Jak2 TYROSINE KINASE: NEW INSIGHTS REGARDING STRUCTURE, FUNCTION, AND PHARMACOLOGY

By

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This dissertation is dedicated to my parents, for their constant love and support.

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The kinase Jak2 is a member of the Janus family of non-receptor tyrosine kinases. One major impediment to understanding the role that Jak2 plays in physiology and pathophysiology is the lack of a specific Jak2 inhibitor. We used several strategies to circumvent this problem. First, using Jak2 -/- cells, we examined the role of Jak2 in regulating angiotensin II-dependent ERK2 activity. We found that, contrary to previously published work, Jak2 is required for inactivation of ERK2 after angiotensin II treatment. In response to angiotensin II, Jak2 induces expression of MAP kinase phosphatase-1 (MKP-1), a protein that dephosphorylates and inactivates ERK2.

Second, using stable expression of a Jak2 dominant negative mutant that specifically suppresses endogenous Jak2 kinase activity, we found that Jak2 mediates oxidative stress-induced apoptosis of vascular smooth muscle cells. In response to hydrogen peroxide treatment, Jak2 induces expression of the pro-apoptotic Bax protein.

This causes a loss of mitochondrial transmembrane potential, cleavage of Caspase-9, and subsequent apoptosis.

Third, we attempted to identify novel Jak2 inhibitors. For this, we used homology modeling to analyze the structure of the Jak2 kinase domain, and we identified a previously unknown amino acid interaction that is required for activation of Jak2. This interaction, consisting of two distinct hydrogen bonds between Jak2 residues Glu 1024 and Arg 1113, may be a suitable target for drug design aimed at disabling Jak2 function. Additionally, we used high-throughput compound docking *in silico* to identify a novel Jak2 inhibitor. This compound, cyclohexane-1,2,3,4,5,6-hexabromo- (designated Compound 7) potently inhibits Jak2 autophosphorylation in a time- and dose-dependent manner.

In conclusion, using Jak2 -/- cells, stable expression of a Jak2 dominant negative mutant, and structure-function studies, we successfully circumvented the problems that lack of a Jak2-specific inhibitor pose. In doing so, we identified novel roles for Jak2 kinase function in regulation of angiotensin II-dependent ERK2 signaling and in oxidative stress-induced apoptosis of vascular smooth muscle cells. Additionally, we improved our understanding of Jak2 structure by identifying a previously unknown amino acid interaction within the Jak2 kinase domain that is required for Jak2 kinase function, and identified a novel Jak2 inhibitor.

CHAPTER 1
INTRODUCTION TO THE Jak-STAT PATHWAY

History of the Jak-STAT Pathway

In 1990, the first member of the Jak family of cytoplasmic tyrosine kinases was cloned and sequenced (1). The gene, termed Tyk2, was unique compared to previously identified tyrosine kinases in that it had a kinase-like, or pseudokinase domain, immediately N-terminal to a highly conserved protein tyrosine kinase domain. The tandem manner by which these two domains adjoined one another was reminiscent of Janus, Roman God of two opposing faces. As such, Tyk2 was classified as the first member belonging to the *Janus* associated kinase family of protein tyrosine kinases (or more simply) the Jaks. Other groups independently cloned the cDNAs encoding Jak1, Jak2, and Jak3 (2-6). Because some of the genes were cloned from hematopoietic tissues, it was hypothesized that the Jak kinases played a critical role in cytokine-mediated signal transduction. This hypothesis was largely correct.

In 1992, Wilks and colleagues (3) were the first to clone and publish the Jak2 cDNA sequence. The gene encoded a protein of about 130 kDa in mass. Like the two previously cloned Jaks (Tyk2 and Jak1) the predicted amino acid sequence of Jak2 contained the kinase and pseudokinase domains adjoining one another on the carboxyl half of the protein. These regions are termed the Jak homology 1 (JH1) and Jak homology 2 (JH2) domains, respectively. Wilks and colleagues also identified five other domains that encompassed the amino half of the molecule. These were designated as the JH3, JH4, JH5, JH6, and JH7 domains. The C-terminal half of the JH4 domain and the

entire JH3 domain were thought to encode a primitive SH2 domain spanning amino acids 412-480. This was significant because Jak family members lack any canonical SH2 or SH3 domains. Aside from this putative SH2 domain, the remaining domains did not possess the characteristics of any other known proteins. Wilks and colleagues found that, like Tyk2 and Jak1, Jak2 was expressed in almost every tissue examined. In contrast, Jak3 is expressed predominantly in hematopoietic cells (5, 7). Interestingly, subsequent work showed that Jak2 homologs exist in animals as diverse as zebrafish (*Danio rerio*) and fruitflies (*Drosophila melanogaster*) (8, 9).

Collectively, these studies showed that a new family of cytoplasmic protein tyrosine kinases existed in animals. These family members shared properties that were unique only unto them. Of these genes, Jak2 is expressed in numerous tissues and in evolutionarily diverse species.

The importance of Jak2 in cellular signaling was realized when it was discovered that Jak2 appeared to be a critical mediator of cytokine-dependent signal transduction (10-15). Subsequent work quickly identified a correlation between activation of Jak2 in the cytoplasm and increased gene transcription in the nucleus. This observation suggested that a specific class of cytokine-responsive transcription factors was mediating this transcriptional effect. This hypothesis was proven correct when concurrent studies identified a new class of cytokine-responsive transcription factors, termed the Signal Transducers and Activators of Transcription (STAT) proteins (16, 17). These proteins (when tyrosine phosphorylated by Jak2 in the cytoplasm) translocate to the nucleus and mediate gene transcription. Thus, within 2 years, the broad framework of the Jak-STAT signaling paradigm was elucidated.

More recently, it was discovered that in addition to mediating cytokine signal transduction, Jak2 also mediates signaling through G protein coupled receptors (GPCRs) (18). In fact, the number of cytokines and GPCR agonists that activate Jak-STAT signaling has grown steadily since the discovery of the pathway. The cytokines currently known to activate Jak2 include IL-2, IL-3, IL-5, IL-6, IL-11, IL-12, granulocyte macrophage colony-stimulating factor, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, granulocyte colony-stimulating factor, interferon-γ, growth hormone, prolactin, erythropoietin, thrombopoietin, and leptin (10-15, 19-27). The GPCR agonists that activate Jak2 include angiotensin II, bradykinin, endothelin, platelet activating factor, α-melanocyte stimulating hormone, isoproterenol, and phenylephrine (28-32). The mechanism of Jak2 activation by these two receptor subtypes differs, but the downstream effects of Jak2 activation are similar. In both cases, Jak2 acts a critical link in coupling ligand binding at the cell surface with gene transcription in the nucleus. Key differences in Jak-STAT signaling through cytokine receptors and GPCRs are shown in Fig. 1-1 and discussed in the next section.

In addition to cytokines and GPCR agonists, Jak2 can also be activated in response to a number of cellular stressors. These include mechanical cell stretch, ischemia-reperfusion, and hydrogen peroxide (33-36). The upstream activators of Jak2 and the downstream effects of Jak2 activation by these stimuli are not well characterized. In Chapter 5, we elucidate the role that Jak2 plays in hydrogen peroxide-induced signaling in vascular smooth muscle cells.

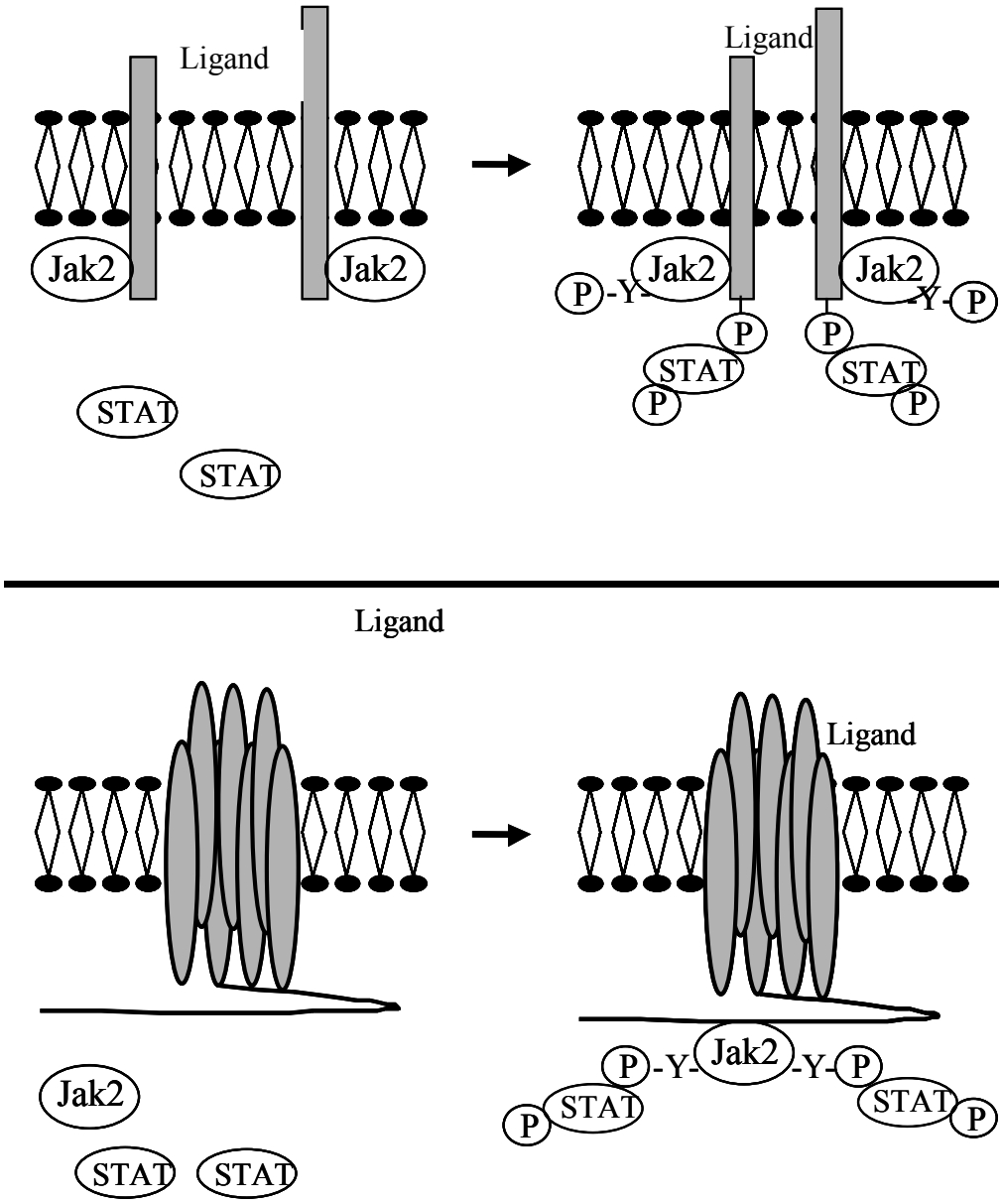


Figure 1-1. Differences in the mechanism of Jak-STAT signaling through cytokine receptors (Top) and GPCRs (Bottom)

Initiation of Jak-STAT Signaling

Jak2 signaling through cytokine receptors is initiated by the binding of an extracellular cytokine to its cognate monomeric receptor on the cell surface, resulting in receptor dimerization. This oligomerization of two distinct receptors results in the

activation of Jak2 molecules bound non-covalently to the receptors before ligand binding. An activated Jak2 then phosphorylates specific tyrosine residues on the cytoplasmic tails of the receptors creating docking sites for SH2 domain-containing proteins, such as the STATs. Once bound to the receptors, STATs are themselves phosphorylated by Jak2 on tyrosine residues. The tyrosine phosphorylated STATs then dissociate from the receptor to form active homo- and hetero-dimer protein complexes. The STAT complexes then translocate into the nucleus where they bind specific DNA sequences in gene promoter elements and modulate gene transcription. Interestingly, the increased tyrosine phosphorylation, nuclear translocation, and DNA binding activity of the STATs occurs in the presence of cycloheximide, suggesting that this signaling pathway uses a post-translational modification of existing proteins and does not require *de-novo* protein synthesis (37). Thus, Jak2 is capable of transducing a signal from the cell surface to the nucleus through a tyrosine phosphorylation signal transduction cascade.

While the downstream consequences of Jak2 signaling through GPCRs are similar to Jak2 signaling through cytokine receptors, there are key differences in how Jak2 signaling is initiated in these two pathways. Similar to Jak2 signaling through cytokine receptors, Jak2 signaling through GPCRs is initiated by binding of an extracellular ligand to its cognate receptor on the cell surface. However, Jak2 is not ubiquitously bound to GPCRs. Instead, it becomes activated in the cytoplasm after ligand binding (18). The exact mechanism by which this occurs has not been elucidated. In the case of angiotensin II (a Jak2-activating GPCR ligand), Jak2 is recruited to the angiotensin II type I receptor (AT_1R) after its activation. Then Jak2 acts as a molecular bridge, linking STAT proteins

to the AT_1R. Jak2 phosphorylates the STAT proteins, and the signal cascade proceeds similar to cytokine-mediated Jak2 signaling (38).

Physiology and Pharmacology of Jak2

Jak2 signaling plays a critical role in normal physiological processes, including development, regulation of other signaling pathways, and cellular stress responses. In addition, Jak2 signaling has been implicated in the pathophysiology of cancer and heart disease. The role that Jak2 plays in physiology and pathophysiology will be discussed in this section.

Target Genes and Regulation of Signal Transduction Pathways

Since its discovery, Jak2 activation has been linked to mediation of gene expression. It accomplishes this through activation of the STAT family of transcription factors. Despite this, the identities of downstream target genes of the Jak-STAT pathway are largely unknown. Additionally, Jak2 has been shown to directly regulate other signal transduction pathways. In fact, data in the results section show that Jak2 can indirectly regulate angiotensin II-induced extracellular signal regulated kinase 2 (ERK2) signaling by mediating expression of an ERK2 regulatory protein.

Jak2 can influence other angiotensin-dependent signaling events as well. For example, when Jak2 is activated by angiotensin II, Jak2 recruits the Src family tyrosine kinase Fyn to the Jak2-based signaling complex (39). Jak2 then activates Fyn, by binding the SH2 domain of Fyn with very high affinity (K_d = 2.36 nM). This strong interaction results in a conformational change within Fyn that allows the Fyn kinase domain to become accessible to substrate. Thus, Jak2 serves as a potent activator of Fyn kinase.

Additionally, Jak2 has been shown to regulate ERK2 activity in response to angiotensin II (40). Jak2 was found to be essential for activation of ERK2. Despite this,

our study (Chapter 4) suggests that these findings may be an artifact of using a nonspecific inhibitor of Jak2 kinase function in the studies.

Other molecules that are recruited into Jak2-based signaling complexes include c-Src, Grb2, PI3 kinase, PP2A, Yes, Raf-1, Shc, Syp, and FAK (41-47). Furthermore, a review of the literature found that more than 50 different cellular proteins associate with Jak2 in some manner. While the precise relationship of each of these proteins to Jak2 is not known, it would seem that each interaction is occurring for a specific cellular and biochemical reason. As such, these studies demonstrate a role for Jak2 as a cellular headquarters for the recruitment, modification, and modulation of numerous signaling pathways. While Jak2 clearly can regulate other intracellular signal transduction pathways, the Jak2 pathway itself is tightly regulated within the cell.

Regulation of Jak2 Signaling

For Jak2 to initially become activated, a single tyrosine within the Jak2 activation loop must be phosphorylated. Site-directed mutagenesis studies showed that phosphorylation of Tyr 1007 is required for ligand-induced Jak2 activation and subsequent phosphorylation of several STATs (48). Activation of Jak2 may depend on its interaction with ancillary molecules such as SH2B-β and SHP-2. The mere expression of SH2B-β in the same cell as Jak2 increases Jak2 tyrosine phosphorylation levels and its catalytic activity (49). It is known that SH2B-β directly binds Jak2, however exactly how SH2B-β biochemically modifies Jak2 to promote its activation has not been determined. The tyrosine phosphatase SHP-2 is a more controversial Jak2-regulatory protein; there are conflicting data regarding whether this protein acts as an activator or an inhibitor of Jak2 function. The discrepancies are due to several factors, including the specific ligand

and cell types used in each experiment. For example, when growth hormone binds to its receptor on transfected COS-7 cells, SHP-2 augments the level by which Jak2 increases *c-fos* expression (50). Jak2 shows higher tyrosine phosphorylation levels in these cells and it is thus believed that SHP-2 augments Jak2 function by elevating Jak2 tyrosine kinase activity. In contrast, when fibroblasts are treated with interferons, SHP-2 inhibits Jak2 function (51).

Equally important to the activators of Jak2 are the inhibitors, that serve to terminate Jak2-dependent signaling. These inhibitors work at different levels of the signal transduction cascade to attenuate Jak2 signaling. In addition to providing a certain level of redundancy in this inhibitory process, the inhibitors also appear to act in a temporal or sequential manner.

Since the Jak2 activation state is dependent on its tyrosine phosphorylation levels, one obvious mechanism of inactivation is tyrosine dephosphorylation of Jak2. This is accomplished by protein tyrosine phosphatases, including SHP-1. The binding of ligands such as growth hormone, erythropoietin, IL-2, and IL-4 to their cognate receptors promotes the binding of SHP-1 to the Jak2-based receptor-signaling complex. The SH2 domain of SHP-1 binds a phosphotyrosine residue on Jak2, thus inhibiting Jak2 activity (52). Not surprisingly, loss of SHP-1 expression in cells lead to a variety of transformed phenotypes, owing to the growth-promoting actions of Jak2 (53, 54).

