and PTP1D (1)], in contrast to SHP-1, which is a negative effector of mitogenic signaling, frequently functions as a positive effector of growth-stimulatory signaling pathways. This important role of SHP-2 has been well documented for the tyrosine kinase growth factor receptors including those of platelet-derived growth factor, epidermal growth factor, insulin-like growth factor, insulin, and nerve growth factor (13, 15, 17, 23–25, 35, 36, 39). In these pathways, SHP-2 appears to interact directly with the mitogenic receptor via its two SH2 domains. It has also been shown for the platelet-derived growth factor and c-kit receptors that SHP-2 acts as an adaptor protein mediating the association of the Grb2-Sos complex with the receptor (5, 18, 30). In addition, SHP-2 has been shown to form a complex with JAK2 in hematopoietic cells in response to interleukins, erythropoietin, and prolactin (4, 10, 31, 34). Furthermore, SHP-2, JAK2, and the prolactin receptor form a trimeric complex in these cells, consistent with the possibility that SHP-2 may also function as an adaptor protein in this context. Further evidence that complex forma-
tion of SHP-2 with JAK2 may serve a function other than to dephosphorylate JAK2 is provided by the observation that interaction is not dependent on either the phosphatase activity or the SH2 domains of SHP-2 (38).

Recently, we reported that ANG II stimulates the tyrosine phosphorylation and activation of SHP-2 in VSMC (2). Whether SHP-2 has a role in ANG II-induced JAK/STAT signaling in VSMC has not been determined. Also unclear is the role, if any, of SHP-1 in this signaling pathway. Therefore, in the present study, we have examined the role of the PTPases, SHP-1 and SHP-2, in ANG II-induced tyrosine phosphorylation and regulation of JAK2 in VSMC.

MATERIALS AND METHODS

Materials. Tween 20, acrylamide, SDS, N,N’-methylenebisacrylamide, N,N,N’,N’-tetramethylethylenediamine, protein assay reagents, and nitrocellulose membranes were purchased from Bio-Rad Laboratories. Molecular weight standards, immunoprecipitin, protein A and G agarose, DMEM, fetal bovine serum, trypsin, and all medium additives were obtained from Life Technologies. Monoclonal antibodies to phosphotyrosine were obtained from Transduction Laboratories (PY20) and Upstate Biotechnology (4G10). Anti-SHP-1 and anti-SHP-2 antibodies were obtained from either Santa Cruz Biotechnology or Transduction Laboratories. PTPase assay kit 1 was purchased from Upstate Biotechnology. CellTiter 96 AQuesous nonradioactive cell proliferation assay kit 1 was purchased from Promega. The enhanced chemiluminescence kit was obtained from Amersham. ANG II, goat anti-mouse IgG, and all other chemicals were purchased from Sigma Chemical.

Cell culture. VSMC from 200–300 g male Sprague-Dawley rat aortas were isolated and maintained in DMEM supplemented with 10% (vol/vol) fetal bovine serum, 10 mg/ml streptomycin, and 100 U/ml penicillin at 37°C in a 5% CO2-enriched, humidified atmosphere as previously described (19). Cells were subcultured at 1:5 or 1:10 at 7-day intervals, and the medium was changed at 2- to 3-day intervals, and the medium was changed at 2- to 3-day intervals. VSMC passages 8–15 were grown to 70% confluence, washed once with serum-free DMEM, and growth-arrested in 5 ml serum-free DMEM for 24–48 h before ANG II stimulation.

Electroporation. Growth-arrested VSMC were electroporated in tissue culture dishes using a petri dish electrode (100 mm in diameter with 2 mm gap) manufactured by BTX as described previously (3, 20, 22, 27). Electroporation was performed in Ca2+-free and Mg2+-free Hank’s balanced salt solution (in mM: 5 KCl, 0.3 KH2PO4, 138 NaCl, 4 NaHCO3, and 0.3 NaHPO4, pH 7.4) containing anti-SHP-1 or anti-SHP-2 polyclonal antibodies or rabbit IgG at a final concentration of 10 µg/ml. Cells were exposed to 1 pulse at 100 V for 40 ms (square wave) using a BTX model T820 ElectrosquarePoration. We have previously shown by flow cytometry that these electroporation parameters efficiently electroinject antibodies into VSMC without adversely affecting their viability (22). The tissue culture plates were then incubated for 30 min at 37°C (5% CO2). Finally, the plates were washed once with serum-free DMEM and then incubated in 5 ml serum-free DMEM for 30 min at 37°C.

