Jak2 Tyrosine Kinase Mediates Angiotensin II-dependent Inactivation of ERK2 via Induction of Mitogen-activated Protein Kinase Phosphatase 1

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Previous work has shown that inhibition of Jak2 via the pharmacological compound AG490 blocks the angiotensin II (Ang II)-dependent activation of ERK2, thereby suggesting an essential role of Jak2 in ERK activation. However, recent studies have thrown into question the specificity of AG490 and therefore the role of Jak2 in ERK activation. To address this, we reconstituted an Ang II signaling system in a Jak2−/− cell line and measured the ability of Ang II to activate ERK2 in these cells. Controls for this study were the same cells expressing Jak2 via the addition of a Jak2 expression plasmid. In the cells expressing Jak2, Ang II induced a marked increase in ERK2 activity as measured by Western blot analysis and in vitro kinase assays. ERK2 activity returned to basal levels within 30 min. However, in the cells lacking Jak2, Ang II treatment resulted in ERK2 activation that did not return to basal levels until 120 min after ligand addition. Analysis of phosphatase gene expression revealed that Ang II induced mitogen-activated protein kinase phosphatase 1 (MKP-1) expression in cells expressing Jak2 but failed to induce MKP-1 expression in cells lacking Jak2. Therefore, our results suggest that Jak2 is not required for Ang II-induced ERK2 activation. Rather, Jak2 is required for Ang II-induced ERK2 inactivation via induction of MKP-1 gene expression.

Extracellular signal-regulated kinase 2 (ERK2)† is a member of the mitogen-activated protein kinase family of serine/threonine protein kinases. These proteins become activated by phosphorylation on tyrosine and threonine residues in response to a variety of ligands binding their cognate receptors at the cell surface (1–4). Angiotensin II is one such ligand; it exerts many of its mitogenic effects by binding to the angiotensin II type 1 (AT1) receptor and thus activating ERK1/2. This occurs via rapid angiotensin II-dependent phosphorylation of the dual specificity kinase MEK, which in turn phosphorylates ERK1/2 on both threonine and tyrosine residues (5).

ERK activity is tightly regulated. The duration of ERK activation is regulated by the intracellular signals that phosphorylate and dephosphorylate it (6). While ERKs are activated by ligand binding at the cell surface, they are inactivated by several dual specificity phosphatases (7). One of these, mitogen-activated protein kinase phosphatase 1 (MKP-1), associates with and is phosphorylated by activated ERK2 and thus protected from proteasomal degradation. The phosphorylated MKP-1 then dephosphorylates ERK2, thereby inactivating it (8).

Evidence shows that Jak2 forms a membrane complex with the intermediate signaling molecules Ras and Raf1 and may therefore play a role in the regulation of ERK activity (9–11). In fact, previous work has suggested that inhibition of Jak2 using the pharmacological compound AG490 blocks the angiotensin II-dependent activation of ERK2 (12). One problem, however, with using AG490 to study the function of Jak2 is its lack of specificity for Jak2. While AG490 is a potent Jak2 inhibitor, it is known to inhibit other tyrosine kinase signaling pathways as well. AG490 inhibits activation of cyclin-dependent kinases and causes growth arrest of cells in G1 phase (13). It also inhibits calf serum-inducible cell growth and DNA synthesis (14) and is a partial blocker of c-Src activity (13). Most critically, AG490 inhibits epidermal growth factor receptor autophosphorylation more potently than it inhibits Jak2 kinase activity (15). In our experiments, AG490 is certainly a potent inhibitor of Jak2. However, we have found that alternate methods such as transfection of dominant negative Jak2, introduction of Jak2 antisense oligonucleotides, or some other method must be used when working with AG490 (16, 17). Clearly new strategies are needed to study Jak2 kinase function.

In this work, we utilized the γ2A cell line, which contains a Jak2 null mutation (18). On this background, stable cell lines were created that expressed either the AT1 receptor alone (γ2A/AT1) or the AT1 receptor and Jak2 (γ2A/AT1-Jak2) via stable integration of cDNA plasmids. Using these cells, we measured the ability of angiotensin II to activate ERK2 as a