Suppressors of Cytokine Signaling (SOCS) also act as potent inhibitors of Jak kinase function. They act in a classical negative feedback mechanism. Cytokine inducible SH2 domain-containing protein (CIS) was the first SOCS family member to be cloned. It was initially identified as a gene that was rapidly induced by IL-3 (55). Based

on sequence homology, subsequent groups cloned and characterized seven additional SOCS family members, termed SOCS1-SOCS7. Accumulation of SOCS mRNA is rapidly induced by a variety of cytokines and growth factors including IL-2, IL-4, IL-6, leukemia inhibitory factor, granulocyte colony stimulating factor, interferon-γ, growth hormone, prolactin, erythropoietin, and leptin. There are multiple mechanisms by which SOCS proteins suppress Jak2 function. For instance, SOCS1 binds Jak2 via a direct interaction between the SH2 domain of SOCS1 and phosphotyrosine residue 1007 on Jak2. Structure-function studies have shown that this interaction is required for suppression of Jak2 kinase activity, as deletion of the SOCS1 SH2 domain results in the inability of SOCS1 to reduce Jak2 kinase function (56). However, the exact biochemical modification(s) that occur on Jak2 after co-association with SOCS1 is not known.

A final group of Jak-STAT inhibitory proteins are termed the Protein Inhibitors of Activated STATs (PIAS). The four members identified so far are PIAS-1, PIAS-3, PIAS-X, and PIAS-Y (57, 58). They differ from protein tyrosine phosphatases and SOCS in that they bind STATs and not Jaks. While they share homology among themselves, they have no previously characterized protein domains. These proteins are constitutively expressed in numerous tissues, and do not appear to be highly specific for which STATs they bind. Unfortunately, the biochemical and cellular mechanisms by which PIAS proteins suppress STAT function are not well understood.

Specific activators and inhibitors of Jak2 signaling have been identified. While the activators allow maximal Jak2 activation and kinase function, the inhibitors work in concert to suppress Jak2 signaling at different levels of the Jak-STAT pathway. These regulatory proteins control the magnitude and duration of Jak-STAT signaling. Although

Jak2 activity is tightly controlled within cells, aberrant regulation of this pathway contributes to the pathological progression of certain diseases.

Jak2 in Cancer

In 1995, studies of the *Drosophila* Jak2 homolog, *hopscotch*, were the first to implicate Jak2 in tumorigenesis (59). Specifically, a Glu 695 to Lys mutation in the Hop^{T42} protein increased the intrinsic tyrosine kinase activity of Jak2, and led to malignant neoplasia of Drosophila blood cells. When the same mutation was introduced into the JH2 domain of mammalian Jak2, the mutant protein similarly had significantly increased kinase activity, and hyperactivated the mammalian Jak-STAT signaling pathway (60). Concurrent work by Meydan and colleagues (61) was the first to implicate Jak2 in human cancer, as they reported an inhibition of acute lymphoblastic leukemia via treatment with the Jak2 pharmacological inhibitor AG490. Collectively, these works suggested a direct link between activated Jak2 and neoplastic cell growth.

Tel-Jak2 fusion proteins result from a translocation event between the kinase domain of Jak2 and the HLH domain of Tel. The first reports describing Tel-Jak2 fusion proteins came from a child with early B-precursor acute lymphoid leukemia, and an adult with atypical chronic myeloid leukemia (62, 63). The tumors of these 2 patients are differ because of distinct translocations within the Jak2 and Tel genes, which then give rise to distinct chimeras. Nonetheless, it appears that all Tel-Jak2 fusions confer constitutive Jak-STAT activity. They have been shown to increase NF-κB signaling and induce growth factor-independent proliferation in Ba/F3 hematopoietic cells (64, 65). More importantly, the creation of Tel-Jak2 transgenic mice revealed a causal relationship

between the Tel-Jak2 gene product and leukemogenesis, as over expression of this fusion protein stimulated development of T-cell leukemia in these animals (66).

While many of the mechanisms involving Jak2 activation during cancer are incompletely understood, striking data have surfaced implicating Jak2 activation in human hepatocellular carcinoma (HCC) cells. Methylation of CpG islands within the SOCS-1 gene, which results in reduced SOCS-1 expression, was directly responsible for constitutive Jak2 activity. Furthermore, restoration of SOCS-1 expression and/or treatment with AG490 reduced Jak2 tyrosine kinase activity and the growth rate of these cells (67). Therefore, increased activation of Jak2 was triggered by inactivation of its inhibitor, and this finding identified a new area of therapeutic research.

Increased Jak2 tyrosine kinase activity is associated with many other types of cancers as well. Specifically, Jak2 has been shown to activate ErbB-2, whose oncogenic activity is associated with various human breast cancers (68-70). Additionally, BRCA1 over expression enhanced the ability of Jak2 to activate STAT3 in human prostate cancer cells (71).

Jak2 in Cardiovascular Disease

Jak2 plays a pivotal role in various cardiovascular signaling systems. Therefore, it is not surprising that activation of this protein has been implicated in the molecular events of certain cardiovascular disease states including cardiac hypertrophy, ischemia-reperfusion injury, and heart failure. A review of the literature presents strong evidence that Jak2 plays a role in the cellular signaling processes associated with these and other pathological disease states.

Cardiac hypertrophy (or the increase of cardiac muscle mass) is a natural defense for coping with cardiovascular diseases, and is a major cause of mortality in the United

States (72-74). Cardiac hypertrophy occurs in response to an increased workload on the heart and/or the secretion of certain humoral factors (75-85). Interestingly, cardiac hypertrophy-inducing stimuli also activate Jak2. For example, acute pressure overload in rats increases Jak2 tyrosine phosphorylation levels by causing autocrine/paracrine secretion of angiotensin II (86, 87). Jak2 is also activated by cardiotrophin-1, another potent activator of cardiomyocyte hypertrophy (88, 89). Cardiotrophin-1, a member of the interleukin-6 related cytokine family, induces cardiomyocyte hypertrophy by increasing expression of angiotensinogen mRNA through the activation of STAT3. Activated STAT3 forms dimer complexes that translocate into the nucleus and bind ST-domains within the angiotensinogen gene promoter (90). Jak2 was implicated in this pathological process, as AG490 treatment suppressed the cardiotrophin-induced STAT3 binding to the angiotensinogen promoter and subsequent gene activation (90). Thus, Jak2 appears to have a key role in the signaling system leading to cardiotrophin-1 induced cardiac hypertrophy via increased expression of the angiotensinogen gene.

Heart failure is another disease that has been linked to Jak2, but in a different fashion. In short, heart failure is defined as inadequate cardiac output. The progression of heart failure is dependent on a balance between cardiomyocyte hypertrophy and apoptosis (91). Podewski et al. (92) examined signaling events associated with one disease that leads to heart failure, dilated cardiomyopathy (DCM). They showed that Jak2 tyrosine phosphorylation levels were decreased in patients with DCM, while levels of the Jak2 inhibitor, SOCS1, were increased. Based on this and subsequent work, they proposed a model in which decreased Jak2 tyrosine phosphorylation levels (attributable to an increase in SOCS1 activity) result in decreased phosphorylated STAT3. The result

of this is that STAT3 fails to increase expression of cardioprotective genes that would save the heart from failure. What makes this intriguing is that during DCM, reduced Jak2 activity is pathological; while during cardiac hypertrophy, increased Jak2 activity is pathological. Clearly, future work will need to better define the signaling pathways leading to cardiac hypertrophy and heart failure. Presently, these and other results show that either too much, or too little Jak2 activity, can have negative consequences.

Jak2 activation is also associated with cardiac injury during ischemia-reperfusion (34). Ischemia-reperfusion is a pathological condition characterized by impeded blood flow to an area of tissue followed by the reestablishment of circulation to that same area. It has been shown that treatment with AG490 leads to a reduction in cardiac infarct size and a reduction in apoptotic cell death of cardiomyocytes after ischemia-reperfusion in an isolated perfused rat heart (34). Furthermore, ischemia-reperfusion leads to STAT5a and STAT6 binding to the angiotensinogen gene promoter. Treatment with either AG490 or the AT_1 receptor antagonist losartan resulted in the loss of STAT/St-domain complex formation, and a subsequent reduction in angiotensinogen mRNA levels. Thus, a positive feedback model in which Jak2 activates Stat5a and Stat6 which bind to the angiotensinogen gene promoter, resulting in an increase in angiotensinogen mRNA, and subsequent angiotensin II production and activation of the AT_1 receptor.

Recent evidence has linked Jak2 to vascular injury. Jak2 and STAT3 protein expression levels are increased after balloon injury of rat carotid arteries, and increased activity of the Jak-STAT pathway is involved in the ensuing vascular smooth muscle cell proliferation and neointima formation seen in this model of vascular injury (93). In addition, oxidative stress in the form of hydrogen peroxide potently activates Jak2 in

vascular smooth muscle cells, suggesting a possible role for Jak2 during oxidative stress (35). Evidence supporting a pro-apoptotic role for Jak2 during oxidative stress in vascular smooth muscle cells is given in Chapter 5.

In conclusion, reports have shown a clear relationship between Jak2 and neoplastic transformation, and between Jak2 and cardiovascular disease. Researchers are now trying to determine the cellular and biochemical mechanisms by which aberrant Jak2 activity leads to cancerous cell growth; and to determine the precise role that Jak2 plays in cardiovascular disease progression. These results show that in the near future, Jak2 may be an attractive target for pharmacological inhibition during cancer and heart disease.

Jak2 Pharmacology

Based on the role that Jak2 plays in cancer and cardiovascular disease, pharmacological inhibition of Jak2 may soon hold therapeutic promise. This section reviews research that highlights Jak2 as a therapeutic target.

Many studies have used the commercially available Jak2 inhibitor, tyrphostin AG490, to demonstrate the benefits of inhibiting this signaling pathway during certain disease states. For instance, AG490 suppressed growth of human hepatocellular carcinoma cells (67). Jak2 is constitutively activated in these cells because of methylation and transcriptional silencing of the SOCS-1 gene, a negative regulator of Jak2 signaling. AG490 prevented constitutive Jak2 activation, and induced apoptosis in these cells (67). Similarly, AG490 sensitized metastatic breast cancer cells to chemotherapy-induced apoptosis (68), induced apoptosis in myeloblastic cells (94), and blocked growth of acute lymphoblastic leukemia cells *in vitro* and *in vivo* by inducing apoptosis (61). AG490 also prevented Jak2-mediated constitutive tyrosine

phosphorylation of ErbB-2 and DNA synthesis in breast cancer cells (68), and abrogated growth of human B-precursor leukemic cells (95).

In addition to the potentially therapeutic effects of inhibiting Jak2 in various types of cancer, Jak2 inhibition via AG490 was shown to be therapeutic in several cardiovascular disease models. For instance, AG490 reduced neointima formation in the carotid artery of rats after balloon injury (92). In cultured cardiomyocytes, AG490 attenuated leukemia inhibitory factor-induced hypertrophy and myofilament reorganization (96). Additionally, AG490 inhibited several signaling pathways rapidly induced after myocardial infarction that are thought to contribute to diastolic dysfunction and arrythmogenicity in the post-myocardial infarcted heart (97). Finally, AG490-treated hearts showed a reduction in myocardial infarct size and in the number of cardiomyocytes undergoing apoptosis after ischemia-reperfusion (34). It is apparent from these studies that inhibition of Jak2 in various types of cancer, heart, and vascular disease holds therapeutic promise.

While AG490 has been used extensively to study the Jak-STAT pathway in health and disease, and has been instrumental in identifying Jak2 as a therapeutic target, it suffers from a lack of specificity. For instance, AG490 inhibits activation of cyclin dependent kinases and causes growth arrest of cells in G1 phase (98). It inhibits calf serum inducible cell growth and DNA synthesis (98), and is a partial blocker of c-Src activity (99). Most critically, AG490 inhibits epidermal growth factor receptor autophosphorylation more potently than it inhibits Jak2 activity (100, 101). Moreover, no assay is available for quantifying tissue AG490 concentrations, and no data exist

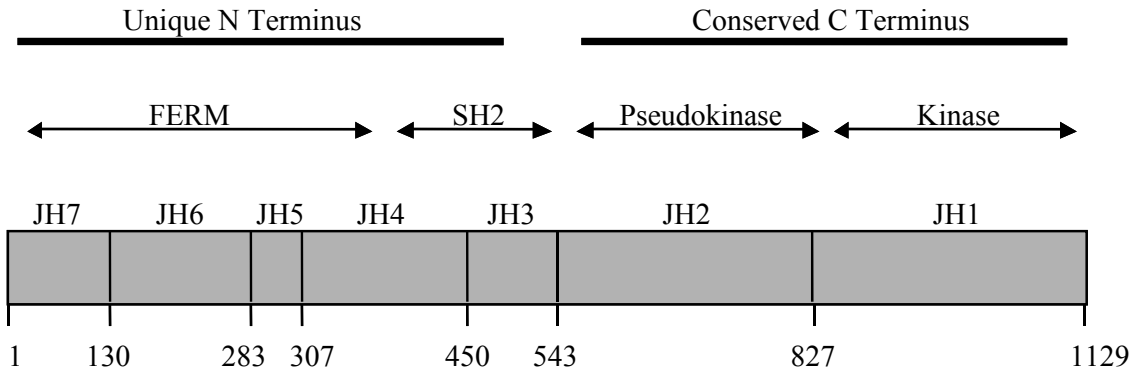
describing the *in vivo* degradation rate of AG490 (93). Thus, the issue of specificity of AG490 for Jak2 is a major concern.

Lack of a specific Jak2 inhibitor has made study of the Jak-STAT pathway difficult. Additionally, Jak2 knockout mice die embryonically, further complicating research efforts (102, 103). Therefore, the identification or development of new, Jak2-specific pharmacological inhibitors would provide useful research tools and potentially therapeutic drugs. As such, investigation into the structural characteristics of the Jak2 protein as they relate to kinase function may be an important step towards achieving this goal.

Structure-Function Relationship of Jak2

When the Jak family of protein kinases was first discovered, their unique structural characteristics were quickly noted. The proteins consist of seven novel protein domains now termed Jak homology (JH) domains. Most of these JH domains have a distinct role in controlling Jak2 function. Figure 1-2 is cartoon summarizing the major domains of Jak2. This section reviews the structural characteristics of the Jak2 protein, and recent research that advanced our understanding of the structure-function relationship of Jak2.

The carboxyl terminus of Jak2 contains the JH1 and JH2 domains. The JH1 domain is the highly conserved kinase domain, which contains the ATP-binding region and the activation loop. Most of the structure-function data available for Jak2 concerns this domain. Studies have shown the requirement for the JH1 domain for Jak2 tyrosine kinase function (104). In addition, a number of individual amino acids within the JH1 domain have bee identified that are critical to this function. Two adjacent tyrosines, Tyr 1007 and Tyr 1008, were shown to be phosphorylation sites within the



Amino Acid	Function
2-240	Critical for Jak2/GHR co-association
231-235	Critical for Jak2/AT_1R co-association
Lys 882	Critical for Jak2 autophosphorylation
994-1024	Activation loop
Try 1007	Phosphorylated upon Jak2 activation
Tyr 1008	Phosphorylated upon Jak2 activation
Trp 1020	Critical for Jak2 autophosphorylation
Glu 1024	Critical for Jak2 autophosphorylation
Glu 1046	Stabilizes activation loop via H bond with Trp 1020

Figure 1-2. Summary of major Jak2 domains and amino acids critical to Jak2 tyrosine kinase activity

Jak2 activation loop. The tyrosine at position 1007 is required for Jak2 tyrosine kinase activity, while the tyrosine at position 1008 seems to be dispensable for the tyrosine kinase function of Jak2 (48). Additionally, Tyr 1007 interacts with a Jak binding protein (JAB), also known as suppressor of cytokine signaling 1 (SOCS1), a protein that negatively regulates the kinase activity of Jak2 (56). Also, mutation of the invariant Lys 882 in the JH1 domain rendered Jak2 catalytically inactive (104, 105).

In 1994, a Jak2 containing mutations at both Trp 1020 and Glu 1024 (W1020G/E1024A) was shown to not only be catalytically inactive, but also dominant negative (106). We recently showed that mutation of either of these amino acids

individually rendered Jak2 catalytically inactive (107). Moreover, we elucidated the requirement for Trp 1020 for Jak2 kinase activity, by demonstrating that Trp 1020 forms a hydrogen bond with Glu 1046, an amino acid that previously had not been shown to be required for Jak2 function. The importance of this interaction is that it appears to stabilize the three dimensional structure of the Jak2 activation loop. More recently, we elucidated the requirement for Glu 1024. These data are presented in Chapter 7.

The JH2 domain of Jak2 is the pseudokinase domain. While it shares conserved motifs with other protein kinases, this region is catalytically inactive. In fact, the active site and activation loop are modified compared to traditional protein tyrosine kinase activation loops (108). These modifications are, however, conserved amongst Jak family members, suggesting a regulatory role for this pseudokinase domain (109).

Other lines of evidence point to a critical role for the JH2 domain. In *Drosophila*, a mutation in the JH2 domain of the Jak homolog *Hop*, rendered the protein hyperactive and caused hematopoietic hyperplasia (59). This was the first evidence of a negative regulatory role for the JH2 domain. Subsequent work by Saharinen et al. showed that the JH2 domain of mammalian Jak2 interacts with the JH1 kinase domain and suppresses the activity of Jak2 at the basal level (109). Following this work, another group constructed a homology model of the JH1 and JH2 domains. Using this model, they predicted two major interactions between the JH1 and JH2 domains of Jak2. The first interaction is between the two α helices of the two domains. The second interaction occurs between a loop that lies between two β stands of the JH2 domain and the activation loop of the JH1 domain (110). Incidentally, another group, Chen et al., showed a similar role for the JH2 domain in Jak3, suggesting functional conservation amongst Jak family members (111).

In addition, Saharinen *et al.* showed that the JH2 domain is also required for the ability of Jak2 to become maximally activated in response to cytokine stimulation (112).