Immunoprecipitation and immunoblotting. VSMC were stimulated with ANG II (10−7 M) for various times ranging from 1 to 60 min. The reaction was terminated by washing the cells twice with ice-cold PBS (in mM: 10 NaHPO4, 1.7 KH2PO4, 136 NaCl, 2.6 KCl, and 1 Na2VO4, pH 7.4). Cells were then lysed in ice-cold lysis buffer [25 mM Tris, 1% NP-40, 10% glycerol, 50 mM NaF, 10 mM Na3VO4, 137 mM NaCl, 2 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotonin, and 10 µg/ml leupeptin, pH 7.5]. The lysate was scraped from the plate and centrifuged for 20 min at 6,000 g at 4°C. Protein concentration was measured in the cleared supernatant by Bio-Rad detergent-compatible protein assay. To immunoprecipitate tyrosine-phosphorylated proteins from the cleared lysate, 10 µg/ml of anti-phosphotyrosine monoclonal antibody was added to the lysate. Antibodies were allowed to equilibrate with the lysate overnight at 4°C followed by the addition of protein A/G plus agarose for an additional 2 h at 4°C. The immunoprecipitates were then recovered by centrifugation and washed three times in 1 ml of ice-cold wash buffer (50 mM Tris·HCl, 150 mM NaCl, and 0.1% Triton X-100, pH 8.0). The immunoprecipitated proteins were dissolved in 80 µl of SDS sample buffer, boiled for 5 min at 95°C, and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane for 16 h at 100 mA. The membrane was blotted with anti-SHP-1, anti-SHP-2, or anti-JAK2 antibodies. Proteins were visualized using a horseradish peroxidase-conjugated goat anti-mouse or donkey anti-rabbit IgG and an enhanced chemiluminescence kit. In other experiments, the addition of antibodies was reversed. That is, VSMC proteins were initially immunoprecipitated with anti-JAK2, anti-SHP-2, or anti-SHP-2 antibodies and nitrocellulose membranes were probed with anti-phosphotyrosine antibody.

In vitro binding assays. AT,glutathione S-transferase (GST) fusion proteins, the construction and expression of which has been described previously (3, 33), were expressed in Escherichia coli DH5α cells and purified by affinity chromatography using immobilized glutathione-Sepharose 4B beads. Glutathione Sepharose beads containing 5 µg of fusion proteins or GST alone were incubated with 1.0 ml of VSMC lysate (0.9–1.0 mg protein) for 2 h at 4°C. The beads were washed three to four times with ice-cold lysis buffer containing 1 M NaCl, and the bound proteins were eluted with SDS sample buffer. Eluted proteins were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with a SHP-2 monoclonal antibody.

Tyrosine phosphatase activity assay. SHP-1 and SHP-2 proteins were immunoprecipitated from VSMC lysates and immunocomplexes were washed three times with ice-cold wash buffer and then three times with phosphate buffer (50 mM HEPES, 60 mM NaCl, 60 mM KCl, 0.1 mM PMSF, 10 µg/ml aprotonin, and 10 µg/ml leupeptin, pH 7.4). Phosphatase activity was assessed by monitoring the rate of formation of p-nitrophenol by dephosphorylation of p-nitrophenyl phosphate. Immunocomplex pellets were thus resuspended in 100 µl of phosphate buffer containing 1 mg/ml BSA, 5 mM EDTA, and 10 mM dithiothreitol. The reaction was initiated by the addition of p-nitrophenyl phosphate (10 mM final concentration). After a 30-min incubation at room temperature, the reaction was stopped by the addition of 1 M NaOH and absorbance of the sample was determined at 410 nm in a spectrophotometer.
RESULTS AND DISCUSSION