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§ The abbreviations used are: ERK, extracellular signal-regulated kinase; Ang II, angiotensin II; HA, hemagglutinin; MKP-1, mitogen-activated protein kinase phosphatase 1; AT1, angiotensin II type 1; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; VSMC, vascular smooth muscle cell; DN, dominant negative; WT, wild type; PBS, phosphate-buffered saline; mAb, monoclonal antibody; STAT, signal transducers and activators of transcription; Jak, Janus tyrosine kinase; PP2A, protein phosphatase 2A; EGFR, epidermal growth factor receptor.
function of Jak2 expression. We show that, in cells expressing Jak2, angiotensin II induced a marked increase in ERK2 activity, which returned to basal levels within 30 min. However, in cells lacking Jak2, angiotensin II treatment resulted in sustained ERK2 activation, which did not return to basal levels until 120 min after ligand stimulation. We hypothesized that Jak2 was playing a role in the inactivation of ERK2 by mediating the expression of one or more phosphatase proteins that target ERK2 for inactivation. Here we provide evidence that angiotensin II stimulates expression of MKP-1 and that Jak2 is essential for this event. Moreover, we provide evidence that Jak2 is essential for angiotensin II-induced co-association of ERK2 and MKP-1. Collectively, these studies show that Jak2 is not required for angiotensin II-dependent ERK2 activation but rather that Jak2 is required for angiotensin II-induced inactivation of ERK2. Furthermore, these studies demonstrate the need for novel strategies to study the function of Jak2 beyond the use of AG490.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—\(\gamma_{A2A/T1}\) and \(\gamma_{A2A/T1+Jak2}\) cells were created as described below. These cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 10% fetal bovine serum and growth arrested in serum-free Dulbecco's modified Eagle's medium for 24 h prior to experiments. The vascular smooth muscle cells (VSMCs) expressing either a Jak2 dominant negative allele (Jak2 DN) or a neomycin resistance cassette (control) have been described previously (16). Cell culture reagents were obtained from Invitrogen. Inhibitor compounds were from either Calbiochem or LC Laboratories. All other reagents were purchased from Sigma or Fisher.

**Generation of Plasmid Constructs and Stable Cell Lines**—The HA-tagged AT1 receptor cDNA cloned into the pcDNA1 vector was kindly provided by Dr. Robert J. Lefkowitz (19). The cDNA was excised from pcDNA1 and cloned into pcDNA3 via HindIII and NotI restriction digestion. A fragment encoding the amino-terminal HA tag and the 5' sequence of the AT1 receptor cDNA up through the internal EcoRI site was then removed via HindIII and EcoRI restriction digestion and cloned into pSec/o/WT (20) that had also been cut with these same enzymes. The resulting construct, pSec/HA-AT1-WT, contained an amnio-terminal HA tag inserted just after the first methionine, \(-1100\) bp encoding the rat AT1 receptor open reading frame, and \(-850\) bp of rat AT1 receptor 3' untranslated region in a zeocin-resistant plasmid.

To generate the stable cell lines, \(\gamma_{2A/AT1}\) cells were transfected with 20 \(\mu\)g of pSec/HA-AT1-WT and 10 \(\mu\)g of pBOS/Jak2-WT encoding the wild type Jak2 cDNA (21). Control cells received 20 \(\mu\)g of pSec/HA-AT1-WT and 10 \(\mu\)g of empty vector control plasmid. Two days after transfection, cells were switched to medium supplemented with 500 \(\mu\)g/ml zeocin to select for stable transfectants. Surviving colonies were eventually ring cloned, and binding assays were performed using [\(\gamma\)]-labeled angiotensin II (where Sar is sarosine) (PerkinElmer Life Sciences) as described previously (20). Nonspecific binding was defined as binding in the presence of 1.0 \(\mu\)M angiotensin II. Scatchard analysis was used to identify respective Ki's of \(\gamma_{2A/AT1}\) and \(\gamma_{2A/AT1+Jak2}\) cells. We show that Jak2 is required for angiotensin II-induced inactivation of ERK2.