This same group further clarified the regulatory role of the JH2 domain by identifying three specific regions within this domain that are involved in the auto-inhibition of Jak2, which are termed IR1, IR2, and IR3 (113). IR3, which spans amino acid residues 758 to 807, directly inhibited the JH1 kinase domain, while IR1 spanning residues 619 to 670 and IR2 spanning residues 725 to 757, enhanced IR3-mediated inhibition of the JH1 domain. In this work, the authors present an explanatory model for the function of JH2 in regulating JH1. In it, they suggest that under basal conditions, JH2 is bound to JH1. Upon cytokine binding and receptor aggregation, the inhibitory JH1-JH2 interaction is displaced, possibly through homotypic interaction of the JH1 domains of the two Jak2 molecules. Why the JH2 domain is required for maximal cytokine-induced Jak2 activation still remains to be determined.

The amino terminal region of Jak2 comprises the JH3 through JH7 domains. This region is largely responsible for receptor interactions and may be responsible for interactions between Jak2 and regulatory molecules. Interestingly, although the Jak family members have traditionally been considered absent of a canonical SH2 domain, it was noted that in Tyk2, the second half of the JH4 domain plus the whole of the JH3 domain weakly resembles an SH2 domain (114). Upon cloning of murine Jak2, it was similarly noted that the sequence GLYVLRWS bore weak homology to the core sequence element of SH2 domains (FLVRES) (3). Using multiple sequence alignments and secondary and tertiary structure predictions, Kampa *et al.* presented for the first time a three dimensional view of a putative Jak family SH2 domain spanning amino acids 412-

480 of Jak3. Furthermore, this group demonstrated binding of phosphorylated proteins to the putative SH2 domain of Jak3, thus proving an SH2-like function for this domain (115, 116).

Despite these results, Giordanetto and Kroemer recently published a predicted Jak2 structure comprising JH domains 1 through 7, whereby they contended that the putative SH2 domain of Jak2 might not be fully functional (117). Comparing the SH2 domain of $p56^{lck}$ with the putative Jak2 SH2 domain, they demonstrated several key differences at amino acids involved in phosphotyrosine binding between the two that may preclude the Jak2 SH2 domain from being fully functional. They stated that despite conservation between the two SH2 domains of one arginine known to interact with phosphate groups (Arg 426 of Jak2, Arg 134 of $p56^{lck}$), another highly conserved arginine known to interact with phosphate groups is replaced by a methionine at the corresponding position in Jak2 (Met 406 of Jak2, Arg 154 of $p56^{lck}$). This, they point out, would abolish interaction with a phosphate group. Furthermore, a lysine within the putative Jak2 SH2 domain corresponds to a highly conserved serine residue in other SH2 domains (Lys 430 of Jak2, Ser 158 of $p56^{lck}$). The authors contend that the longer side chain of lysine would present difficulties in allowing it to interact with a phosphotyrosine residue. Finally, they show that there are key differences in the phosphotyrosine binding pockets of the two SH2 domains. Specifically, Glu 157 of $p56^{lck}$ is replaced by Pro 429 in Jak2. In the predicted model, this proline appears to restrict access to the phosphotyrosine binding pocket, because of the rigid side chain of proline. Whether an SH2 domain exists within Jak2 and whether it is fully functional may be clarified in the future by determining the crystal structure of Jak2.

Adjacent to the putative SH2 domain of Jak2 lays the FERM domain, which spans from the middle of the JH4 domain through the JH7 domain (118). This domain is present in a number of other proteins including the band 4.1 protein from erythrocytes, some protein tyrosine phosphatases, and the focal adhesion kinases (119-121). Often, this domain is involved in stable association with membrane bound proteins (122). In fact, the amino terminal region of Jak2, especially the JH6 and JH7 domains (comprising part of the FERM domain), has been shown to be crucial for Jak2/receptor interactions.

For example, deletion of amino acids 2-239, which deletes the JH7 domain and part of the JH6 domain, abrogated Jak2 association with the growth hormone receptor (123). It was then shown, though, that neither the JH6 and JH7 domains alone, nor the entire Jak2 FERM domain (comprising the JH6 and JH7 domains with the JH5 domain and half of the JH4 domain) were sufficient for Jak2 association with the growth hormone receptor. This suggested that multiple JH domains must interact to allow Jak2 association with cytokine receptors (124). Additionally, amino acids 1-294 were found to be essential for Jak2 binding to the granulocyte-macrophage colony stimulating factor (GM-CSF)-β_c receptor (125). More recently, our lab found that the YRFRR motif within the JH6 domain of Jak2, spanning amino acids 231-235, was essential for both Jak2 association with the angiotensin II type 1 receptor (AT_1 receptor) and STAT1 translocation to the nucleus in response to angiotensin II treatment (126). Similar requirements for the amino terminal region for receptor association have been shown for the other Jak family members (127, 128). The Jak2 FERM domain interacts with cytokine receptors via a proline rich box1 motif on the cytoplasmic chain of the cytokine receptor (129, 124). A recent paper describing a homology model of Jak2 comprising the

JH1-7 domains highlighted hydrophobic amino acids within the FERM domain predicted to be essential for Jak2 association with the box1 motif of cytokine receptors (117). The analysis predicted that Met 181, Phe 236, Phe 240, and Iso 223 within the Jak2 FERM domain would mediate interaction with receptors. These results elucidated the requirement of the FERM domain for receptor interaction, but mutational studies on the predicted amino acids are required for confirmation of these predictions.

Collectively, structure-function studies involving the Jak family of non-receptor tyrosine kinases identified seven conserved Jak homology domains in each protein. Studies have described essential functions for each of these domains, including kinase activation for the JH1 domain, autoregulation for the JH2 domain, and interactions with substrates, regulatory proteins, and membrane-bound receptors for the JH3-7 domains. Future structure-function studies will be critical in advancing our understanding of Jak2, and may lead to new ways to control its function, including Jak2-specific pharmacological inhibition.

CHAPTER 2
EXPERIMENTAL METHODS

Cell Culture

All cells were cultured at 37^{o}C in a 5% CO_2 humidified atmosphere and were maintained in DMEM containing 10% fetal bovine serum. Ample stocks of these cells are stored in liquid nitrogen.

Vaccinia Virus Transfection/Infection

BSC-40 cells were transfected in serum free media with Jak2 cDNA cloned into the pRC-CMV plasmid. After 4 h of transfection, cells were infected with 1.0 MOI of vaccinia virus vTF7-3. After 1 h of incubation with the virus, complete media was put on the cells to stop transfection, and the cells were allowed to become infected with virus for 16 h. The virus expresses a T7 RNA polymerase, which drives Jak2 expression from a T7 promoter upstream of the Jak2 cDNA in the pRC-CMV plasmid, allowing overexpression of the Jak2 protein.

Immunoprecipitation/Western Blotting

To prepare lysates, cells were washed with two volumes of ice-cold PBS containing 1 mM Na_3VO_4 and lysed in 1.0 mL of ice-cold RIPA buffer containing protease inhibitors. The samples were sonicated and incubated on ice for 30 min. Samples were spun at 16,000 x g for 5 min at 4°C, and supernatants were normalized for protein content using the Bio-Rad D_c assay. Normalized lysates (approx. 400 μg/mL) were immunoprecipitated for 2-4 h at 4°C with 2 μg of antibody and 20 μl of Protein A/G Plus agarose beads (Santa Cruz Biotechnology). After centrifugation, protein complexes were

washed 3 times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) and resuspended in SDS sample buffer. Bound proteins were boiled, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. After blocking membrane for 1 h in 5% dry milk/TBST (100 mM Tris, pH 7.5, 0.9% NaCl and 0.05% Tween 20) at room temperature, nitrocellulose membranes were probed with primary antibody for 1-2 h at room temperature in 5% milk/TBST. Blots were washed with TBST and proteins were visualized using enhanced chemiluminescence (ECL) following the manufacturers instructions (Amersham).

Immunocytochemistry

Cells were grown on microscope slides. After treatment, cells were washed twice with K^+ free PBS and fixed for 60 min at room temperature with 4% paraformaldehyde. After fixation, cells were washed 4 times with K^+ free PBS, permeabilized for 10 min at room temperature with 0.2% Triton X-100 in K^+ free PBS (vol/vol), washed an additional 4 times, and then blocked with 5 mg/mL BSA in K^+ free PBS for 4 h at room temperature. After blocking, cells were incubated with primary antibody overnight at 4ºC in K^+ free PBS containing 5 mg/mL BSA. The next day, cells were washed 5 times and incubated with secondary antibody conjugated to Texas Red for 4 h at room temperature. The cells were then dehydrated through increasing concentrations of ethanol, dipped into xylene, and mounted. The next day, the cells were visualized with a fluorescent microscope.

***In Vitro* Kinase Assays**

ERK2 immunoprecipitates were washed twice with wash buffer, followed by two washes in kinase reaction buffer (25 mM HEPES pH 7.4 and 20 mM $MgCl_2$). The precipitates were resuspended in 50 μl of the same kinase buffer containing 50 μM ATP,

2 μCi ^{32}P γ-ATP, and 5 μg myelin basic protein (MBP). The samples were incubated for 15 min at 30°C. Reactions were terminated by adding sample buffer. The samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to autoradiography.

Site-Directed Mutagenesis

Mutations of Jak2 amino acids were generated using the QuikChange site-directed mutagenesis system (Stratagene). All mutations were confirmed by DNA sequence analysis.

Luciferase Assays

100 mm dishes of 70% confluent COS-7 cells were transfected with 10 μg *c-fos*/luciferase in 12 μl Lipofectin for 5 h. The cells were then trypsinized and seeded into 6-well plates at 4.5 x 10^5 cells per well, and allowed to attach overnight in serum containing medium. The next morning, the cells were washed and placed into serum free DMEM. The next morning, the cells were stimulated with angiotensin II, and luciferase activity was measured from detergent extracts in the presence of ATP and luciferin using the Reporter Lysis Buffer System (Promega) and a luminometer (Moonlight 3010).

Molecular Modeling

A structural homology model of the Jak2 kinase domain ranging from amino acid 814 to the stop codon (position 1129) was generated using the program Swiss Model. The model was based on the known crystal structure of the kinase domain of the fibroblast growth factor tyrosine kinase receptor. The program HBPLUS Hydrogen Bond Calculator, version 3.15, was used to determine hydrogen bond interactions and bond lengths.

Analysis of DNA Laddering

Genomic DNA was isolated using the Easy-DNA kit from Invitrogen. 20 μg of DNA was separtated on a 1.8% agarose gel and stained with ethidium bromide. Laddering was analyzed under ultraviolet light using the GelDoc system.

Hoechst 33342 Staining

Cells were grown on microscope slides, serum starved for 48 h, and treated. Cells were washed twice with PBS and incubated for 30 min at room temperature with 50 μg/mL Hoechst 33342 nuclear stain. Cells were then washed, fixed, and mounted as described in the immunocytochemistry section. The cells were visualized using a fluorescent microscope with appropriate filters. Apoptotic cells were counted as those showing condensed and/or fragmented nuclei.

Propidium Iodide Staining

Cells were grown on microscope slides and stained with 1 μg/mL propidium iodide for 10 min at 37^{o}C. Live cells were examined using confocal microscopy. Same field images were captured under phase contrast and fluorescent conditions.

Analysis of Mitochondrial Membrane Integrity

Mitochondrial membrane integrity was analyzed using the MitoCapture Apoptosis Detection Kit from BioVision. Cells were treated with 1 mM hydrogen peroxide for 2 h and stained according to the manufacturer's protocol. Live cells were then visualized using appropriate filters on a confocal microscope. Predominant green staining occurs in cells with a disrupted mitochondrial membrane potential, while predominant red staining occurs in cells with an intact mitochondrial membrane potential.

CHAPTER 3
MEANS OF CIRCUMVENTING PROBLEMS WITH AG490

Introduction

As discussed in Chapter 1, one of the central problems facing researchers studying Jak2 tyrosine kinase function is the lack of a specific Jak2 inhibitor. While AG490 is a useful research tool, results acquired using AG490 must be corroborated using other methodology. Here we will briefly discuss *in vitro* strategies that we use to circumvent the problems with using AG490.

Primary Rat Aortic Smooth Muscle Cells

To study the role that Jak2 tyrosine kinase plays in VSMC physiology, we use primary rat aortic smooth muscle cells (RASM cells) stably transfected with a Jak2 dominant negative mutant (RASM-DN). As controls, we use the same cells expressing a neomycin resistant cassette (RASM-Control). Creation of these cells has been described previously (39). In the RASM-DN cells, Jak2 tyrosine kinase function is suppressed by the Jak2 dominant negative mutant, allowing us to reliably study the function of Jak2.

γ2A Cell Line

To study the role that Jak2 plays in intracellular Ang II-dependent signaling, we used γ2A cells, which are human fibrosarcoma cells that were gamma-irradiated and screened to identify Jak2-deficient cells. The cells were then stably transfected with the angiotensin II type I receptor (γ2A/AT_1). Controls are γ2A/AT_1 cells stably transfected with Jak2 cDNA to reconstitute Jak2 signaling (γ2A/AT_1+Jak2) (130).

CHAPTER 4
Jak2 REGULATES ANGIOTENSIN II-DEPEDENDENT ERK2 SIGNALING

Introduction

Extracellular signal regulated kinase 2 (ERK2) is a member of the mitogen-activated protein (MAP) 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 (131-134). Angiotensin II is one such ligand; it exerts many of its mitogenic effects by binding to the angiotensin II type 1 (AT_1) receptor and 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 tyrosine and threonine residues (135).

ERK activity is tightly regulated. The duration of ERK activation is regulated by the intracellular signals that phosphorylate and dephosphorylate it (136). While ERKs are activated by ligand binding at the cell surface, they are inactivated by several dual-specificity phosphatases (137). One of these, MAP 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 (138).

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 (44, 139-140). In fact, previous work suggested that inhibition of Jak2 using the pharmacological compound AG490, blocks the angiotensin II-dependent activation of

ERK2 (18). One problem, though, with using AG490 to study the function of Jak2 is its lack of specificity for Jak2. To examine the role that Jak2 plays in the regulation of Ang II-induced ERK2 signaling, we used the γ2A cells described in Chapter 3. Briefly, these are Jak2 -/- cells that stably express the AT_1 receptor (γ2A/AT_1). Controls cells are the γ2A cells stably transfected with both the AT_1 receptor and Jak2 (γ2A/AT_1+Jak2), to reconstitute Ang II-dependent Jak2 signaling.

Results

ERK2 Activity is Sustained in γ2A/AT_1 Cells Compared to γ2A/AT_1+Jak2 Cells after Angiotensin II Treatment

We sought to determine the role that Jak2 plays in angiotensin II-dependent ERK2 activity using the γ2A-derived cells. γ2A/AT_1 and γ2A/AT_1+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-ACTIVE-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. 4-1A, Top). However, in the γ2A/AT_1+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 then stripped and reprobed with an anti-ERK2 antibody to show constant ERK2 expression at all time points (Fig. 4-1A, Bottom).

The 3 different membranes representing Fig. 4-1A were scanned for densimetric analysis, and ERK2 phosphorylation was plotted as a function of angiotensin II treatment (Fig. 4-1B). The graph shows that in the cells expressing Jak2, ERK2 phosphorylation transiently increased, peaking 6 min after angiotensin II treatment. However, in cells

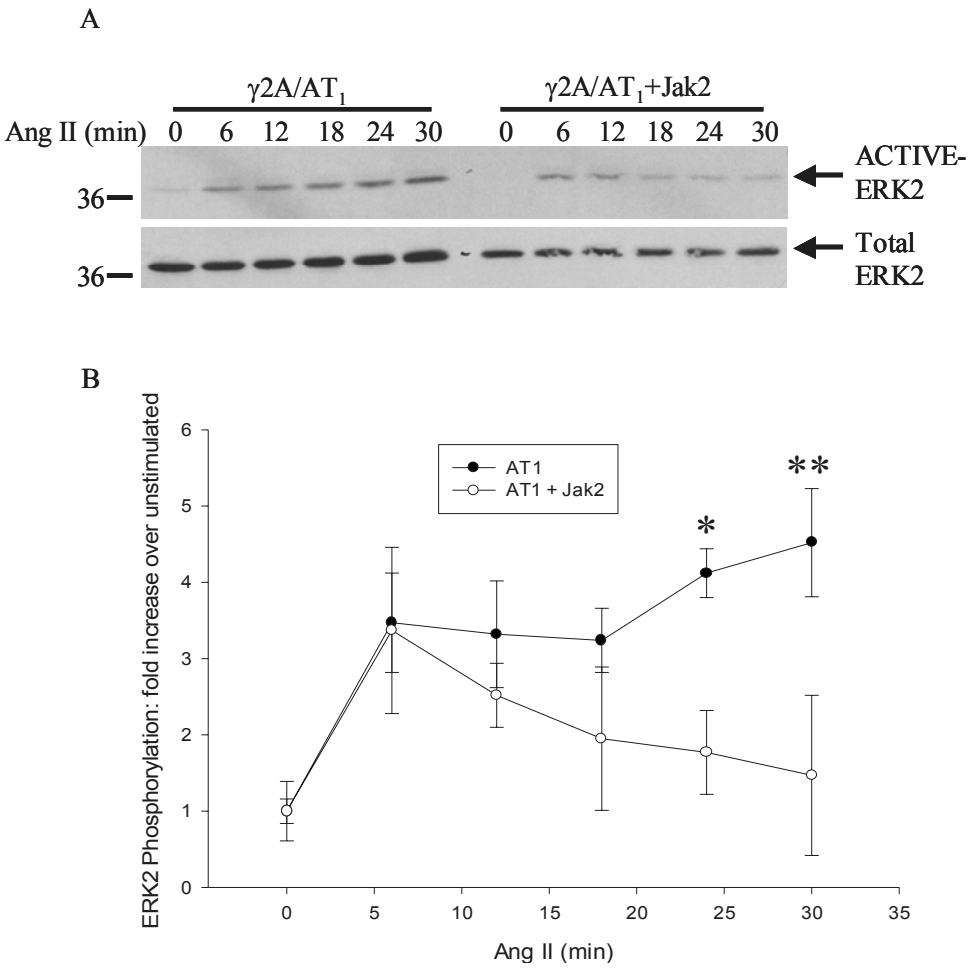


Figure 4-1. ERK2 activity is sustained in γ2A/AT$_1$ cells compared to γ2A/AT$_1$+Jak2 cells after angiotensin II treatment A) γ2A/AT$_1$ and γ2A/AT$_1$+Jak2 cells were treated with 100 nM angiotensin II for the indicated times. Whole cell lysates were prepared and Western blotted with anti-phospho-ERK2 antibody to detect activated ERK2 (Top). The membrane was then stripped and reprobed with anti-ERK2 antibody to confirm total ERK2 protein levels (Bottom). B) The three membranes representing Fig. 5-1A were subjected to densimetric analysis. Anti-phospho-ERK2 signal was plotted as a function of both angiotensin II treatment and Jak2 expression. Values are expressed as the mean +/- SD. * $p<0.01$, **$p<0.05$ (Student's *t* test). Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1960, Fig. 3A and 3B with permission from publisher.

lacking Jak2, ERK2 phosphorylation was significantly elevated 30 min after angiotensin II stimulation. Thus, the data in Fig. 4-1 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 (18).