We have shown previously that ANG II induces the rapid tyrosine phosphorylation and activation of both JAK2 and SHP-2 in VSMC (2, 21). We have also shown previously that electroinjection into VSMC of specific antibodies against cellular signaling proteins is an effective means of interrupting ANG II-induced signal transduction cascades (2, 20, 22, 27). Under the conditions of electroporation used in these previous studies, ~80% of electroporated cells survive and, of these, >95% contain electroporated antibody (22). To examine the role of SHP-2 in ANG II-induced JAK2 phosphorylation, we electroporated VSMC under the previously established conditions with anti-SHP-2 antibodies before stimulation of the cells for various times with ANG II (10⁻⁷ M). Cells were lysed, and JAK2 was immunoprecipitated from lysates with anti-JAK2 antibody. Immunoprecipitated proteins were separated by gel electrophoresis, transferred to nitrocellulose, and then immunoblotted with anti-phosphotyrosine antibody. To examine the role of SHP-2 in ANG II-induced JAK2 phosphorylation, similar experiments were also carried out following electroporation of VSMC with anti-SHP-1 antibodies. As a control, cells were also electroporated with rabbit IgG (mock electroporation). As shown in Fig. 1, ANG II stimulated the tyrosine phosphorylation of JAK2 in mock-electroporated cells with a time course that reached a maximum at 10 min and returned to a low, basal level by 60 min. We have shown previously that a similar time course of JAK2 tyrosine phosphorylation occurs in response to ANG II stimulation in non-electroporated cells (21). However, when cells were electroporated with anti-SHP-2 antibody, the ANG II-induced tyrosine phosphorylation of JAK2 was completely blocked. In contrast, when cells were electroporated with anti-SHP-1 antibody, JAK2 dephosphorylation was completely blocked for at least 60 min. Time-dependent differences in the amounts of tyrosine-phosphorylated JAK2 observed in these experiments were not due to differences in the amounts of JAK2 immunoprecipitated by the anti-JAK2 antibody because, when JAK2 immunoprecipitates were subjected to immunoblotting with anti-JAK2 antibody, equal amounts of JAK2 were detected for all time points in all conditions (n = 3, data not shown). These results suggest that SHP-1 is the PTPase that dephosphorylates JAK2 following ANG II-induced JAK2 phosphorylation in VSMC. Thus, like erythropoietin receptor signaling in hematopoietic cells (14, 16), G protein-coupled ATP₁ receptor signaling in VSMC is dependent on a key role of SHP-1 in terminating the JAK/STAT cascade. SHP-2, on the other hand, appears to have a critical positive role in JAK2 signal transmission induced by the ATP₁ receptor similar to that described previously for growth factor and cytokine receptor signaling (13, 15, 17, 23–25, 35, 36, 39).

The conclusion that SHP-1, but not SHP-2, is the PTPase responsible for JAK2 dephosphorylation in ANG II-stimulated VSMC is further supported by the results of experiments designed to determine the time course of ANG II-induced SHP-1 and SHP-2 tyrosine phosphorylation and activation in VSMC. Cells were exposed to ANG II (10⁻⁷ M) for various times, and anti-phosphotyrosine antibody was used to immunoprecipitate tyrosine-phosphorylated proteins. Immunoblotting of the precipitated proteins with anti-SHP-2 antibody revealed a transient ANG II-induced tyrosine phosphorylation of SHP-2 that was maximal at 5 min and that returned to near basal levels by 30 min (Fig. 2A). In additional experiments, the order of antibody addition was reversed and anti-SHP-2 immunoprecipitates were probed on blots with anti-phosphotyrosine antibody. These experiments also showed a transient tyrosine phosphorylation of SHP-2 that was maximal at 5 min. When blots were stripped and reprobed with anti-SHP-2 antibody, equal amounts of SHP-2 were confirmed to be immunoprecipitated for each time point (n = 3, data not shown). When the time course of ANG II-induced tyrosine phosphorylation of SHP-1 was determined by immunoprecipitation with anti-phosphotyrosine antibody and immunoblotting with anti-SHP-1 antibody, a clearly different time dependency of phosphorylation was observed. In the case of SHP-1, a significant decrease in tyrosine phosphorylation was observed during the first 5 min followed by an increase after 30 min that reached levels significantly higher than control (Fig. 2A). Similar to experiments for SHP-2, experiments were also carried out for SHP-1 in which the order of antibody addition was reversed and anti-SHP-1 immunoprecipitates were immunoblotted with anti-phosphotyrosine. These experiments also
showed a decrease in tyrosine phosphorylation during the first 5 min followed by an increase after 30 min. When blots of anti-SHP-1 immunoprecipitates were stripped and reprobed with anti-SHP-1 antibody, equal amounts of the SHP-1 protein were confirmed to be immunoprecipitated for each time point (n = 3, data not shown). To determine whether ANG II-induced changes in the tyrosine phosphorylation state of SHP-1 and SHP-2 were correlated with changes in SHP-1 and SHP-2 catalytic activities, the phosphatase activities of anti-SHP-1 and anti-SHP-2 immunoprecipitates were assayed by measuring the rate of formation of p-nitrophenol from p-nitrophenyl phosphate. U/mg represents µmol of p-nitrophenol released per min per mg of total protein in the sample. Results represent means ± SE from 3 different experiments. PTPase, protein tyrosine phosphatase.