**RESULTS**

**\(\gamma_{A2A/AT1}\) Cells Lack Functional Jak2-dependent Signaling**—The \(\gamma_{A2A/AT1}\) cell line was originally created by irradiating HT-1080 human fibrosarcoma cells to create mutations within single genes and then selecting for cells containing a mutation that ablated the expression of Jak2 (18). To constitute angiotensin II signaling in these cells, the AT1 receptor, which was cloned into a zeocin-resistant vector, was stably transfected into cells either alone (\(\gamma_{A2A/AT1}\)) or with a wild type Jak2 cDNA plasmid (\(\gamma_{A2A/AT1+Jak2}\)). We performed binding assays, and respective \(\gamma_{2A}\) clones were identified in which the binding parameters were similar. \(\gamma_{A2A/AT1}\) (clone 4) had a \(K_d\) of 0.44 nM and a \(B_{max}\) of 201 fmol/mg of protein, while \(\gamma_{A2A/AT1+Jak2}\) (clone 1) had a \(K_d\) of 0.41 nM and a \(B_{max}\) of 226 fmol/mg of protein. To demonstrate that Jak2 is expressed in \(\gamma_{A2A/AT1+Jak2}\) cells, but not in \(\gamma_{2A/AT1}\) cells, both cell types were grown to about 70% confluency and lysed, and protein was extracted. The protein extracts were immunoblotted with an anti-Jak2 antibody (Fig. 1, top). The blot shows that Jak2 is only expressed in the \(\gamma_{2A/AT1+Jak2}\) cells. By stripping the membrane and reprobing with an anti-STAT1 antibody, we demonstrated equal sample loading by detecting endogenous STAT1 protein (Fig. 1, bottom). Thus, while both clones expressed AT1 receptor at similar affinity and abundance, only the \(\gamma_{2A/AT1+Jak2}\) clone expressed Jak2 protein.

We next wanted to determine whether angiotensin II-stimulated, Jak2-dependent signaling was in fact lost in the cells lacking Jak2 but intact in the cells expressing Jak2. Upon angiotensin II stimulation, Jak2 becomes phosphorylated and associates with the AT1 receptor (20). To show that these events do not occur in \(\gamma_{2A/AT1}\) cells but do occur in \(\gamma_{2A/AT1+Jak2}\) cells, both sets of cells were stimulated with 100 nM angiotensin II for 0, 5, and 15 min. The cells were then lysed, and protein was extracted. The protein extracts were immunoprecipitated with an anti-phosphotyrosine antibody and then immunoblotted with an anti-Jak2 antibody to detect phosphorylated Jak2 protein. In the \(\gamma_{2A/AT1}\) cells, angiotensin II treatment failed to induce Jak2 tyrosine phosphorylation (Fig. 2A). However, in \(\gamma_{2A/AT1+Jak2}\) cells, angiotensin II stimulation rapid tyrosine phosphorylation of Jak2.

After Jak2 is activated, it binds to the AT1 receptor in response to angiotensin II (20). To detect Jak2 co-association with the AT1 receptor, \(\gamma_{2A/AT1}\), and \(\gamma_{2A/AT1+Jak2}\) cells were next stimulated with 100 nM angiotensin II for 0, 2, 4, and 8 min. The cells were lysed, protein was extracted, and the pro-
The results show that in both cell types, paxillin was rapidly tyrosine-phosphorylated in response to angiotensin II treatment, thereby indicating that angiotensin II signaling events, which are independent of Jak2, function normally in both cell types.

Collectively, the data in Fig. 2 demonstrate that, in γ2A/AT1 cells, functional Jak2-dependent signaling has been lost, while Jak2-independent signaling events have been preserved. As such, these cells are good vehicles for studying Jak2-dependent signaling.

**ERK2 Activity Is Sustained in γ2A/AT1 Cells Compared with γ2A/AT1 + Jak2 Cells following Angiotensin II Treatment**—Previous work demonstrated that inhibition of Jak2 phosphorylation using AG490 blocks angiotensin II-dependent activation of ERK2, thus implicating Jak2 as essential in ERK2 activation (12). Recently, however, the specificity of AG490 for Jak2 has come under scrutiny (13, 14). We therefore sought to determine the role that Jak2 plays in angiotensin II-dependent ERK2 activity using the γ2A-derived cells. γ2A/AT1 and γ2A/AT1 + Jak2 cells were stimulated with 100 nM angiotensin II for 0, 6, 12, 18, 24, and 30 min. The cells were lysed, and protein was extracted. The protein extracts were immunoblotted with an anti-phospho-ERK2 antibody that detects phosphorylated ERK2 protein. In the cells lacking Jak2, angiotensin II stimulation resulted in a rapid and sustained increase in ERK2 activation that persisted for 30 min (Fig. 3A, top). However, in the γ2A/AT1 + Jak2 cells, angiotensin II caused an increase in ERK2 activity that peaked at 6–12 min and returned to basal levels 18–24 min after angiotensin II stimulation. The membrane was subsequently stripped and reprobed with an anti-ERK2 antibody to show constant ERK2 expression at all time points (Fig. 3A, bottom).