To verify our results using an alternate protocol, *in vitro* kinase assays were performed using myelin basic protein (MBP) as a substrate for ERK2 phosphorylation. In γ2A/AT_1 cells, phosphorylation of MBP remained elevated 20 min after angiotensin II treatment, suggesting that ERK2 was catalytically active at this time point (Fig. 4-2).

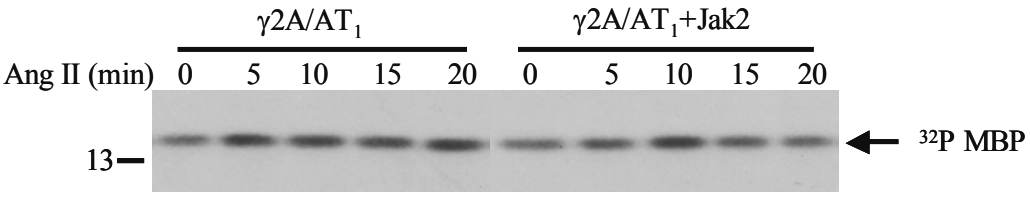


Figure 4-2. In vitro kinase assay confirms that ERK2 activity is sustained in γ2A/AT_1 cells compared to γ2A/AT_1+Jak2 γ2A/AT_1 cells and γ2A/AT_1+Jak2 cells were treated with 100 nM angiotensin II for the indicated times. Lysates were prepared and immunoprecipitated with anti-ERK2-mAb and then resuspended in kinase reaction buffer. Phosphorylation of myelin basic protein was detected by autoradiography. Shown is one of three independent results. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1960, Fig. 3C with permission from publisher.

However, in γ2A/AT_1+Jak2 cells, angiotensin II stimulated ERK2 activity peaked at 5-10 min. After 15-20 min, the ^{32}P-labeled MBP signal was similar to that of basal conditions.

Collectively, the results in Fig. 4-1 and 4-2 clearly show that loss of Jak2 expression via a Jak2 null mutation results in enhanced ERK2 activation in response to angiotensin II when compared to Jak2-expressing control cells.

AG490 Suppresses Angiotensin II-Dependent ERK2 Activation Independent of Jak2 Inhibition

We next wanted to determine whether treating the Jak2 expressing cells with AG490 could recapitulate the observations seen in Fig. 4-1. 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/AT_1+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/AT_1 cells were treated with angiotensin II to reproduce the enhanced ERK2 activation seen 30 min after angiotensin II treatment (Fig. 4-3A, lanes 1-3). In γ2A/AT_1+Jak2 cells pretreated with the AG9 control compound, Ang II-induced ERK2 phosphorylation levels were high at 5 min, but returned to basal levels by 30 min, similar to that shown in Fig. 4-1A (Fig. 4-3A, lanes 4-6). However, in γ2A/AT_1+Jak2 cells pretreated with AG490, the ability of angiotensin II to induce ERK2 phosphorylation appeared to be lost (Fig 4-3A, 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 suggests that one mechanism by which G protein coupled receptors activate ERK1/2 is via transactivation of the epidermal growth factor receptor (EGFR) (14-143). This is important because, as indicated in Chapter 1, AG490 inhibits epidermal growth factor receptor autophosphorylation more potently that it inhibits Jak2 kinase activity (100, 101). To determine whether this mechanism of action is responsible for the angiotensin II-dependent activation of ERK2 in the γ2A-derived cells, γ2A/AT_1+Jak2 cells were treated with the EGFR tyrosine kinase inhibitor, AG1478, before measuring angiotensin II-dependent ERK2 activation. AG1478 treatment, at a dose that has

previously been shown to fully suppress EGFR kinase activity (144, 145), failed to inhibit the angiotensin II-mediated activation of ERK2 in γ2A/AT_1+Jak2 cells, when

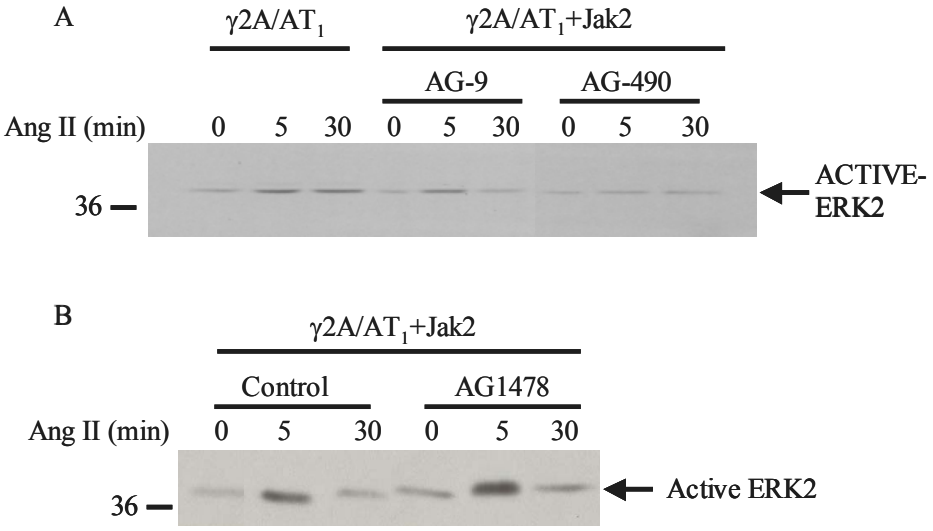


Figure 4-3. AG490 suppresses angiotensin II-dependent ERK2 activation independent of Jak2 inhibition A) Cells were pretreated for 16 hrs with either 100 μM AG9 or 100 μM AG490 and then stimulated with 100 nM angiotensin II for the indicated times. Whole cell lysates were prepared and then Western blotted with anti-phospho-ERK2 antibody to detect activated ERK2 protein. B) Cells were pretreated for 1 hr with 10 μM AG1478 and then stimulated with 100 nM angiotensin II for the indicated times. Whole cell lysates were prepared and then Western blotted with anti-phospho-ERK2 antibody to detect activated ERK2 protein. Shown is one of three representative results for each. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1961, Fig. 4A and 4B with permission from publisher.

compared to similarly treated control cells (Fig. 4-3B). This result suggests that in the γ2A-derived cells, angiotensin II mediates activation of ERK2 via a mechanism that is independent of EGFR tyrosine kinase activity.

In summary, the data in Fig. 4-3A show that AG490 blocks the angiotensin II-dependent activation of ERK2 via a mechanism that is independent of Jak2. The data in Fig. 4-3B show that this nonspecific effect of AG490 is not because of reduced EGFR tyrosine kinase activity, as direct suppression of EGFR tyrosine kinase activity with

AG1478 does not inhibit angiotensin II-dependent activation of ERK2. 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/AT$_1$ and γ2A/AT$_1$+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 (136). We next wanted to determine if a difference in nuclear accumulation of activated ERK2 existed between γ2A/AT$_1$ and γ2A/AT$_1$+Jak2 cells. For this, both cell types were immunostained with an anti-ACTIVE-ERK2 antibody. The cells were then visualized using a fluorescent microscope to measure angiotensin II-dependent ERK2 nuclear accumulation, as a function of Jak2 expression (Fig. 4-4). There was a marked difference in the nuclear accumulation pattern of phospho-ERK2 between the two cell types. Specifically, in the cells lacking Jak2, angiotensin II treatment facilitated a rapid nuclear accumulation of activated ERK2. This staining persisted for at least 30 min, but also appeared to be perinuclear in nature at this time point (arrows). For the Jak2 expressing cells however, angiotensin II treatment promoted a transient nuclear accumulation of activated ERK2 that was visible at 5 min, but virtually gone at 30 min. Collectively, these data suggest that the sustained levels of phospho-ERK2 seen in Fig. 4-1 correlate with increased phospho-ERK2 immunoreactivity in the nucleus of the cell.

ERK2-Dependent Gene Transcription in the γ2A/AT$_1$ and γ2A/AT$_1$+Jak2 Cells

To determine if differences in ERK2-mediated gene expression existed between the γ2A/AT$_1$ and γ2A/AT$_1$+Jak2 cells, we measured angiotensin II-dependent, ERK2

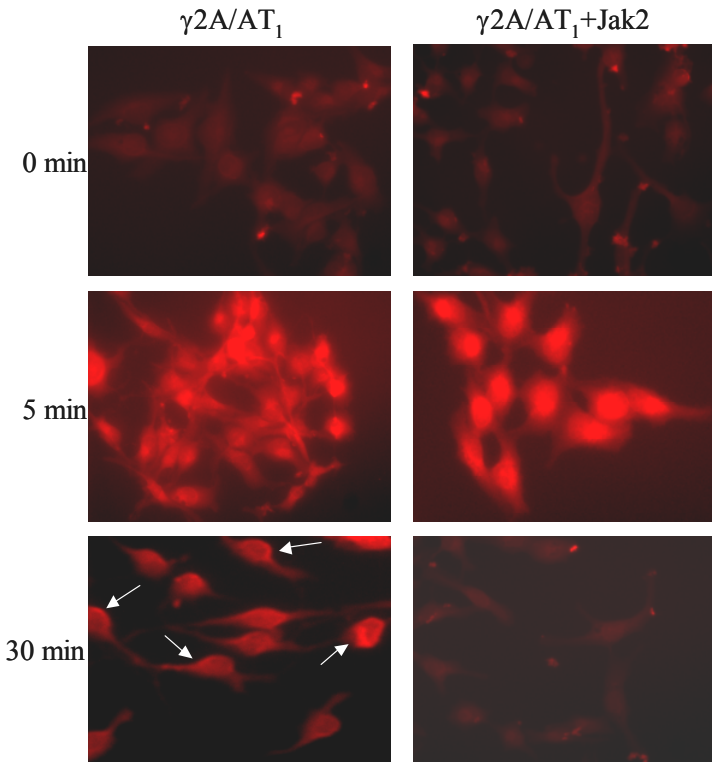


Figure 4-4. Difference in the angiotensin II-dependent nuclear accumulation of phospho-ERK2 between γ2A/AT_1 and γ2A/AT_1+Jak2 cells γ2A/AT_1 cells and γ2A/AT_1+Jak2 cells were treated with 100 nM angiotensin II for either 0, 5 or 30 min. The cells were incubated with anti-phospho-ERK2-pAb and immunostained with goat-anti-rabbit antibody conjugated to Texas Red. The cells were visualized using a fluorescent microscope to detect nuclear accumulation of activated ERK2 protein. Arrows indicate apparent perinuclear staining. Shown is one of three independent results. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1962, Fig. 5 with permission from publisher.

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. 4-5). Although the γ2A/AT_1 cells repeatedly generated higher fold changes in luciferase activity

compared to 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. 4-1 and 4-2 show that in the cells lacking Jak2, ERK2 has sustained levels of activation when compared to the Jak2-expressing cells. We hypothesized that the signal transduction pathways leading to the induction of ERK2

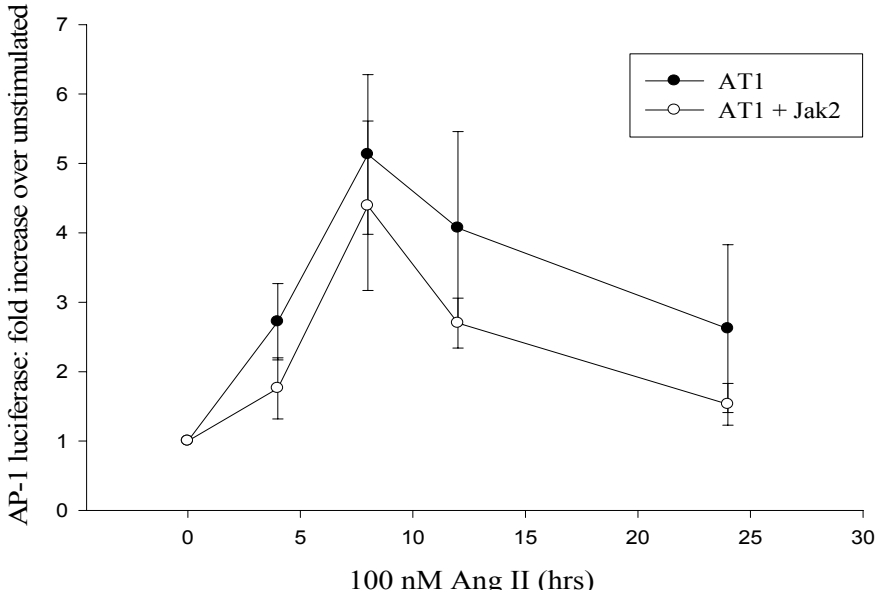


Figure 4-5. No difference in angiotensin II-dependent ERK mediated gene transcription between γ2A/AT_1 and γ2A/AT_1+Jak2 cells γ2A/AT_1 and γ2A/AT_1+Jak2 cells were transfected with 10 μg of a luciferase reporter whose expression is driven by a synthetic promoter containing seven copies of the AP-1 binding element to measure ERK-mediated gene transcription. The cells were treated with 100 nM angiotensin II for the indicated times. Lysates were prepared and luciferase activity was measured. Shown is one of three independent results. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1963, Fig. 6 with permission from publisher.

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. 4-6A). The results show that there was no marked difference in PP2A protein expression between the γ2A/AT$_1$ and γ2A/AT$_1$+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/AT$_1$ cells, angiotensin II induced very little MKP-1 expression (Fig 4-6B). In the γ2A/AT$_1$+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 (138). We next investigated if Jak2 is required for co-association of MKP-1 with ERK2. γ2A/AT$_1$ and γ2A/AT$_1$+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 anti-ERK2 antibody and then immunoblotted with anti-MKP-1 antibody. The results show that no increase in co-association of ERK2 and MKP-1 was

observed in γ2A/AT_1 cells, whereas in γ2A/AT_1+Jak2 cells, angiotensin II induced a substantial increase in co-association of ERK2 and MKP-1 (Fig. 4-6C).

The data in Fig. 4-6B 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 (137, 138). To determine the relative contribution of ERK2 and Jak2 in mediating angiotensin II-dependent MKP-1 gene expression, both cell types were treated with angiotensin II in the presence or absence of the MEK specific inhibitor, PD98059 (Fig. 4-6D). 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. 4-6 suggest that Jak2 is not only required for induction of MKP-1 expression in response to angiotensin II, but also for co-association of MKP-1 and ERK2. Additionally, the data indicate that that both Jak2 and ERK2 are required for maximal angiotensin II-mediated MKP-1 protein expression.