Fig. 2. ANG II-induced tyrosine phosphorylation and activation of SHP-2 and SHP-1 in VSMC. Cells were treated with ANG II (10^{-7} M) for the times indicated and then lysed. A: tyrosine-phosphorylated proteins were immunoprecipitated from lysates; precipitated proteins were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with either anti-SHP-2 or anti-SHP-1 antibody. Densitometric analysis of blots from 3 separate experiments (mean ± SE) is shown. B: SHP-2 and SHP-1 were immunoprecipitated from lysates, and phosphatase activity of the precipitated enzymes was assayed by measuring the rate of formation of p-nitrophenol from p-nitrophenyl phosphate. U/mg represents µmol of p-nitrophenol released per min per mg of total protein in the sample. Results represent means ± SE from 3 different experiments. PTPase, protein tyrosine phosphatase.

A role for SHP-1, but not SHP-2, in ANG II-induced JAK2 dephosphorylation is also suggested by experiments designed to determine the time course of ANG II-induced JAK2 complex formation with SHP-2 and SHP-1. VSMC were treated with ANG II (10^{-7} M) for various times, cells were lysed, and SHP-1 and SHP-2 were immunoprecipitated from lysates with anti-SHP-1 and anti-SHP-2 antibodies. Equal amounts of SHP-1 and SHP-2 were confirmed to be immunoprecipitated at each time point by immunoblotting samples with the same antibody used in immunoprecipitation experiments (n = 3, data not shown). Immunoprecipitates were then immunoblotted with anti-JAK2. As shown in Fig. 3A, the anti-SHP-2 antibody coimmunoprecipitated a small amount of JAK2 (130 kDa) at time zero. Within 1 min after ANG II exposure, however, the amount of coprecipitating JAK2 was greatly increased. SHP-2 thus appears to form a complex very rapidly with JAK2 in VSMC in response to ANG II, which continues to exist as a stable complex to at least 30 min. ANG II-induced complex formation of JAK2 and SHP-1, on the other hand, followed a completely different time course (Fig. 3B). In this case, the amount of JAK2 complexed with SHP-1 was ini-
The COOH-terminal tail of the AT1 receptor is also bound by recently, we have shown that this same motif in the platelet-derived growth factor-JAK2 (3). The YIPP motif was originally identified in unable to mediate ligand-dependent phosphorylation of wild-type receptor can induce JAK2 tyrosine phosphorylation, a mutant receptor lacking the YIPP motif is (-1–5 min) and then significantly increased to well above basal levels at 10–30 min. Complex formation of JAK2 with SHP-1 following ANG II treatment is thus temporally correlated with JAK2 dephosphorylation, again consistent with the possibility that SHP-1 is the PTPase that dephosphorylates JAK2 in VSMC. In contrast, maximal association of SHP-2 with JAK2 occurs within 1 min, during the time when JAK2 tyrosine phosphate content is actually increasing and well in advance of the time frame in which dephosphorylation occurs (10–30 min, see Fig. 1).