The three different membranes representing Fig. 3A were scanned for densitometric analysis, and ERK2 phosphorylation was plotted as a function of angiotensin II treatment (Fig. 3B). The graph shows that in the cells expressing Jak2, ERK2 phosphorylation transiently increased, peaking 6 min after angiotensin II treatment. However, in cells lacking Jak2, ERK2 phosphorylation was significantly elevated 30 min after angiotensin II stimulation. Longer time course studies using the γ2A/AT1 cells demonstrated that ERK2 phosphorylation did not return to basal level until about 120 min after angiotensin II treatment (data not shown). Thus, the data in Fig. 3A suggest that loss of Jak2 expression via a null mutation results in sustained ERK2 phosphorylation in response to angiotensin II. This is contrary to previously published data, which suggested that inhibiting Jak2 kinase function using AG490 results in diminished angiotensin II-dependent ERK2 phosphorylation (12). To verify our results using an alternate protocol, *in vitro* kinase assays were performed using myelin basic protein as a substrate for ERK2 phosphorylation. In γ2A/AT1 cells, phosphorylation of myelin basic protein remained elevated 20 min after angiotensin II treatment, suggesting that ERK2 was catalytically active at this time point (Fig. 3C). However, in γ2A/AT1 + Jak2 cells, angiotensin II stimulated ERK2 activity with peak activity occurring around 5–10 min. By 15–20 min, the 32P-labeled myelin basic protein signal was similar to that of γ2A-derived cells.
Angiotensin II-mediated signaling in the γ2A/AT1 cells and γ2A/AT1+Jak2 cells. γ2A/AT1 cells and γ2A/AT1+Jak2 cells were treated with 100 nM angiotensin II for the indicated times, and lysates were prepared. A, lysates were immunoprecipitated with anti-Tyr(P) mAb and Western blotted with anti-Jak2 mAb to detect tyrosine-phosphorylated Jak2. B, lysates were immunoprecipitated with anti-HA mAb to immunoprecipitate the HA-tagged AT1 receptor and then Western blotted with anti-Jak2 polyclonal antibody to detect Jak2/AT1 co-association. C, lysates were immunoprecipitated with anti-Tyr(P) mAb and Western blotted with anti-STAT1 mAb to detect tyrosine-phosphorylated STAT1. D, lysates were immunoprecipitated with anti-Tyr(P) mAb and Western blotted with anti-STAT3 mAb to detect tyrosine-phosphorylated STAT3. E, lysates were immunoprecipitated with anti-Tyr(P) mAb and Western blotted with anti-paxillin mAb to detect tyrosine-phosphorylated paxillin. Shown is one of four (A, C, and D) or three (B and E) independent results for each. IP, immunoprecipitation; IB, immunoblot.

| Time (min) | γ2A/AT1 | γ2A/AT1+Jak2 |
|------------|---------|--------------|
| 0          |         |              |
| 5          |         |              |
| 15         |         |              |

**A.** IP:αTyr(P)-mAb  
IB:αJak2-mAb

**B.** IP:αHA-mAb  
IB:αJak2-mAb

**C.** IP:αTyr(P)-mAb  
IB:αStat1-mAb

**D.** IP:αTyr(P)-mAb  
IB:αStat3-mAb

**E.** IP:αTyr(P)-mAb  
IB:αPaxillin-mAb
recapitulate the observations seen in Fig. 3. In other words, could we reproduce the enhanced ERK2 activation phenomenon seen in the Jak2-deficient cells by treating the Jak2-expressing cells with AG490? For this, γ2A/AT₁+Jak2 cells were treated with either AG490 or the inert control compound AG9. The cells were then treated with angiotensin II, and phospho-ERK2 levels were directly measured via Western blot analysis. First γ2A/AT₁ cells were treated with angiotensin II to once again reproduce the enhanced ERK2 activation seen 30 min after angiotensin II treatment (Fig. 4A, lanes 1–3). Next, when the γ2A/AT₁+Jak2 cells were treated with the AG9 control compound, the ERK2 phosphorylation levels were high at 5 min but returned to basal levels by 30 min, similar to that shown in Fig. 3A (Fig. 4A, lanes 4–6). Finally, however, when
the γ2A/AT₁+Jak2 cells were treated with AG490, the ability of angiotensin II to induce ERK2 phosphorylation appeared to be lost (Fig. 4A, lanes 7–9). As such, this result indicates that pharmacological suppression of Jak2 via AG490 does not recapitulate the effect of the Jak2 null mutation and suggests that AG490 is blocking angiotensin II-dependent ERK2 activation
via a mechanism that is independent of Jak2.