MPK-1 is Required for Angiotensin II-Dependent Inactivation of ERK2

The data in Fig. 4-6 show 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

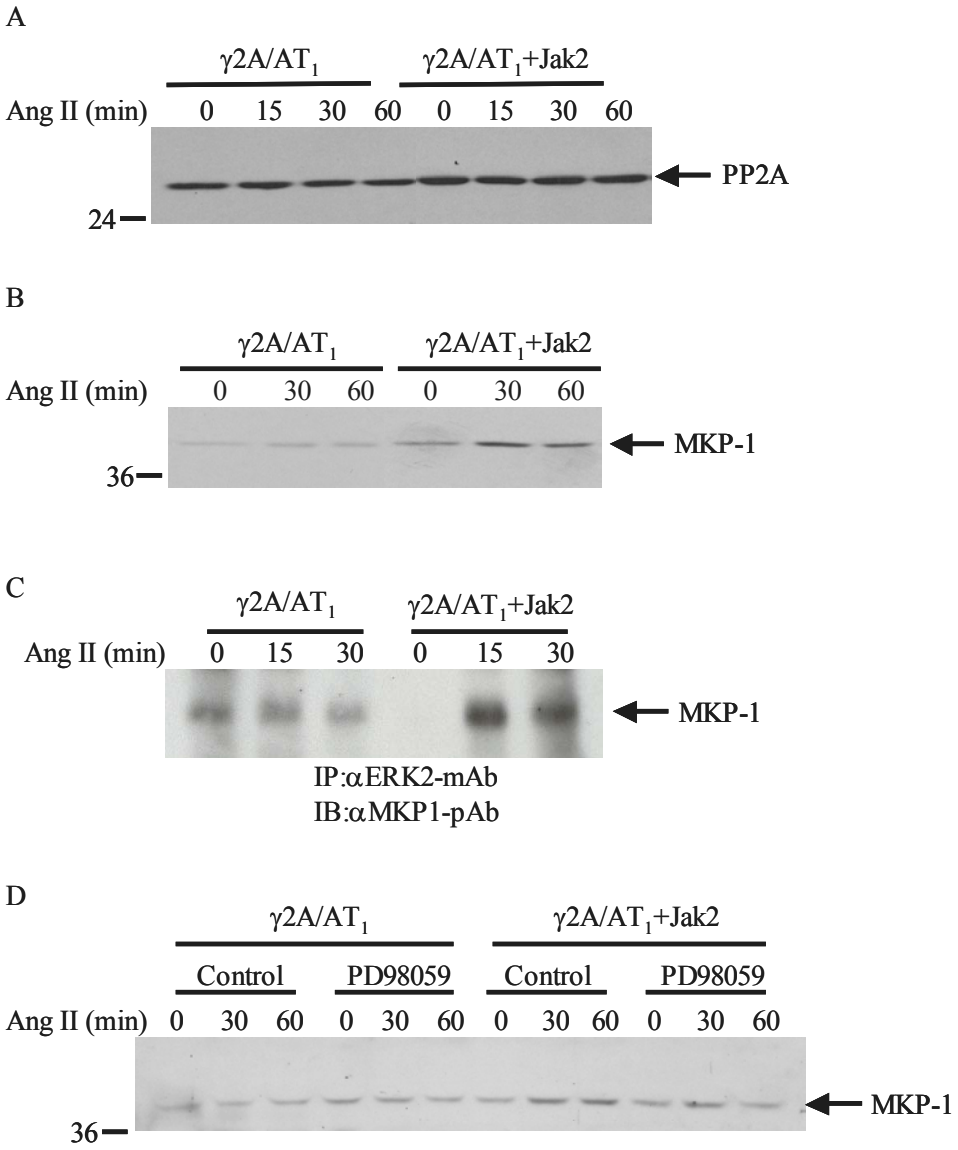


Figure 4-6. Jak2 is essential for angiotensin II-induced MKP-1 expression $\gamma 2A/AT_1$ and $\gamma 2A/AT_1$+Jak2 cells were treated with angiotensin II for the indicated times and cell lysates were prepared. A) Whole cell lysates were Western blotted with anti-PP2A-mAb to detect PP2A expression. B) Whole cell lysates were Western blotted with anti-MKP-1-mAb to detect MKP-1 expression. C) Lysates were immunoprecipitated with anti-ERK2-mAb and Western blotted with anti-MKP-1-mAb to detect co-association of MKP-1 with ERK2. D) Cells were pre-treated for 60 min with either DMSO or 50 μM PD98059 and then stimulated with 100 nM angiotensin II for the indicated times. Whole cell lysates were prepared and then Western blotted with anti-MKP-1-mAb to detect MKP-1 protein. Shown is one of four (A) or three (B, C, D) independent results for each. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1964, Fig. 7 with permission from publisher.

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, $\gamma 2A/AT_1$ and $\gamma 2A/AT_1$+Jak2 cells were pretreated with the PP2A specific inhibitor, okadaic acid (146). The cells were then treated with angiotensin II and ERK2 phosphorylation was measured via Western blot analysis (Fig. 4-7 A). In the $\gamma 2A/AT_1$ cells, before ligand treatment, ERK2 was already phosphorylated, possibly because of the presence of a phosphatase inhibitor. Addition of angiotensin II did not significantly increase the signal at either the 5 or 30 min time points, thereby suggesting that the signal was at, or near, maximal phosphorylation levels before ligand treatment. Additionally, the data suggest that angiotensin II treatment did not induce the necessary cellular factors, such as Jak2, that are required for dephosphorylating ERK2, since ERK2 remained phosphorylated 30 min after ligand treatment. For the $\gamma 2A/AT_1$+Jak2 cells, ERK2 was also basally phosphorylated before ligand treatment. In contrast however, 30 min of angiotensin II treatment promoted its relative dephosphorylation. This observation suggests two important things. First, it indicates that the $\gamma 2A/AT_1$+Jak2 cells contain the necessary component(s) to promote the dephosphorylation of ERK2 (ie. 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 (147). The cells were then treated with angiotensin II and ERK2 phosphorylation was again measured via Western blot analysis with anti-ACTIVE-ERK2 antibody (Fig. 4-7B).

In the γ2A/AT_1 cells, before ligand treatment, ERK2 showed some basal phosphorylation. Addition of angiotensin II modestly increased the signal at both the 5 and 30 min time points. These cells were once again unable to dephosphorylate ERK2 after 30 min of ligand treatment. Similarly, in the γ2A/AT_1+Jak2 cells, ERK2 was basally phosphorylated before ligand treatment and 5 min of angiotensin II treatment

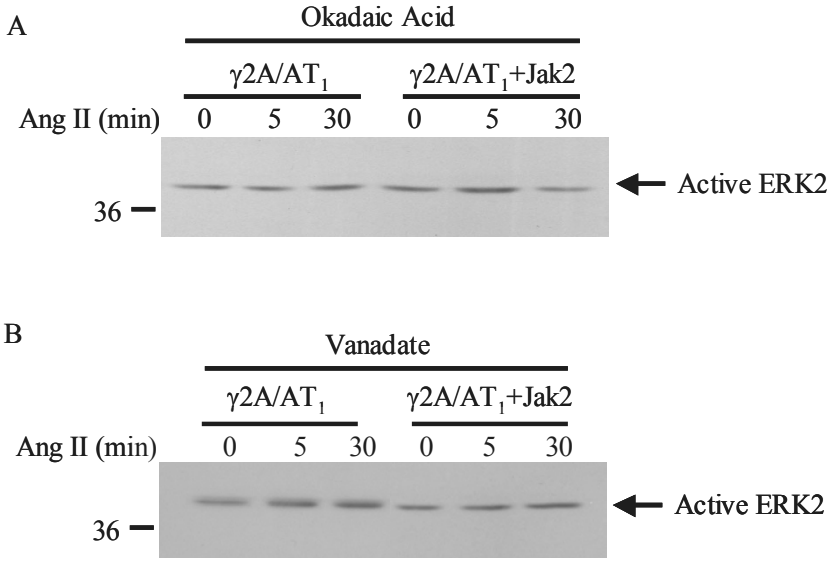


Figure 4-7. Angiotensin II-dependent inactivation of ERK2 requires Jak2 and MKP-1 A) γ2A/AT_1 and γ2A/AT_1+Jak2 cells were pretreated for 1 hr with 500 nM okadaic acid and then stimulated with 100 nM angiotensin II for the indicated times. Whole cell lysates were prepared and then Western blotted with anti-phospho-ERK2 antibody to detect activated ERK2 protein. B) γ2A/AT_1 and γ2A/AT_1+Jak2 cells were pretreated for 1 hr with 100 μM sodium ortho-vanadate and then stimulated with 100 nM angiotensin II for the indicated times. Whole cell lysates were prepared and then Western blotted with anti-phospho-ERK2 antibody to detect activated ERK2 protein. Shown is one of three independent results for each. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1965, Fig. 8 with permission from publisher.

modestly increased its signal. However, unlike the previous experiments in the Jak2 expressing cells, ERK2 phosphorylation levels remained elevated at the 30 min time point. These 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. 4-7 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 requires Jak2.

Discussion

Here, we report several new observations. First, lack of Jak2 signaling in a cell increases the duration of ERK2 activity after angiotensin II stimulation. This was shown by both Western blot analysis and in vitro kinase assays. This observation is contrary to what has been reported previously in studies using AG490 to study the role that Jak2 plays in ERK activation. One possible reason for this discrepancy is that AG490 has been shown to inhibit other tyrosine kinases nonspecifically (98-101). A second observation that we report is that angiotensin II induces rapid induction of the MKP-1 gene. We report that angiotensin II not only causes activation of ERK2, but also simultaneously induces upregulation 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 upregulation of MKP-1 gene expression is dependent on Jak2 expression. We believe that this is the reason that in γ2A/AT$_1$ cells lacking Jak2, ERK2 activity is sustained after angiotensin II stimulation compared to γ2A/AT$_1$+Jak2 cells. Without Jak2 present, angiotensin II is simply unable to upregulate MKP-1 expression, and thus the cell is slower to inactivate ERK2.

Interestingly, in the γ2A/AT$_1$ cells lacking Jak2, after 30 min of angiotensin II treatment the active ERK2 showed predominantly perinuclear localization. There was

not as much nuclear localization of active ERK2 after 30 min of treatment compared to 5 min of treatment. Therefore, despite the sustained ERK2 activation in the $\gamma 2A/AT_1$ cells lacking Jak2, there is not a sustained ERK2 nuclear localization. This is probably the reason that no significant difference in gene transcription between the two cell types was observed. Furthermore, it suggests that ERK2 nuclear localization is not dependent only upon ERK2 activation. Instead, ERK2 nuclear localization appears to be controlled in a temporal manner, so that even though a cell may have sustained ERK2 activity, it may not show sustained ERK2 nuclear localization and ERK2-mediated gene transcription. This presents an interesting possibility. Perhaps in cells with sustained ERK2 activity, the cytosolic actions, rather than the nuclear actions, of ERK2 are sustained. This could be particularly relevant in cancer cells showing constitutive ERK activity. Further testing is needed to determine this.

Fig. 4-8 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 AT_1 receptor. Jak2 induces expression of MKP-1, presumably through activation of one or more STAT proteins. ERK2 also acts 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 (148-150). 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 (151, 152). 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 shows that Jak2 plays an important role in determining whether ERK2 activation is transient or sustained. As such, we may have

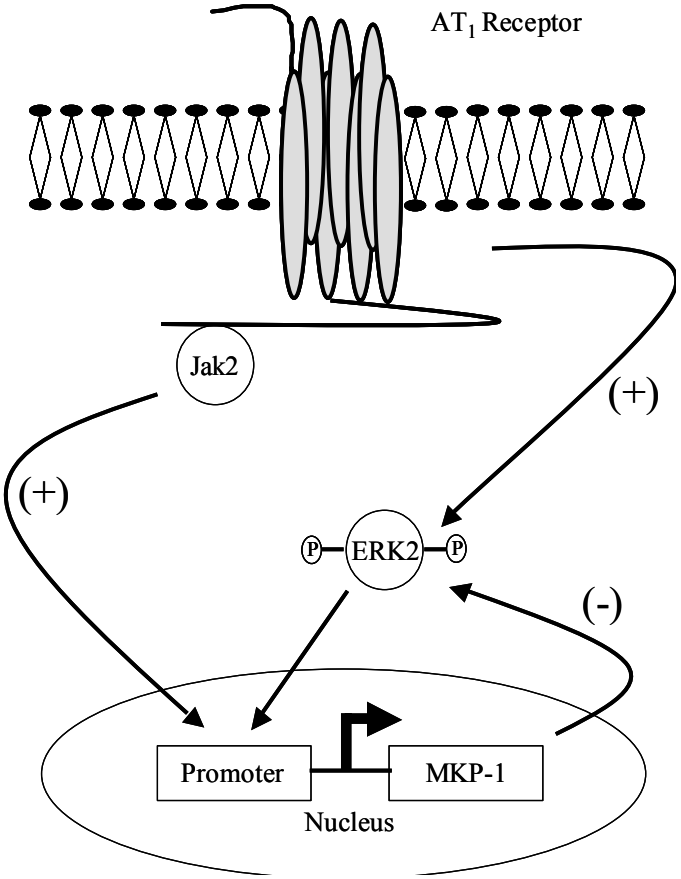


Figure 4-8. Proposed model of the mechanism by which Jak2 mediates ERK2 inactivation after angiotensin II treatment Angiotensin II binding to its type 1 receptor activates ERK2, while simultaneously activating Jak2. Jak2, probably through the action of STAT proteins, increases expression of MKP-1. ERK2 also increases MKP-1 expression. The expressed MKP-1 protein then associates with and dephosphorylates ERK2, thus inactivating the signal. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 1966, Fig. 9 with permission from publisher.

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.

CHAPTER 5
Jak2 PROMOTES OXIDATIVE STRESS-INDUCED APOPTOSIS IN VASCULAR SMOOTH MUSCLE CELLS

Introduction

In 1998, it was demonstrated that Jak2 is activated in response to oxidative stress (36). While the signaling cascade responsible for oxidative stress-induced Jak2 activity was at least partially elucidated in vascular smooth muscle cells, no physiological role has been ascribed to this pathway. Runge's group (35) showed that Jak2 activation by oxidative stress caused up regulation of heat-shock protein 70, a protein that can protect cells from oxidative stress. These data were generated using AG490 to inhibit Jak2 function. Based on these data, they suggested that Jak2 might help vascular smooth muscle cells adapt to oxidative stress (35).

Oxidative stress in vascular smooth muscle cells can cause proliferation, contraction, or apoptosis (153-155). How the same stimulus can result in such opposing endpoints is unknown, but is probably dependent on oxidant dose. Furthermore, the signaling proteins that mediate these different responses are unknown. Oxidative stress-induced apoptosis of vascular smooth muscle cells contributes to the progression of a number of vascular pathologies, including atherosclerosis and restenosis (156, 157). Identifying the mediators of oxidant-induced apoptosis may therefore uncover novel therapeutic targets. Interestingly, both pro- and anti-apoptotic roles have been ascribed to Jak2 tyrosine kinase activity in a variety of signaling systems (34, 97, 158). We therefore

sought to determine what role, if any, Jak2 plays in oxidative stress-induced apoptosis in vascular smooth muscle cells.

Results

Jak2 Activation by Hydrogen Peroxide is Suppressed in RASM-DN Cells

To study the role that Jak2 activation plays in oxidative stress-induced apoptosis, we used rat aortic smooth muscle cells stably expressing a Jak2 dominant negative protein (RASM-DN). We used the same cells, expressing only a neomycin resistant cassette, as controls (RASM-Control). To show that Jak2 activation by hydrogen peroxide is inhibited in RASM-DN cells, we treated RASM-Control and RASM-DN cells with 0.2, 0.5, or 1.0 mM hydrogen peroxide for 0, 5, or 10 min (Fig. 5-1, Top). Cellular lysates were immunoprecipitated with anti-phosphotyrosine antibody, and immunoblotted with anti-Jak2 antibody, to detect tyrosine phosphorylated Jak2. Aliquots from the cellular lysates were Western blotted with anti-Jak2 antibody to demonstrate equal protein loading amongst all samples (Fig. 5-1, Bottom).

The results show that Jak2 is strongly activated by hydrogen peroxide in RASM-Control cells, but Jak2 activation is greatly reduced in RASM-DN cells. This shows that RASM-Control and RASM-DN cells are good models for studying the physiological role of Jak2 during oxidative stress in vascular smooth muscle cells.

Jak2 Activation is Required for Oxidative Stress-Induced Apoptosis

To determine what role, if any, Jak2 plays in oxidative stress-induced apoptosis, RASM-Control and RASM-DN cells were treated with 1 mM hydrogen peroxide for 24 h. We assessed apoptosis by analyzing genomic DNA isolated from each cellular condition (Fig. 5-2A). Genomic DNA isolated from RASM-Control cells that were treated with hydrogen peroxide was fragmented into approximately 200 base pair bands,

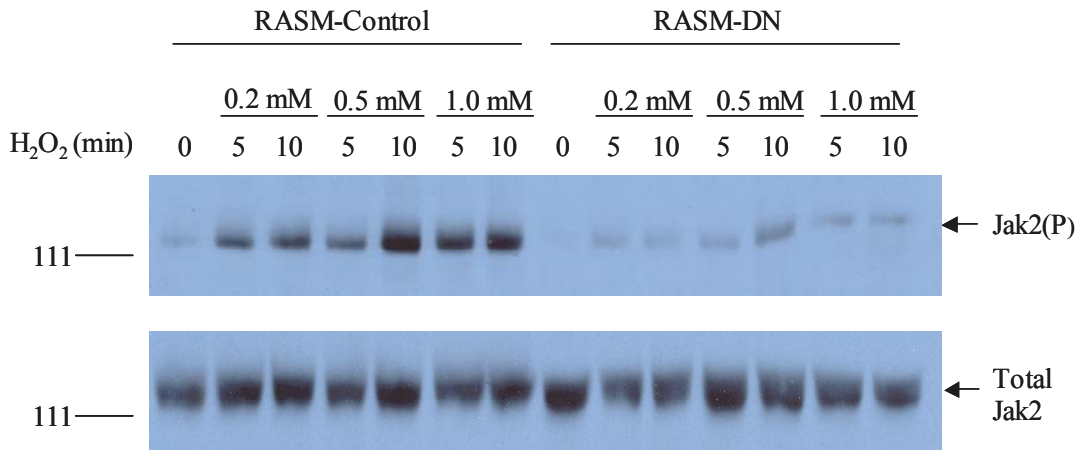


Figure 5-1. Hydrogen peroxide-induced Jak2 activity is suppressed in RASM-DN cells RASM-Control and RASM-DN cells were treated with 0.2 mM, 0.5 mM, or 1.0 mM hydrogen peroxide for 0, 5, or 10 min. Cellular lysates were immunoprecipitated with anti-phosphotyrosine-mAb antibody and immunoblotted with anti-Jak2-pAb antibody to detect tyrosine phosphorylated Jak2 (Top). An aliquot from each lysate was Western blotted with anti-Jak2-pAb to demonstrate equal Jak2 expression amongst all samples (Bottom). Shown is one of three representative experiments. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34548, Fig. 1 with permission from publisher.

characteristic of apoptosis. In contrast, genomic DNA from RASM-DN cells treated with hydrogen peroxide showed no evidence of DNA fragmentation. Furthermore, AG490 prevented genomic DNA fragmentation in RASM-Control cells treated with hydrogen peroxide. Next, to show that RASM-DN cells could undergo apoptosis in response to another pro-apoptotic stimulus, genomic DNA was isolated from RASM-DN cells treated for 4 h with 5 μM staurosporine, a potent activator of the intrinsic apoptosis pathway (Fig. 5-2B). The banding pattern characteristic of fragmented DNA was clearly seen in these cells. Finally, to determine the effect of different doses of hydrogen peroxide on vascular smooth muscle cell apoptosis, we treated RASM-Control and RASM-DN cells with 0.2, 0.5, or 1.0 mM hydrogen peroxide for 24 h and examined genomic DNA for fragmentation (Fig. 5-2C). The results show that, as expected no DNA fragmentation

occurs in RASM-DN cells. In RASM-Control cells, 1.0 mM hydrogen peroxide was required to induce DNA fragmentation. Collectively, these results show that Jak2 activation is required for oxidative stress-induced apoptosis in vascular smooth muscle cells.