The combined results of the electroporation experiments and the experiments defining the relative time courses of SHP-1 and SHP-2 activation and complexation with JAK2 suggest that SHP-1 is responsible for dephosphorylation of JAK2 in VSMC. In addition, the electroporation experiments also suggest that SHP-2, rather than being required for JAK2 dephosphorylation, may actually be required for JAK2 phosphorylation. Recently, we have shown that ANG II-induced association of JAK2 with the AT1 receptor is mediated by a YIPP motif in the COOH-terminal tail of the receptor. Furthermore, although stimulation of the wild-type receptor can induce JAK2 tyrosine phosphorylation, a mutant receptor lacking the YIPP motif is unable to mediate ligand-dependent phosphorylation of JAK2 (3). The YIPP motif was originally identified in the platelet-derived growth factor-α and β-receptors as a tyrosine-phosphorylated binding site for the SH2 domains of phospholipase C-γ1 (PLC-γ1) (9, 32). Recently, we have shown that this same motif in the COOH-terminal tail of the AT1 receptor is also bound by PLC-γ1 in a tyrosine phosphorylation- and ANG II-dependent manner. Binding is mediated by the most COOH terminal of two SH2 domains in PLC-γ1 (33). Because JAK2 does not contain any SH2 domains, we have considered the possibility that the SH2 domain-containing SHP-2 phosphatase is required for ANG II-induced tyrosine phosphorylation of JAK2 because it functions as an adaptor protein that mediates the interaction of JAK2 with the receptor, an association necessary for ANG II-induced JAK2 phosphorylation. This hypothesis predicts that SHP-2 will bind to the YIPP motif in an ANG II-dependent manner and that JAK2 binding to this motif requires the presence of SHP-2. To determine whether ANG II induces binding of SHP-2 to the COOH-terminal tail of the AT1 receptor, we utilized an approach similar to that used previously to study JAK2 binding (3). VSMC were treated with ANG II (10⁻⁷ M) for various times, and cell lysates were prepared and mixed with a GST fusion protein (pre-bound to agarose beads) containing the COOH-terminal 54 residues of the rat AT1 receptor (GST-AT1, 306–359). As a control, lysates were also incubated with GST alone, pre-bound to beads. After a 2-h incubation, the beads were washed extensively, and bound proteins were eluted by boiling in SDS sample buffer. The amount of SHP-2 bound to the fusion protein was then quantified by immunoblotting with anti-SHP-2 antibody. As shown in Fig. 4, at time zero no SHP-2 binding to the fusion protein was detected. However, significant binding was detected after 1 min of ANG II exposure and was maximal after 5 min. In parallel experiments, no SHP-2 binding was observed for the GST alone negative control. When a similar protocol was used to test for possible ANG II-induced binding of SHP-1 to the AT1 receptor COOH-terminal tail, no complex formation was detected (n = 3, data not shown).

To determine whether ANG II-induced binding of SHP-2 is dependent on the YIPP motif (residues 319–322) of the AT1 receptor, we prepared a series of GST-fusion proteins in which we introduced deleterional or point mutations into the wild-type fusion protein. Each of the mutant fusion proteins was then tested for its ability to bind SHP-2 in ANG II-treated (10⁻⁷ M for 1 min) VSMC lysates. Binding was detected by immunoblotting with anti-SHP-2 as before. Fusion proteins of AT1 receptor residues 306–348, 306–329, and 318–359 (each of which contain the YIPP sequence) were bound by SHP-2 (n = 3, data not shown). However, fusion proteins of residues 323–359, 336–359, and 306–318 (which lack the YIPP motif) were not bound by SHP-2 (n = 3, data not shown). We have shown previously that tyrosine 319 within the YIPP motif is essential for

Fig. 3. ANG II-induced complex formation of JAK2 with SHP-2 and SHP-1 in VSMC. Cells were treated with ANG II (10⁻⁷ M) for the times indicated and then lysed. SHP-2 and SHP-1 were immunoprecipitated (IP) with anti-SHP-2 (A) and anti-SHP-1 (B) antibodies; immunoprecipitated proteins were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-JAK2 antibody. Results shown are representative of 3 separate experiments.

Fig. 4. ANG II-induced binding of SHP-2 to a glutathione S-transferase (GST)-AT1-306–359 fusion protein. VSMC were treated with ANG II (10⁻⁷ M) for the times indicated and then lysed. Lysates were subjected to an in vitro binding assay with either GST alone or a GST-AT1-306–359 fusion protein. Binding was quantitated by immunoblotting with anti-SHP-2 antibody. Results shown are representative of 3 different experiments.
JAK2 binding to the AT₁ receptor (3). To determine whether this residue is also important for SHP-2 binding, we carried out in vitro binding assays with a GST fusion in which tyrosine 319 was mutated to a phenylalanine. In contrast to the wild-type fusion protein (GST-AT₁-306–359), the mutant protein [GST-AT₁-306–359 (Y319-F)] did not bind SHP-2 in VSMC lysates (Fig. 5).