Recent work has suggested that a possible mechanism by which G protein-coupled receptors activate ERK1/2 is via transactivation of the EGFR (27–29). This is important because, as indicated earlier, AG490 inhibits epidermal growth factor receptor autophosphorylation more potently than it inhibits Jak2 kinase activity (15). To determine whether this mechanism of action is responsible for the angiotensin II-dependent activation of ERK2 in the γ2A-derived cells, γ2A/AT1+Jak2 cells were treated with the EGFR tyrosine kinase inhibitor AG1478 prior to measuring angiotensin II-dependent ERK2 activation. AG1478 treatment, at a dose and time that has previously been shown to fully suppress EGFR kinase activity (30, 31), failed to inhibit the angiotensin II-mediated activation of ERK2 in γ2A/AT1+Jak2 cells when compared with similarly treated control cells (Fig. 4B). This result suggests that in the γ2A-derived cells, angiotensin II mediates the activation of ERK2 via a mechanism that is independent of EGFR tyrosine kinase activity.

It is possible that the pattern of ERK2 activation seen in the cells lacking Jak2 is due to abnormal signaling properties in these cells compared with other cell types. Therefore, we chose to use an alternate means to eliminate Jak2 function and subsequently measure angiotensin II-mediated ERK2 activation. Specifically, we utilized VSMCs that stably express a Jak2 dominant negative cDNA. Expression of the dominant negative protein blocks function of wild type Jak2 normally found in the VSMCs. The full characterization of these cells has been reported previously (16). In short, Jak2-dependent signaling in the dominant negative expressing cells is reduced by about 90% when compared with the controls. The control cells are vascular smooth muscle cells that express only a neomycin resistance cassette. Both sets of cells were treated with 100 nM angiotensin II for the indicated times, and whole cell lysates were subsequently Western blotted with anti-phospho-ERK2 antibody (Fig. 4C, top). In the control cells, angiotensin II elicited a marked increase in ERK2 phosphorylation with peak activation occurring about 10 min after ligand addition. In the Jak2 dominant negative cells, angiotensin II similarly activated ERK2 at the 5-, 10-, and 15-min time points, but the magnitude of the phosphorylation was greatly increased when compared with the control cells. Interestingly there was no phosphorylation signal detected at the 30-min time point in the Jak2 dominant negative cells. Overall, while this result is not identical to that obtained in the γ2A cells, it is very similar. In both cases, loss of Jak2 function, either by null mutation or by expression of a Jak2 dominant negative allele, results in sustained or enhanced ERK2 activation. The differences in these two results may be due to several factors including cell type (γ2A versus VSMCs), method by which Jak2 function is inhibited (null mutation versus dominant negative), or the degree to which Jak2 function is reduced (100% in the γ2A cells versus −90% in the VSMCs). Finally the membrane was Western blotted with anti-ERK2 antibody to show constant ERK2 expression at all time points (Fig. 4C, bottom).

In summary, the data in Fig. 4A demonstrate that AG490 blocks the angiotensin II-dependent activation of ERK2 via a mechanism that is independent of Jak2. The data in Fig. 4B demonstrate that this nonspecific effect of AG490 is not due to reduced EGFR tyrosine kinase activity, as direct suppression of EGFR tyrosine kinase activity with AG1478 does not inhibit the angiotensin II-dependent activation of ERK2. The data in Fig. 4C demonstrate that, when Jak2 function is blocked via expression of a dominant negative Jak2 allele, angiotensin II treatment of cells results in enhanced ERK2 activation similar to that seen in the Jak2 null cell. Collectively the data suggest that AG490 is inhibiting ERK2 activation via nonspecific suppression of a tyrosine kinase that is not the EGFR.

There Is a Marked Difference in the Angiotensin II-induced Nuclear Accumulation Pattern of Activated ERK2 between γ2A/AT1 and γ2A/AT1+Jak2 Cells—ERK2, when activated by angiotensin II, accumulates in the nucleus of cells and modulates the expression of a variety of genes by activating nuclear transcription factors such as AP-1 (6). We next wanted to determine whether a difference in nuclear accumulation of activated ERK2 existed between γ2A/AT1 and γ2A/AT1+Jak2 cells. For this, both cell types were immunostained with an anti-phospho-ERK2 antibody. The cells were then visualized using a fluorescence microscope to measure angiotensin II-dependent phosphorylation occurring about 10 min after ligand addition. In the AT1, AT1, and γ2A/AT1+Jak2 cells, angiotensin II treatment promoted a transient nuclear accumulation of activated ERK2 protein. Arrows indicate apparent perinuclear staining. Shown is one of three independent results.