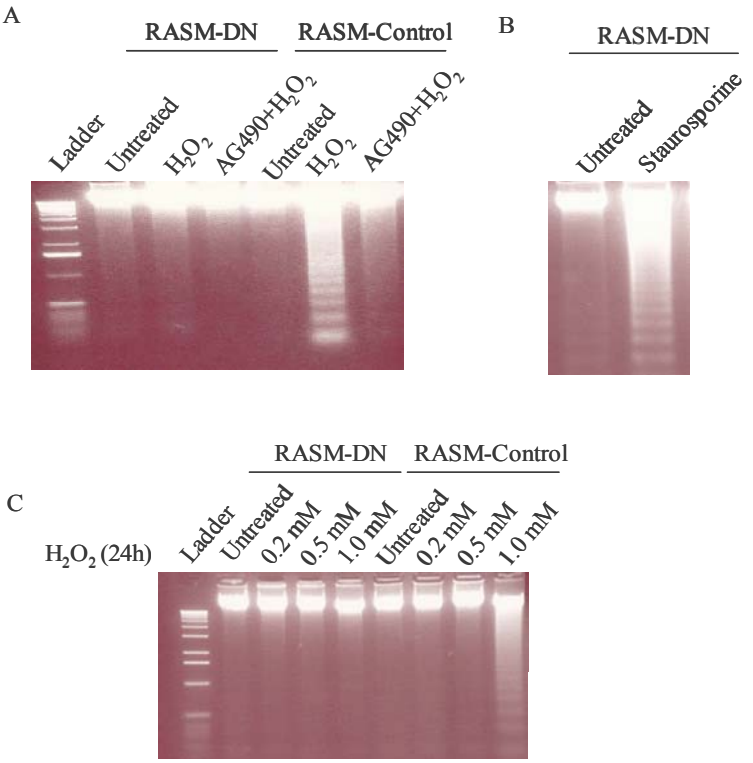


Figure 5-2. Jak2 is essential for hydrogen peroxide-induced apoptosis of vascular smooth muscle cells A) RASM-DN and RASM-Control cells were either left untreated, or treated with 1 mM hydrogen peroxide for 24 h, or with 1 mM hydrogen peroxide for 24 h after 16 h of pretreatment with 100 μM AG490. Genomic DNA was isolated and separated on a 1.8% agarose gel. The gel was stained with EtBr and visualized under U.V. light to detect genomic DNA laddering. B) RASM-DN cells were either left untreated or treated with 5 μM staurosporine for 4 h. Genomic DNA was isolated and separated on a 1.8% agarose gel. The gel was stained and visualized to detect genomic DNA laddering. C) RASM-Control and RASM-DN cells were treated with 0.2 mM, 0.5 mM, or 1.0 mM hydrogen peroxide for 24 h. Genomic DNA was isolated and separtated on a 1.8% agarose gel. The gel was stained with EtBr and visualized under U.V. light to detect genomic DNA laddering. Shown is one of four (A and B) or three (C) representative experiments for each. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34548, Fig. 2 with permission from publisher.

Quantification of Jak2-Mediated Apoptosis

We next quantified the amount of apoptosis occurring in RASM-Control and RASM-DN cells treated with hydrogen peroxide. The cells were grown on microscope slides and treated with 1 mM hydrogen peroxide for either 0 or 24 h. The cells were then stained with the nucleus-specific Hoechst 33342 dye. Representative photomicrographs of RASM-Control and RASM-DN cells either left untreated, or treated with hydrogen peroxide, or hydrogen peroxide after pretreatment with the Jak2 inhibitor AG490, are shown (Fig. 5-3). RASM-Control cells treated with hydrogen peroxide had shrunken nuclei that fluoresced more intensely than untreated cells, which is indicative of apoptotic cells. In contrast, RASM-Control cells treated with AG490 before hydrogen peroxide treatment appeared similar to untreated cells. Similarly, RASM-DN cells either treated with hydrogen peroxide alone, or treated with both AG490 and hydrogen peroxide showed nuclear staining akin to untreated cells.

Four replicate experiments were performed for each condition, and a minimum of 100 cells were counted from each replicate. Cells clearly showing condensed and/or fragmented nuclei were counted as apoptotic. Data are presented as percentage of cells undergoing apoptosis (Fig. 5-4). These data show that untreated RASM-Control and RASM-DN cells showed very low levels of apoptosis (2.4+/-0.54% and 2.9+/-0.85% respectively). RASM-Control cells treated with hydrogen peroxide showed 54.1+/-3.71% of total cells undergoing apoptosis, while only 12.3+/-1.93% of RASM-DN cells treated with hydrogen peroxide were apoptotic. Finally, 5.7+/-0.81% of RASM-Control cells and 4.4+/-0.98% of RASM-DN cells that were pretreated with AG490 before hydrogen peroxide treatment were apoptotic.

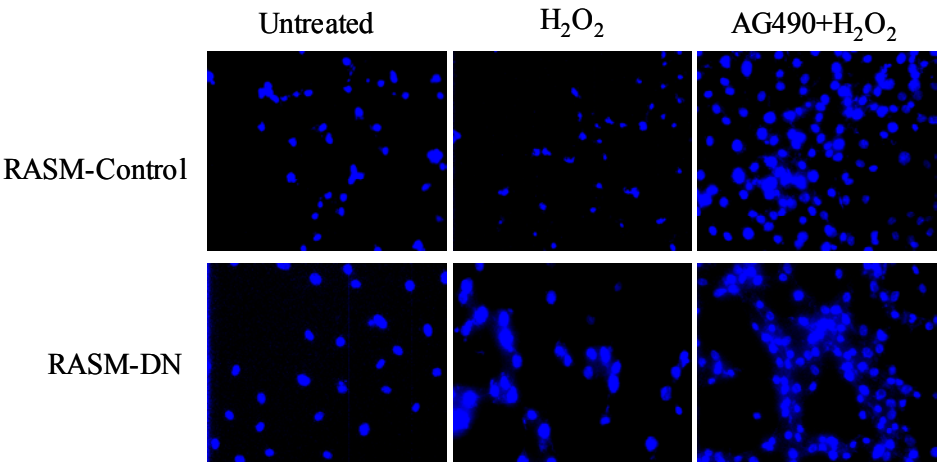


Figure 5-3. Hoechst staining to detect nuclear condensation RASM-Control and RASM-DN cells were grown on microscope slides and treated with 1 mM hydrogen peroxide for 0 or 24 h, or with 1 mM hydrogen peroxide for 24 h after 16 h of pretreatment with 100 μM AG490. The cells were then stained with 50 μg/mL Hoechst 33342 nuclear stain, fixed, mounted, and visualized using a florescent microscope to detect nuclear condensation. Shown is one of four representative photographs of each condition. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34549, Fig. 3A with permission from publisher.

Jak2 Activation by Oxidative Stress Mediates Bax Expression

We hypothesized that Jak2 was promoting apoptosis by regulating expression of a pro-apoptotic protein. Since Bax is a pro-apoptotic protein required for oxidative stress-induced apoptosis, we used Western blotting to determine whether Jak2 was required for oxidative stress-mediated up regulation of Bax expression. For this, RASM-Control and RASM-DN cells were treated with 1 mM hydrogen peroxide for 0, ½, 1, 2, or 3 h (Fig. 5-5A). Cellular lysates were Western blotted with anti-Bax antibody (Fig. 5-5A, Top). The data show that in RASM-Control cells, hydrogen peroxide induced rapid and transient induction of Bax expression, which peaked at 1-2 h. In contrast, hydrogen peroxide did not induce Bax expression in RASM-DN cells.

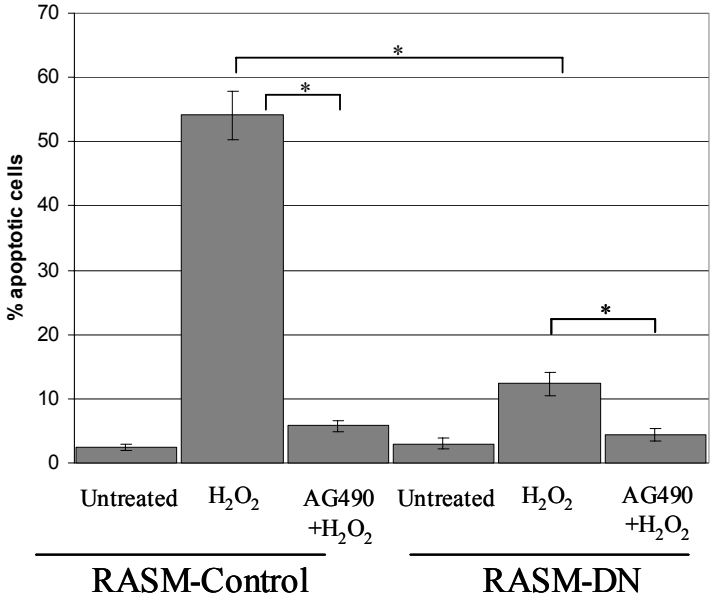


Figure 5-4. Quantification of apoptosis in RASM-Control and RASM-DN cells. A minimum of 100 cells from each of 4 replicates for each of the 6 treatment conditions represented in Fig 5-3 was counted. Apoptotic cells were counted as those showing condensed and/or fragmented nuclei. Data are presented as percentage of cells undergoing apoptosis +/- S.D. *, $p<0.001$. Statistical analysis was performed using Student's *t* test. printed with Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34549, Fig. 3B with permission from publisher.

We next stripped and re-probed the membrane with anti-BCL-2 antibody to determine whether Jak2 influences expression of theanti-apoptotic BCL-2 protein (Fig. 5-5A, Middle). We found that BCL-2 expression increased slightly in both cell types in response to hydrogen peroxide. Because there was no difference in the two cell types, we concluded that Jak2 does not effect expression of BCL-2. To demonstrate equal protein loading amongst all samples, the membrane was stripped and re-probed with anti-STAT1 antibody (Fig. 5-5A, Bottom). These data show that Jak2 activation is required for induction of the pro-apoptotic Bax protein in response to oxidative stress. To confirm this result, RASM-Control cells were pretreated with either the Jak2 inhibitor AG490 or its inactive analog AG9, and then treated with hydrogen peroxide for 0, 1/2, 1, 2, or 3 h

(Fig. 5-5B). The data show that AG490 attenuates hydrogen peroxide-mediated Bax induction in RASM-Control cells (Fig. 5-5B, Top). The membrane was stripped and reprobed with anti-STAT1 antibody to demonstrate equal loading amongst all samples (Fig. 5-5B, Bottom). Collectively, the data in Fig. 5-5 present strong evidence that Jak2 activation promotes oxidative stress-induced apoptosis by mediating an increase in Bax protein expression levels in vascular smooth muscle cells.

Jak2 Activation by Oxidative Stress Promotes Mitochondrial Dysfunction

During oxidative stress, the pro-apoptotic Bax protein localizes to the outer mitochondrial membrane and increases mitochondrial membrane permeability (159, 160). This is an essential step in the intrinsic apoptosis pathway (161, 162). Since we showed in Fig. 5-5 that Jak2 tyrosine kinase mediates induction of Bax expression, we hypothesized that activation of Jak2 by hydrogen peroxide may also contribute to mitochondrial dysfunction. To test this, we used the MitoCapture reagent to stain live cells treated with hydrogen peroxide and visualized the cells using confocal microscopy (Fig. 5-6). In healthy, non-apoptotic cells, the dye accumulates predominantly in the mitochondria where it forms aggregates, and fluoresces red. In contrast, in apoptotic cells, because of the change in mitochondrial trasnsmembrane potential, the dye remains predominantly in the cytosol as a monomer, and fluoresces green. The data show that in untreated RASM-Control and RASM-DN cells, the MitoCapture dye fluoresces predominantly red, indicating that these cells are non-apoptotic. After 1 mM hydrogen peroxide treatment for 2 h, the RASM-Control cells show a decrease in red staining and a dramatic increase in green staining, indicative of mitochondrial dysfunction in these cells. In contrast, the RASM-DN cells treated with hydrogen peroxide actually show increased

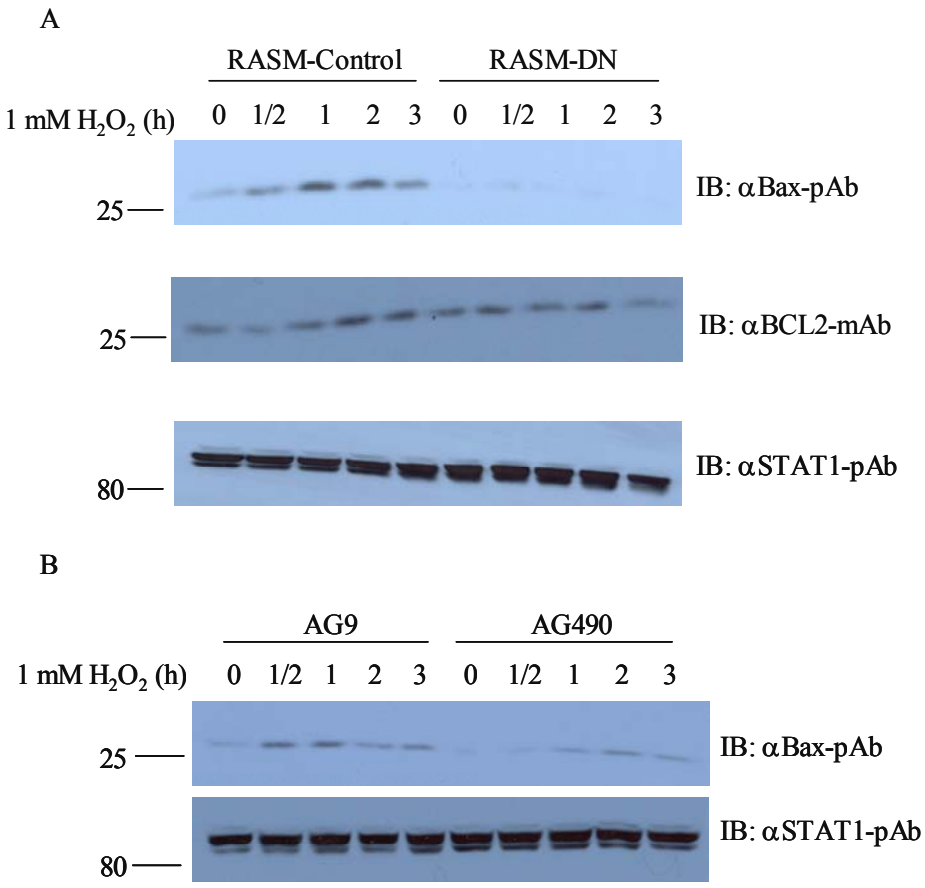


Figure 5-5. Jak2 mediates hydrogen peroxide-induced up regulation of Bax expression A) RASM-Control and RASM-DN cells were treated with 1 mM hydrogen peroxide for 0, ½, 1, 2, or 3 h. Cellular lysates were Western blotted with anti-Bax-pAb antibody (Top). The membrane was stripped and reprobed with anti-BCL2-mAb (Middle). Finally, the membrane was stripped and re-probed with anti-STAT1-pAb to demonstrate equal loading amongst all samples (Bottom). B) RASM-Control cells were pretreated with 100 μM AG9 or 100 μM AG490 for 16 h, followed by treatment with 1 mM hydrogen peroxide for 0, ½, 1, 2, or 3 h. Cellular lysates were Western blotted with anti-Bax-pAb (Top). The membrane was stripped and re-probed with anti-STAT1-pAb to demonstrate equal loading amongst all samples (Bottom). Shown is one of three representative experiments for each. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34550, Fig. 4 with permission from publisher.

red staining, and only marginally increased green staining compared to control cells. This indicates that hydrogen peroxide-induced Jak2 activation promotes mitochondrial dysfunction in vascular smooth muscle cells. To quantify these results, we used

morphometric scanning software to determine the ratio of green staining intensity to red staining intensity (Green:Red) (Fig. 5-7). A higher Green:Red staining intensity is indicative of a greater loss of mitochondrial transmembrane potential. The results indicate that the Green:Red staining intensity was significantly higher in RASM-Control

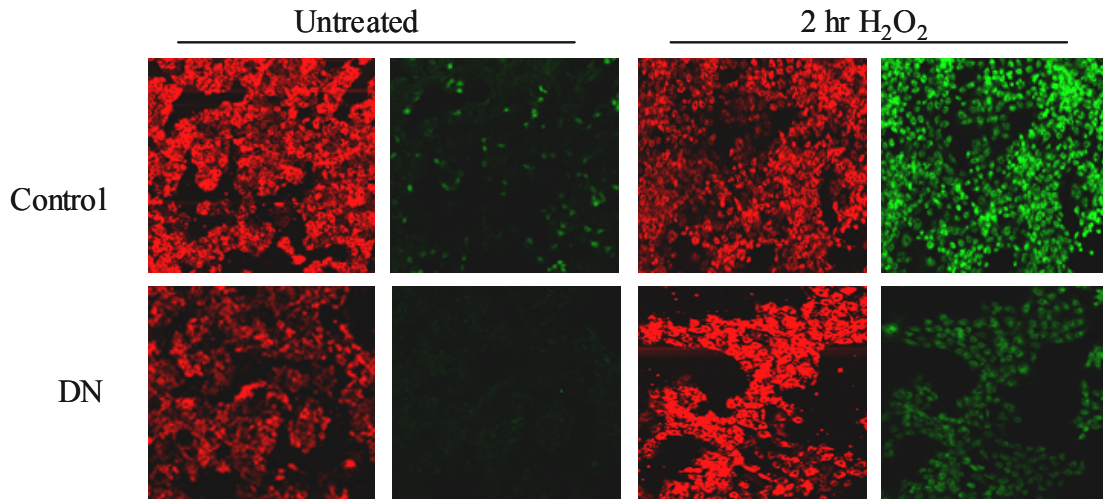


Figure 5-6. Jak2 is essential for hydrogen peroxide-induced mitochondrial membrane dysfunction RASM-Control and RASM-DN cells were grown on microscope slides and treated with 1 mM hydrogen peroxide for 0 or 2 h. Cells were stained with the MitoCapture reagent and visualized using a confocal microscope. Predominant red staining is indicative of healthy cells, while predominant green staining is indicative of apoptotic cells. Shown is one of three representative experiments. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34551, Fig. 5A with permission from publisher.

cells treated with hydrogen peroxide than in similarly treated RASM-DN cells, showing that relative to RASM-Control cells, RASM-DN cells maintained their mitochondrial transmembrane potential.