SHP-2 binding to the AT₁ receptor thus appears to depend on the same YIPP motif that mediates JAK2 binding. Because SHP-2 and JAK2 form a complex very rapidly and because SHP-2 binds to the receptor very rapidly in response to ANG II stimulation of VSMC, we considered the possibility that the JAK2 association with the receptor is an indirect association mediated by SHP-2 acting as an adaptor protein. To determine whether SHP-2 is required for JAK2 binding to the AT₁ receptor, we carried out in vitro binding assays with lysates from ANG II-treated VSMC (10⁻⁷ M for 1 min) in which lysates were specifically depleted of SHP-2 before determining the extent of JAK2 binding to the wild-type AT₁ receptor COOH-terminal tail GST fusion protein. SHP-2 was thus quantitatively immunodepleted from VSMC lysates (confirmed by immunoblotting) before carrying out the JAK2 binding assay. As a negative control, lysates were also quantitatively immunodepleted with anti-SHP-1 or with A/G plus agarose. As a positive control, lysates were quantitatively immunodepleted with anti-JAK2. As shown in Fig. 6, SHP-2 depletion was as effective as JAK2 depletion in blocking completely the association of JAK2 with the AT₁-306–359 GST fusion protein. SHP-2 thus appears to be required for JAK2 association with the AT₁ receptor, likely by acting as an adaptor protein that mediates the interaction between the two other proteins. In contrast, SHP-1 depletion or A/G plus agarose treatment had little or no effect on the JAK2 to GST-AT₁-306–359 fusion protein interaction.

ANG II-induced activation of the JAK/STAT pathway in VSMC is required for the ANG II-induced proliferative response (20). To determine whether SHP-2 is involved in this response, we have also carried out studies examining the effect of electroporation of anti-SHP-2 into cultured VSMC on ANG II-induced proliferation. VSMC were electroporated with anti-SHP-2 antibody before treatment or no treatment of cells with ANG II (10⁻⁷ M). Cell proliferation was then quantitated over a period of 48 h. As shown in Fig. 7, the anti-SHP-2 antibody completely blocked the ANG II stimulation of VSMC proliferation, suggesting that SHP-2 may have a key role in mediating ANG II stimulation of VSMC proliferation. SHP-1 antibody electroporation, on the other hand, had no effect (n = 3, data not shown). ANG II-stimulated JAK2 phosphorylation and activation is accompanied by tyrosine phosphorylation of STAT1, translocation of STAT1 to the nucleus, and activation of genes involved in cell proliferation (22). Termination of this signaling pathway could occur through several alternate mechanisms, one of which is the dephosphorylation of JAK2. However, the lack of an
effect of the anti-SHP-1 antibody on ANG II-stimulated cell proliferation suggests that ANG II signaling through the JAK/STAT pathway can also be terminated by a mechanism other than JAK2 dephosphorylation. For example, signaling could be terminated through AT1 receptor internalization. Another possible explanation for the lack of an effect of SHP-1 antibody electroporation on ANG II stimulation of cell proliferation is that transient activation of JAK2 produces the maximum effect of ANG II on proliferation, which is not further enhanced by sustained JAK2 activation.

In summary, the present study provides the first evidence that SHP-1 and SHP-2 have essential and opposite roles in regulation of JAK2 tyrosine phosphorylation by the G protein-coupled AT1 receptor. Blockade of ANG II-induced JAK2 dephosphorylation by anti-SHP-1 antibody as well as the time dependencies of ANG II-induced JAK2 activation and complexation with JAK2 suggest that the SHP-1 PTPase is responsible for JAK2 dephosphorylation and thus termination of the ANG II-induced JAK/STAT cascade in VSMC. In contrast, blockade of ANG II-induced JAK2 phosphorylation by anti-SHP-2 antibody as well as the time dependencies of SHP-2 phosphorylation and complexation with JAK2 suggest that the SHP-2 PTPase has a crucial role in JAK2 phosphorylation and thus initiation of the ANG II-induced JAK/STAT cascade leading to cell proliferation in VSMC. Because both SHP-2 and JAK2 interact very rapidly with the same YIPP motif in the COOH-terminal tail of the AT1 receptor and because JAK2-receptor association is dependent on the presence of SHP-2, we propose that SHP-2 has a novel noncatalytic role in VSMC, functioning as an adaptor protein for JAK2 association with the AT1 receptor, thereby facilitating JAK2 phosphorylation and activation.

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REFERENCES