Fig. 5. Angiotensin II-dependent nuclear accumulation of phospho-ERK2 in the γ2A/AT1 and γ2A/AT1+Jak2 cells. γ2A/AT1 and γ2A/AT1+Jak2 cells were treated with 100 nM angiotensin II for either 6, 5, or 30 min. The cells were incubated with anti-phospho-ERK2 polyclonal antibody and immunostained with goat anti-rabbit antibody conjugated to Texas Red. The cells were visualized using a fluorescence microscope to detect nuclear accumulation of activated ERK2 protein. Arrows indicate apparent perinuclear staining. Shown is one of three independent results.
the γ2A/AT1 and γ2A/AT1 + Jak2 cells, we measured angiotensin II-dependent, ERK2-mediated gene transcription in the two cell types. This was accomplished using a synthetic promoter containing seven copies of the AP-1 binding element upstream of a luciferase reporter. After transfection with this construct, the cells were treated with 100 nM angiotensin II for the indicated times and then lysed. Angiotensin II treatment elicited a marked increase in luciferase activity in both cell types (Fig. 6). Although the γ2A/AT1 cells repeatedly generated higher-fold changes in luciferase activity compared with the Jak2-expressing control cells, the difference failed to reach statistical significance at any time point.

**Jak2 Is Essential for Angiotensin II-induced MKP-1 Expression and Co-association of MKP-1 with ERK2**—The data in Fig. 3 demonstrate that, in the cells lacking Jak2, ERK2 has sustained levels of activation when compared with the Jak2-expressing cells. We hypothesized that the signal transduction pathways leading to the induction of ERK2-specific phosphatases were different in the two cell types. Specifically we hypothesized that, in the cells lacking Jak2, there would be little to no angiotensin II-mediated induction of the phosphatases that inactivate ERK2. We therefore tested the ability of angiotensin II to induce gene expression of two such phosphatases, PP2A and MKP-1, in both cell types. Quiescent cells were treated with 100 nM angiotensin II for 0, 15, 30, or 60 min, and protein lysates were prepared. The whole cell lysates were first immunoblotted with an anti-PP2A antibody (Fig. 7A). The results show that there was no marked difference in PP2A protein expression between the γ2A/AT1 and γ2A/AT1 + Jak2 cells in response to angiotensin II. Based on these results, we concluded that angiotensin II was not modulating gene expression of PP2A.

We next immunoblotted similarly prepared protein extracts with an anti-MKP-1 antibody and found that, in γ2A/AT1 cells, angiotensin II induced very little MKP-1 expression (Fig. 7B). In the γ2A/AT1 + Jak2 cells, however, angiotensin II induced marked MKP-1 expression, demonstrating that maximal angiotensin II-induced MKP-1 protein expression requires Jak2.

MKP-1 associates with ERK2 in response to angiotensin II treatment and is activated by ERK2. MKP-1 in turn dephosphorylates ERK2 (8). We next investigated whether Jak2 is required for co-association of MKP-1 with ERK2. γ2A/AT1 and γ2A/AT1 + Jak2 cells were treated with 100 nM angiotensin II for 0, 15, and 30 min. The cells were lysed, and cellular protein was extracted. The protein extracts were immunoprecipitated with an anti-ERK2 antibody and then immunoblotted with an anti-MKP-1 antibody. The results show that no increase in co-association of ERK2 and MKP-1 was observed in γ2A/AT1 cells, whereas in γ2A/AT1 + Jak2 cells, angiotensin II induced a substantial increase in co-association of ERK2 and MKP-1 (Fig. 7C).

The data in Fig. 7B suggest that Jak2 is playing a key role in the angiotensin II-dependent increased expression of MKP-1. Previous work has shown that ERK2 itself can also be a critical mediator of MKP-1 gene expression (7, 8). To determine the relative contribution of ERK2 and Jak2 in mediating angiotensin II-dependent MKP-1 gene expression, both sets of cells were treated with angiotensin II in the presence or absence of the MEK-specific inhibitor PD98059 (Fig. 7D). For the cells lacking Jak2, ligand treatment only modestly induced MKP-1 expression (lanes 1–3), and this was completely blocked with PD98059 (lanes 4–6). However, for the cells expressing Jak2, ligand treatment again induced marked MKP-1 expression (lanes 7–9), and this was partially blocked with PD98059 (lanes 10–12). Thus, the data indicate that there is both a Jak2-dependent component and an ERK2-dependent component to the angiotensin II-mediated induction of MKP-1 as maximal MKP-1 expression is only attained when cells have both functional Jak2 and ERK2.