Jak2 is Required for Caspase-9 Cleavage During Oxidative Stress

Caspase-9 is the primary initiator caspase of the intrinsic apoptosis pathway, and is involved in hydrogen peroxide-induced apoptosis. Caspase-9 is cleaved to its active form following disruption of mitochondrial integrity (163). We therefore examined caspase-9

cleavage after hydrogen peroxide treatment in RASM-Control and RASM-DN cells (Fig. 5-8). The two cell types were treated with 1 mM hydrogen peroxide for 0, ½, 1, 2, or 3 h, and cellular lysates were Western blotted with anti-CLEAVED-Caspase-9 antibody (Fig. 5-8, Top). The data show that in RASM-Control cells, hydrogen peroxide treatment causes accumulation of cleaved caspase-9. In contrast, little caspase-9 cleavage occurred

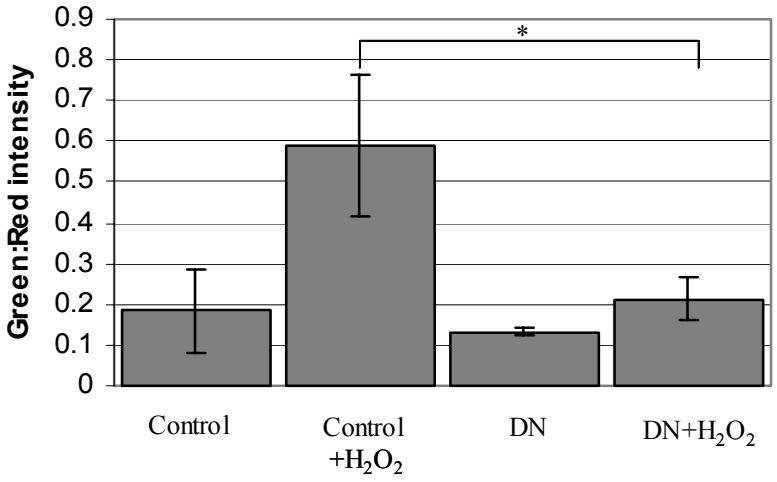


Figure 5-7. Pixel intensity of each of 4 photographs from each condition in Fig. 5-6 was determined, and intensity of green and red staining was determined for each. Data are presented as average ratio of green to red pixel intensity +/- S.D. for each condition. *, $p<0.01$. Statistical analysis was performed using Student's *t* test Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34451, Fig. 5B with permission from publisher.

in RASM-DN cells. The membrane was stripped and re-probed with anti-STAT1 antibody to show equal protein loading amongst all samples (Fig. 5-8, Bottom). Jak2 tyrosine kinase activity is therefore required for cleavage and activation of caspase-9 in vascular smooth muscle cells during oxidative stress.

Discussion

Although Jak2 is traditionally considered a mediator of cytokine signaling, other ligands and stimuli can activate this signaling pathway (18, 36). Oxidative stress is one

such stimulus; Jak2 is potently activated by hydrogen peroxide in a number of cell types, yet no physiological endpoint has been attributed to hydrogen peroxide-induced Jak2 activity (35, 36). Additionally, hydrogen peroxide induces apoptosis in vascular smooth muscle cells, yet few intracellular mediators of hydrogen peroxide-induced apoptosis have been identified (164).

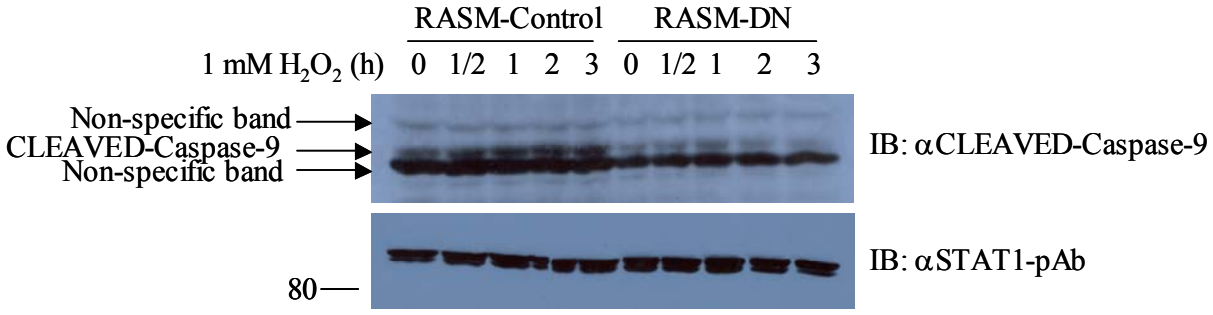


Figure 5-8. Jak2 is required for oxidative stress-induced caspase-9 cleavage RASM-Control and RASM-DN cells were treated with 1 mM hydrogen peroxide for 0, ½, 1, 2, or 3 h. Cellular lysates were Western blotted with anti-CLEAVED-Caspase-9-pAb to detect Caspase-9 activation (Top). The membrane was stripped and re-probed with anti-STAT1-pAb to demonstrate equal loading amongst all samples (Bottom). Shown is one of three representative experiments. Adapted from Sandberg et al. Journal of Biological Chemistry. (2004) 279, pg 34551, Fig. 5C with permission from publisher.

Here, we report that Jak2 activation by oxidative stress in the form of hydrogen peroxide mediates apoptosis of vascular smooth muscle cells. We demonstrated fragmentation of genomic DNA, a characteristic of apoptosis, in control rat aortic smooth muscle cells treated with hydrogen peroxide (RASM-Control), but that fragmentation was non-existent in the same cells expressing a Jak2 dominant negative protein (RASM-DN), or in RASM-Control cells treated with the Jak2 inhibitor AG490. We observed a significant decrease in the percentage of cells undergoing apoptosis in response to hydrogen peroxide in the RASM-DN cells compared to RASM-Control cells. Moreover, pretreatment with AG490 reduced the percentage of cells undergoing hydrogen peroxide-

induced apoptosis nearly to the level of untreated cells, in both cell types, again suggesting a critical role for Jak2 in apoptosis. Finally, we provided evidence of the mechanism by which this occurs. In RASM-Control cells, expression of the pro-apoptotic Bax protein was rapidly induced. There was no such Bax induction in the RASM-DN cells. Furthermore, in RASM-Control cells, AG490 attenuated hydrogen peroxide-mediated Bax induction. This is the first evidence that Jak2 mediates Bax protein expression in response to hydrogen peroxide.

During apoptosis, Bax is largely responsible for loss of mitochondrial transmembrane potential and subsequent increase in mitochondrial membrane permeability. This mitochondrial dysfunction allows translocation of macromolecules such as cytochrome c from the inner mitochondrial membrane to the cytosol, leading to cleavage and activation of caspase-9. We found that mitochondrial membrane integrity was compromised and caspase-9 was cleaved in RASM-Control cells, but not in RASM-DN cells, indicating an essential role for Jak2 in these events.

This report has therefore identified apoptosis as a physiological endpoint of Jak2 activation by hydrogen peroxide. Moreover, this work shows that Jak2 is a novel mediator of hydrogen peroxide-induced apoptosis in vascular smooth muscle cells. These results could have profound consequences for the treatment of a number of vascular diseases in which oxidative stress-mediated cell death plays a prominent role. As such, this work identifies Jak2 as a potential therapeutic target in vascular diseases associated with oxidative stress. Atherosclerosis is one such disease. During the development of an atherosclerotic plaque, a fibrous cap forms over the plaque. Vascular smooth muscle cells that have migrated from the medial layer of the blood vessel are

found within the fibrous cap. These cells are exposed to large amounts of oxidative stress derived largely from circulating macrophages. This oxidative stress can cause apoptosis of the vascular smooth muscle within the fibrous cap, leading to cap weakening, and accelerating the time to plaque rupture. If Jak2 plays a role in oxidative stress-induced apoptosis in vivo during atherosclerosis, inhibition of Jak2 could stabilize the plaque.

Since Jak2 knockout mice die embryonically, investigating the role that Jak2 plays during atherosclerosis is difficult. One possibility is to use vascular smooth muscle cell restricted expression of the Jak2 dominant negative protein to study the in vivo role of Jak2. Mice expressing Jak2 only in vascular smooth muscle cells could then be crossed with apolipoprotein deficient mice, which develop atherosclerosis when fed a high fat diet, to examine the role of Jak2 during atherosclerosis.

Interestingly, Jak2 can play either pro- or anti-apoptotic roles depending on the signaling system and apoptotic stimulus examined. Where, then, is the specificity of the response controlled? One possibility is that the specific STAT proteins that are activated by Jak2 determine whether Jak2 plays a pro- or anti-apoptotic role. For instance, activation of STAT3 is usually associated with inhibition of apoptosis, while STAT1 activation has been associated with induction of apoptosis. It is possible that STAT1 is the predominant STAT activated during oxidative stress. Studies using dominant negative mutants of the STAT1 and STAT3 proteins could be used to determine which STAT protein is responsible for the pro-apoptotic role for Jak2.

Finally, whether Jak2 plays a role in oxidative stress-induced apoptosis in other cell types should be examined. Many cell types are exposed to high oxidative stress, especially during disease. These include endothelial cells and cardiomyocytes. It will be

interesting to determine if the pro-apoptotic role of Jak2 is restricted to vascular smooth muscle cells, or if it is a ubiquitous role.

CHAPTER 6
Jak2 RESIDUES GLU 1024 AND ARG 1113 FORM HYDROGEN BONDS, AND ARE ESSENTIAL FOR Jak-STAT SIGNAL TRANSDUCTION

Introduction

Structure-function studies have identified several specific amino acid residues within Jak2 that are essential for its activation. For example, conversion of Lys 882 to Glu (K882E) within subdomain II rendered Jak2 catalytically inactive (104, 105). Similarly, conversion of Tyr 1007 to Phe (Y1007F) prevented ligand-mediated Jak2 activation (48). A double mutation of W1020G/E1024A within subdomain VIII not only inactivated Jak2, but also rendered the molecule dominant negative (106). This double mutant is of interest to our laboratory because we have shown that expression of it in cells inhibits angiotensin II-mediated Jak2 activation, Jak2/AT_1 receptor co-association, STAT1 tyrosine phosphorylation, and ligand-dependent gene transcription (38, 39). We recently showed that mutation of either Trp 1020 or Glu 1024 individually renders Jak2 catalytically inactive (107). We are interested in elucidating the requirement of these two amino acids for Jak2 kinase function. Recently, we showed that Trp 1020 forms a hydrogen bond with Glu 1046 that is critical to maintain the structural integrity of the Jak2 activation loop (107). The role that Glu 1024 plays in Jak2 kinase function, however, is not known.

Here, we investigated the requirement for Glu 1024 for Jak2 kinase function using homology modeling of the Jak2 kinase domain and site-directed mutagenesis. Our data indicate that Glu 1024 forms an interaction with an arginine at position 1113, via two

distinct hydrogen bonds, and is essential for angiotensin II-dependent activation of the Jak-STAT signaling pathway. Conversion of Arg 1113 to lysine, alanine, or glutamic acid renders Jak2 catalytically inactive. Consequently, this is the first report describing Arg 1113 as being essential for Jak2 kinase activity.

Results

Molecular Modeling Identified a Putative Interaction between Jak2 Residues Glu 1024 and Arg 1113

Previously, we showed that mutation of Glu 1024 to Ala rendered Jak2 catalytically inactive (107). To understand how Glu 1024 contributes to Jak2 kinase function, we generated a molecular model of the Jak2 kinase domain (Fig. 6-1).

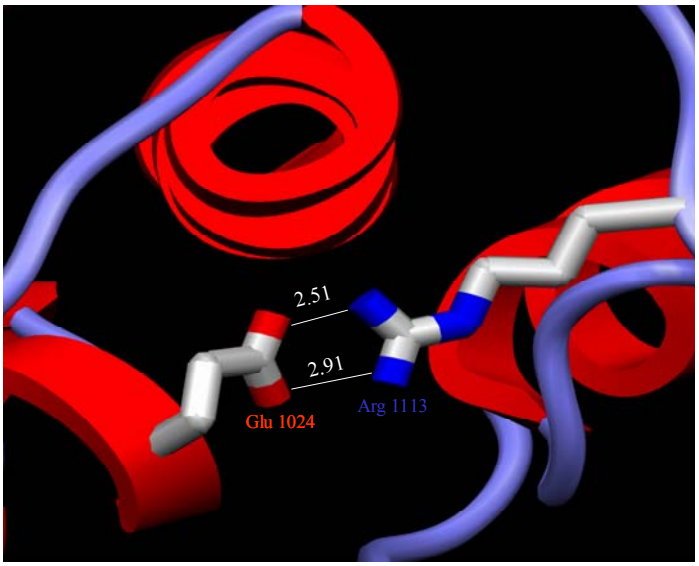


Figure 6-1. Molecular modeling of the Jak2 kinase domain suggested a putative interaction between Glu 1024 and Arg 1113 The model was designed using the program Swiss Model and was based on the known crystal structure of the kinase domain of the fibroblast growth factor tyrosine kinase receptor. Shown are bond distances in angstroms.

The model was based on the known crystal structure of the basic fibroblast growth factor receptor. Our model indicated that Glu 1024 forms a critical interaction with Arg 1113, an amino acid that thus far has not been shown to be essential for Jak2 function. The

model predicted that the activation loop of the Jak2 kinase domain is maintained in its proper conformation via critical interactions between the oxygen groups on the side chain of Glu 1024 and the terminal amino groups on the side chain of Arg 1113. Using the program HBPLUS Hydrogen Bond Calculator, we determined that these interactions were hydrogen bonds, based on the bond lengths (2.91 angstroms and 2.51 angstroms, respectively) between the atoms involved. In each bond, the amino group is the electron donor, while the oxygen group is the electron acceptor. Furthermore, our model suggested that permutation of Arg 1113 would render Jak2 catalytically inactive.

Mutation of Jak2 Residue Glu 1024 or Arg 1113 Abolishes Jak2 Kinase Activity

We tested the ability of a Jak2 protein containing point mutations at either Glu 1024 or Arg 1113 to autophosphorylate, to determine if indeed an interaction between these two amino acids, as predicted by our model, exists. For this, we transfected BSC-40 cells with 10 μg of Jak2 cDNA containing E1024R, E1024D, R1113E, R1113A, or R1113K mutations. BSC-40 cells transfected with 5 μg of wild type Jak2 cDNA served as a positive control, while cells transfected with empty plasmid (pRC) served as a negative control. Cells were lysed and protein was extracted. Protein extracts were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect tyrosine phosphorylated Jak2. Previously, we showed that conversion of Glu 1024 to Ala (E1024A), a neutrally charged amino acid, rendered Jak2 catalytically inactive (107). Here, we show that conversion of Glu 1024 to Asp (E1024D), a negatively charged amino acid, or Arg (E1024R), a positively charged amino acid, similarly abolishes Jak2 tyrosine kinase activity (Fig. 7.2, Top). Likewise, conversion of Arg 1113 to Glu (R1113E), a negatively charged amino acid, Ala

(R1113A), a neutrally charged amino acid, or Lys (R1113K), a positively charged amino acid, rendered Jak2 catalytically inactive (Fig. 2, Top). In addition, a double mutation whereby Glu 1024 was converted to Arg, and Arg 1113 was converted to Glu, thus effectively switching the positions of these two amino acids, also rendered Jak2 catalytically inactive (Fig. 6-2, Top). We confirmed expression of all transfected constructs by Western blotting the same membrane with anti-Jak2 antibody (Fig. 6-2, Bottom). These data show that both Glu 1024 and Arg 1113 are critical for the ability of Jak2 to autophosphorylate, thus supporting the prediction that these two amino acids form a critical interaction.