Collectively the data in Fig. 7 suggest that Jak2 is not only required for induction of MKP-1 expression in response to angiotensin II but also for co-association of ERK2 and MKP-1. Additionally the data indicate that both Jak2 and ERK2 are required for maximal angiotensin II-mediated MKP-1 protein expression.

**MKP-1 Is Required for the Angiotensin II-dependent Inactivation of ERK2**—The data in Fig. 7 demonstrate that Jak2 is required for angiotensin II-dependent induction of MKP-1, but not PP2A. However, the data clearly show that both proteins are present in the cell. To determine the extent to which these two phosphatases regulate the dephosphorylation of ERK2, angiotensin II-mediated ERK2 phosphorylation was measured in the presence, or absence, of PP2A- and MKP-1-specific inhibitors.

To inhibit PP2A, which is a serine/threonine-specific phosphatase, γ2A/AT1 and γ2A/AT1 + Jak2 cells were pretreated...
with the PP2A-specific inhibitor okadaic acid (32). The cells were then treated with angiotensin II, and ERK2 phosphorylation was measured via Western blot analysis (Fig. 8A). A, whole cell lysates were Western blotted with anti-PP2A mAb to detect PP2A expression. B, whole cell lysates were Western blotted with anti-MKP-1 mAb to detect MKP-1 expression. C, lysates were immuno-precipitated with anti-ERK2 mAb and Western blotted with anti-MKP-1 mAb to detect co-association of MKP-1 with ERK2. D, cells were pretreated for 60 min with either Me2SO or 50 μM PD98059 and then stimulated with 100 nM angiotensin II for the indicated times. Whole cell lysates were prepared and subsequently Western blotted with anti-MKP-1 mAb to detect MKP-1 protein. Shown is one of four (A) or three (B–D) independent results for each. IP, immunoprecipitation; IB, immunoblot.
ment. In contrast, however, 30 min of angiotensin II treatment promoted its relative dephosphorylation. This observation suggests two important things. First, it indicates that the \( \gamma 2A/AT_1 \) cells contain the necessary component(s) to promote the dephosphorylation of ERK2 (i.e. Jak2). Second, the data suggest that this ligand-dependent dephosphorylation does not require PP2A since the dephosphorylation occurs in the presence of okadaic acid.

To inhibit MKP-1, which is a threonine/tyrosine dual specificity phosphatase, \( \gamma 2A/AT_1 \) and \( \gamma 2A/AT_1 +Jak2 \) cells were pretreated with the MKP-1 inhibitor vanadate (33). The cells were then treated with angiotensin II, and ERK2 phosphorylation was again measured via Western blot analysis with the anti-phospho-ERK2 polyclonal antibody (Fig. 8B). In the \( \gamma 2A/AT_1 \) cells, prior to ligand treatment, ERK2 showed some basal phosphorylation. Addition of angiotensin II modestly increased the signal at both the 5- and 30-min time points. Interestingly these cells were once again unable to dephosphorylate ERK2 after 30 min of ligand treatment. Similarly, in the \( \gamma 2A/AT_1 +Jak2 \) cells, ERK2 was also basally phosphorylated prior to ligand treatment, and 5 min of angiotensin II treatment modestly increased its signal. However, unlike the previous experiments in the Jak2-expressing cells, 5 min of angiotensin II treatment did not increase ERK2 phosphorylation. This data thereby suggest that MKP-1 is critical for mediating the angiotensin II-dependent dephosphorylation of ERK2 as vanadate treatment of these cells blocks the angiotensin II-dependent dephosphorylation of ERK2.

Collectively the data in Fig. 8 suggest that PP2A and MKP-1 play distinct roles in the dephosphorylation of ERK2; PP2A appears to be largely responsible for the basal phosphorylation state of ERK2, while MKP-1 appears to regulate angiotensin II-dependent dephosphorylation. Moreover, this ligand-dependent dephosphorylation of ERK2 by MKP-1 appears to require Jak2.

**DISCUSSION**

ERK signaling is critical to numerous cellular events including embryogenesis, cell differentiation, cell proliferation, and cell death. Moreover ERK1/2 become activated in response to a variety of stimuli including extracellular ligands such as angiotensin II. ERK activity is tightly controlled and depends on the action of activating and inactivating signals (6). It was previously reported that Jak2 was essential for angiotensin II-dependent ERK2 activation (12). This observation, however, was made using AG490 to inhibit Jak2 kinase function. While AG490 is a potent inhibitor of Jak2, it inhibits several other kinase signaling pathways as well (13–15). We therefore utilized \( \gamma 2A \) cells that contain a Jak2 null mutation to study the role that Jak2 plays in regulating ERK2 activity.

In this study, we report several novel observations. First, lack of Jak2 signaling in a cell increases the duration of ERK2 activity following angiotensin II stimulation. This was shown by both Western blot analysis and in vitro kinase assays. Again this observation is contrary to what has been reported previously in studies utilizing AG490 to study the role that Jak2 plays on ERK activation. One possible reason for this discrepancy is that AG490 has been shown to inhibit other tyrosine kinases as well (13–15). Our data in Fig. 4, however, suggest that this nonspecific effect is independent of the ability of AG490 to inhibit the EGFR. A second observation that we report is that angiotensin II induces rapid up-regulation of MKP-1, a phosphatase that inactivates ERK2. This is an interesting example of the tight regulation of ERK signaling within a cell. Furthermore we report that angiotensin II-induced up-regulation of MKP-1 gene expression is dependent on Jak2 expression. We believe that this is the reason that, in
Finally the aforementioned studies were conducted using the \( \gamma 2A \) cell line, which are human fibrosarcoma cells lacking Jak2 expression. In this report, we characterized these cells and showed that, while angiotensin II-dependent Jak2-mediated signaling is ablated, Jak2-independent signaling remains intact. For this reason, we believe that these \( \gamma 2A \)-derived cells are a very useful model for studying the intracellular function of Jak2 tyrosine kinase given the lack of an adult knock-out animal or a Jak2-specific inhibitor. These studies prove that \( \gamma 2A \)-derived cells are valuable tools for studying Jak2-dependent signaling events and identifying novel signaling pathways. Furthermore this research demonstrates that new strategies for studying Jak2 tyrosine kinase signaling should be developed.

In summary, this work provides novel findings on the mechanism of regulation of ERK activity in response to angiotensin II. These results are significant given the critical roles that both Jak2 and ERK play in health and disease.

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**Fig. 9. Proposed model of the mechanism by which Jak2 mediates ERK2 inactivation following angiotensin II treatment.**

Angiotensin II binding to its type I receptor activates ERK2 while simultaneously activating Jak2. Jak2, probably through the action of STAT proteins, increases expression of MKP-1. ERK2 also increases MKP-1 expression. The expressed MKP-1 protein then associates with and dephosphorylates ERK2, thus inactivating the signal.

\( \gamma 2A/AT1 \) cells lacking Jak2, ERK2 activity is sustained following angiotensin II stimulation compared with \( \gamma 2A/AT1 \)-Jak2 cells. Without Jak2 present, angiotensin II is simply unable to up-regulate MKP-1 expression, and thus the cell is slower to inactivate ERK2.

Fig. 9 shows a model depicting what we believe is occurring in normal cells. Upon angiotensin II stimulation, ERK2 becomes phosphorylated on threonine and tyrosine residues. Simultaneously, Jak2 becomes tyrosine-phosphorylated and associates with the AT1 receptor. Jak2 induces expression of MKP-1 presumably through activation of one or more STAT proteins. ERK2 is also acting on the MKP-1 promoter to increase its expression. The MKP-1 protein that is generated then associates with and inactivates ERK2.

Previous work has shown that the duration of ERK activation is critical for determining cell fate (34–36). In some cell signaling systems for instance, transient activation of ERK2 is a common feature of cell proliferation. Sustained activation on the other hand is associated with very different cellular events such as apoptosis or senescence. Previously published work has shown that Jak2 can promote both cellular proliferation and apoptosis (37, 38). The reasons as to how Jak2 elicits such different cellular responses is presumably dependent on the specific cell type and ligand used in each experiment. However, the exact cellular and biochemical mechanism(s) by which Jak2 accomplishes this is not fully known. Our work here demonstrates that Jak2 has an important role in determining whether ERK2 has transient or sustained activation. As such, we may have determined that one mechanism by which Jak2 influences cell fate is by altering the duration of ERK2 activation via induction of MKP-1. Clearly further studies are needed to fully address this issue.
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