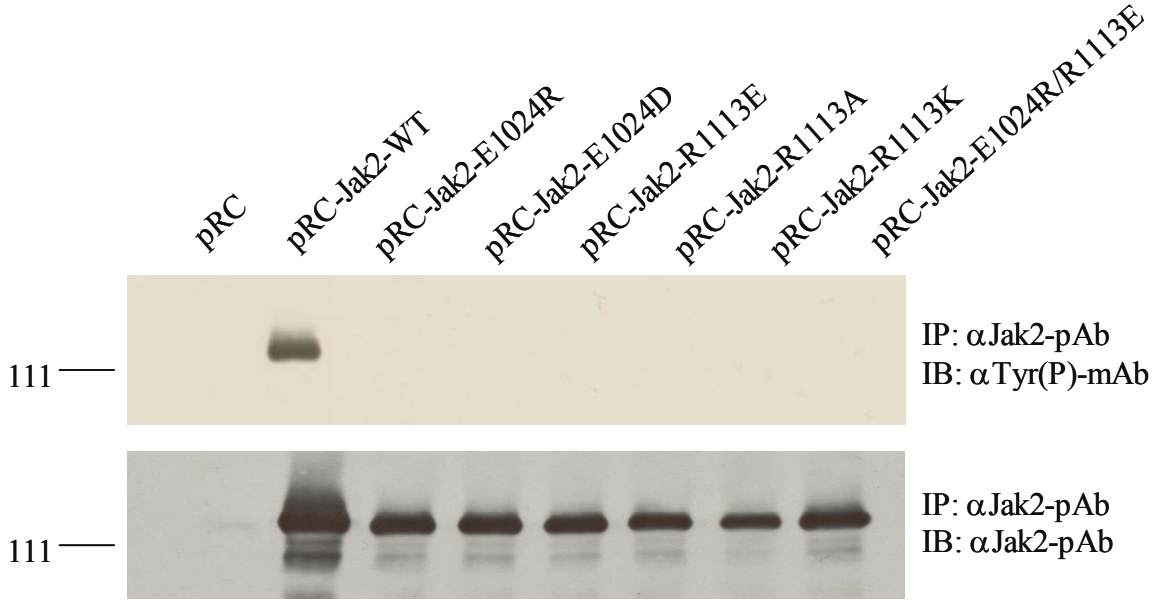


Figure 6-2. Mutation of Glu 1024 or Arg 1113 abolishes the ability of Jak2 to autophosphorylate. BSC-40 cells were transfected with 10 μg of the indicated plasmids and infected with vaccinia virus clone vTF7-3 to induce high-level protein expression. Lysates were prepared, immunoprecipitated with anti-Jak2-pAb, and Western blotted with anti-phosphotyrosine-mAb to assess the ability of Jak2 containing mutations to autophosphorylate (top). The nitrocellulose membranes used for Western blotting were stripped and reprobed with anti-Jak2-pAb to assess Jak2 protein expression (bottom). Shown is one of three independent experiments.

Individual Mutations of W1020G or E1024A Render Jak2 Dominant Negative

It is known that double mutation of W1020G/E1024A renders Jak2 dominant negative (106). This means that when these two amino acids are both mutated, the resulting mutant Jak2 can inhibit the ability of wild type Jak2 to autophosphorylate. We recently showed that mutation of either Trp 1020 or Glu 1024 individually renders Jak2 catalytically inactive (107). We therefore sought to determine if mutation of either of these amino acids individually would render Jak2 dominant negative, as well as catalytically inactive. For this, we transfected BSC-40 cells with either 5 μg of wild type Jak2 cDNA alone, or with 5 μg of Jak2 cDNA plus increasing amounts of mutant Jak2 cDNAs. If the mutation tested does render Jak2 dominant negative, then upon co-transfection with increasing amounts of mutant cDNA, the ability of the wild type Jak2 to autophosphorylate will be inhibited. Addition of wild type plasmid alone results in increased Jak2 tyrosine phosphorylation levels (Fig. 6-3A, Top, lane 2 vs. lane 1). Addition of increasing amounts of the previously characterized Jak2 dominant negative mutant (W1020G/E1024A) inhibits wild type phosphorylation (lanes 3 and 4). Mutation at either Trp 1020 (W1020G) or at Glu 1024 (E1024A) alone is also sufficient to render Jak2 dominant negative in a dose-dependent manner (lanes 5-8). It is clear, though, that the dominant negative that results from mutation of W1020G or E1024A alone is not nearly as potent an inhibitor as the double W1020G/E1024A mutation. For instance, when 5 μg of wild type Jak2 was co-transfected with 5 μg of the Jak2-W1020G or the Jak2-E1024A mutant, we observed a diminished, but existent degree of Jak2 autophosphorylation. In comparison, when 5 μg of wild type Jak2 was co-transfected with 5 μg of Jak2 containing the W1020G/E1024A double mutation, the ability of wild

type Jak2 to autophosphorylate was almost completely lost. Therefore, individual mutations at either Trp 1020 or Glu 1024 can render Jak2 both catalytically inactive and dominant negative. The individual mutants, though, do not show as strong a dominant negative character as the double Jak2-W1020G/E1024A mutant.

We next tested the ability of the R1113E mutant to render Jak2 dominant negative. Again, we transfected BSC-40 cells with either 5 μg of wild type Jak2 cDNA alone, or with increasing amounts of the R1113E mutant plasmid. The results show that even when 5 μg of wild type Jak2 cDNA is co-transfected with 15 μg of Jak2 containing the R1113E mutation, wild type Jak2 is able to autophosphorylate (Fig. 6-3B). Therefore, the data suggest that mutation of Arg 1113, while disrupting Jak2 kinase function, does not render a dominant negative phenotype.

Jak2-R1113E is Unable to Become Tyrosine Phosphorylated by Angiotensin II

The data in Figs. 6-2 and 6-3 were generated using ligand-independent experimental conditions. To determine whether Arg 1113 is critical for proper Jak2 function in a ligand-dependent signaling system, we used COS-7 cells to study the role that Arg 1113 plays in angiotensin II-induced activation of the Jak-STAT signaling pathway. We first tested the ability of angiotensin II to activate Jak2 containing the R1113E point mutation. For this, COS-7 cells were transfected with 10 μg of AT_1 receptor, and either 10 μg of wild type Jak2 cDNA or 12.5 μg of Jak2-R1113E cDNA. The cells were treated with 100 nM angiotensin II for either 0, 5, or 10 min. Cells were lysed and protein was extracted. Protein extracts were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with anti-Jak2 antibody to detect tyrosine phosphorylated Jak2. The results show that in response to angiotensin II, wild type Jak2

becomes increasingly tyrosine phosphorylated over the time course, while Jak2-R1113E is unable to become activated in response to ligand treatment (Fig. 6-4).

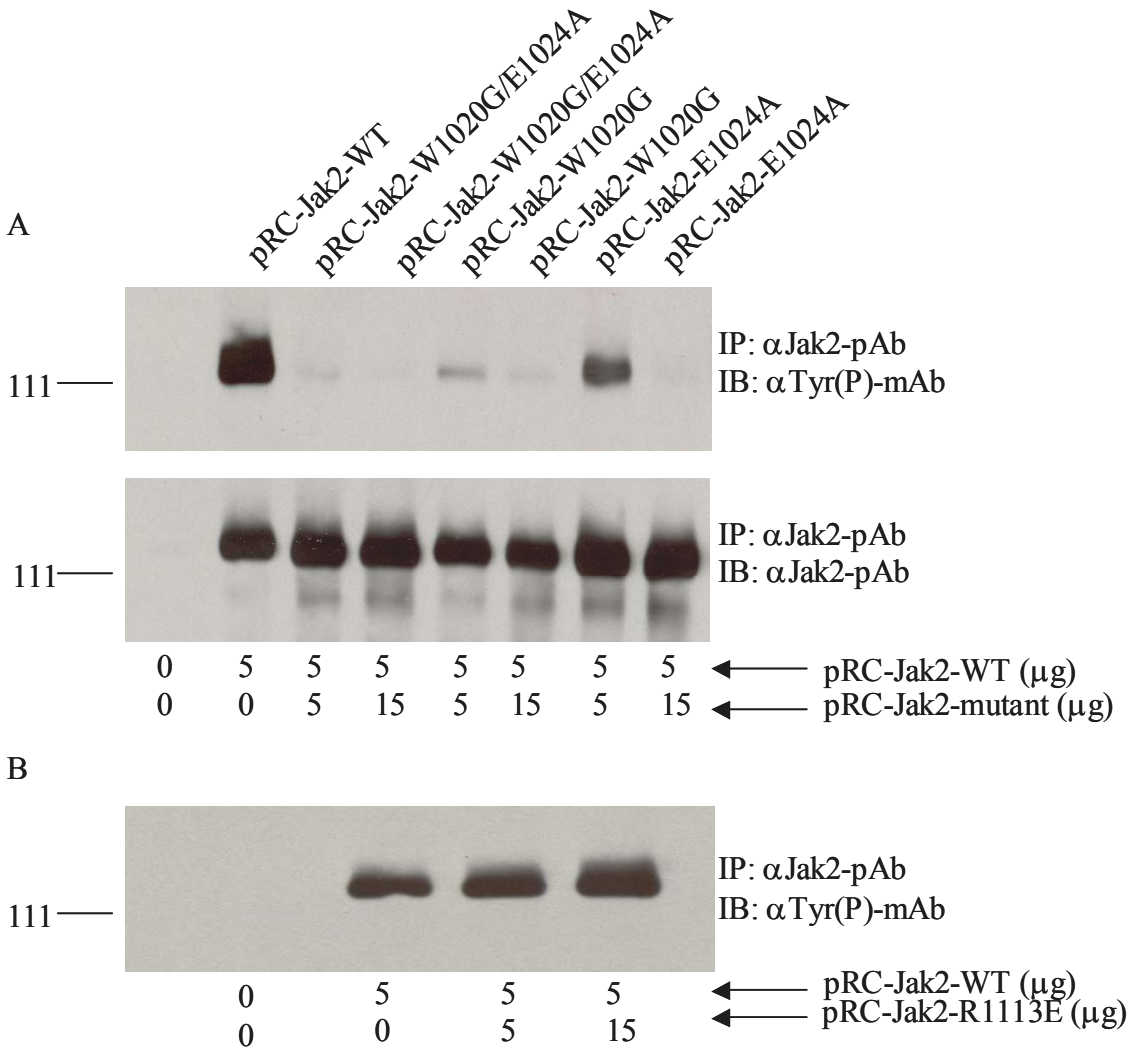


Figure 6-3. The Jak2-W1020G and E1024R mutations render Jak2 dominant negative BSC-40 cells were transfected with the indicated plasmids and infected with vaccinia virus clone vTF7-3. A) Lysates were prepared, immunoprecipitated with anti-Jak2-pAb, and Western blotted with anti-phosphotyrosine-mAb to assess the ability of Jak2-W1020G or Jak2-E1024A to act as a dominant negative (top). The membrane was stripped and reprobed with anti-Jak2-pAb to assess Jak2 protein expression in each sample (bottom). B) Using the same experimental paradigm, the ability of Jak2-R1113E was tested for its ability to act as a dominant negative. Shown is one of two independent experiments for each.

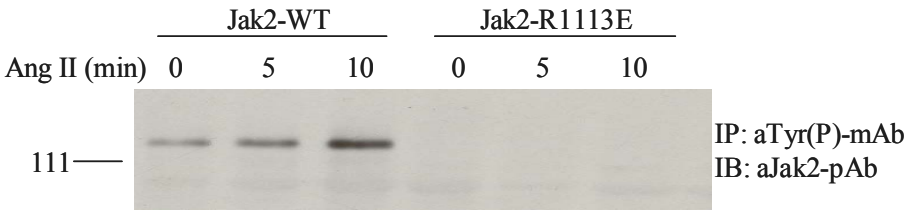


Figure 6-4. Jak2-R1113E mutant cannot become tyrosine phosphorylated in response to angiotensin II COS-7 cells were transfected with 10 μg of AT_1 receptor and either 10 μg of wild type Jak2 or 12.5 μg Jak2-R1113E cDNA. The cells were treated with 100 nM angiotensin II for the indicated times and lysates were prepared to assess Jak2 tyrosine phosphorylation. Lysates were immunoprecipitated with anti-Tyr(P)-mAb and Western blotted with anti-Jak2-pAb. Shown is one of three independent experiments.

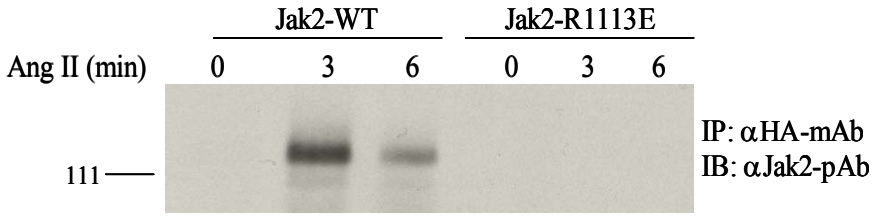


Figure 6-5. Angiotensin II-dependent Jak2/AT_1 receptor co-association does not occur in cells expressing Jak2-R1113E COS-7 cells were transfected with HA-tagged AT_1 receptor and either wild type Jak2 or Jak2-R1113E cDNA. The cells were treated with 100 nM angiotensin II for the indicated times and lysates were prepared to assess Jak2/AT_1 receptor co-association. Lysates were immunoprecipitated with anti-HA-mAb and Western blotted with anti-Jak2-pAb. Shown is one of three independent experiments.

Following angiotensin II-induced Jak2 tyrosine phosphorylation, Jak2 associates with the AT_1 receptor (18). To determine if Jak2 containing the R1113E mutation could associate with the AT_1 receptor, we transfected COS-7 cells similar to those described above. The cells were then treated with 100 nM angiotensin II for either 0, 3, or 6 min, lysed, and protein was extracted. Protein lysates were immunoprecipitated with anti-HA antibody to immunoprecipitate the HA-tagged AT_1 receptor, and immunoblotted with anti-Jak2 antibody to detect Jak2/AT_1 receptor co-association (Fig. 6-5). The results show that in response to angiotensin II, wild type Jak2 rapidly associates with the AT_1

receptor, while Jak2-R1113E is unable to do so, thus demonstrating that Arg 1113 is also essential for Jak2/AT_1 receptor co-association.

Jak2-R1113E is Unable to Tyrosine Phosphorylate STATs in Response to Angiotensin II

Once Jak2 associates with the AT_1 receptor in response to angiotensin II, STAT proteins are recruited to the Jak2/AT_1 receptor complex (38). Jak2 then tyrosine phosphorylates these receptor-bound STATs (18). To examine angiotensin II-dependent STAT activation in cells expressing Jak2-R1113E, we again transfected COS-7 cells similar to those described above. The cells were then treated with 100 nM angiotensin II for either 0, 5, or 10 min, lysed, and protein was extracted. The protein lysates were immunoprecipitated with anti-STAT1 antibody and immunoblotted with anti-Jak2 antibody to detect STAT1/Jak2 co-association (Fig. 6-6, Top).

We show that in cells expressing wild type Jak2, angiotensin II induced STAT1/Jak2 co-association, whereas in cells expressing Jak2-R1113E, no STAT1/Jak2 co-association was seen in response to angiotensin II. We stripped the same membrane and reprobed it with anti-phosphotyrosine antibody to detect tyrosine phosphorylated STAT1 protein (Fig. 6-6, Middle). The results show that in cells expressing wild type Jak2, angiotensin II induces marked STAT1 tyrosine phosphorylation, whereas in cells expressing Jak2-R1113E, STAT1 does not become tyrosine phosphorylated in response to angiotensin II. Finally, we stripped the same membrane and reprobed it with anti-STAT1 antibody to demonstrate equal sample loading (Fig. 6-6, Bottom). Collectively, the data in Fig. 6-6 demonstrate that in cells expressing Jak2-R1113E, angiotensin II-dependent activation of STAT1 is disrupted, as measured by reduced STAT1/Jak2 co-association and STAT1 tyrosine phosphorylation.

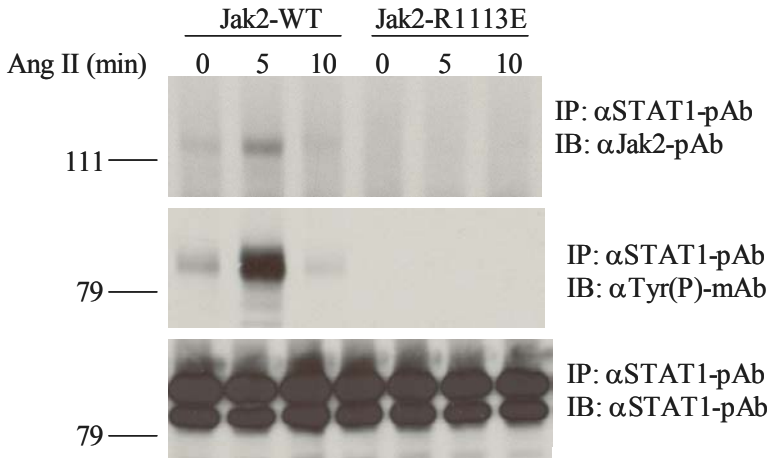


Figure 6-6. Jak2-R1113E is unable to activate STAT1 in response to angiotensin II Transfected COS-7 cells were treated with angiotensin II for the indicated times and lysates were prepared. Lysates were immunoprecipitated with anti-STAT1-pAb and Western blotted with anti-Jak2-pAb to assess Jak2/STAT1 co-association (Top). The membrane was stripped and reprobed with anti-Tyr(P)-mAb to assess STAT1 tyrosine phosphorylation (Middle). The membrane was again stripped, and then reprobed with anti-STAT1-pAb to demonstrate equal sample loading (Bottom). Shown is one of three independent experiments.

Jak2-R1113E is unable to mediate angiotensin II-dependent gene expression

We tested the ability of cells expressing the Jak2-R1113E mutant to activate STAT-mediated gene transcription. For this, COS-7 cells were transfected with 10 μg of AT_1 receptor, 10 μg of a luciferase reporter plasmid encoding a Stat1-binding *sis*-inducible element, and either 10 μg of wild type Jak2 cDNA or 12.5 μg of Jak2-R1113E cDNA. The luciferase reporter plasmid contains a tandem repeat of a minimal DNA enhancer element, the thymidine kinase TATA-containing promoter, and the firefly luciferase cDNA. Each copy of the DNA enhancer contains a STAT1-inducible SIE element, a serum response element, and an AP-1 binding site. Others and we previously showed that this plasmid is a good indicator of Jak-STAT-mediated gene transcription (108, 126). The cells were serum-starved for 24 h, and then treated with 100 nM angiotensin II for 0, 4, 8, or 12 h. We show that in cells expressing wild type Jak2,

angiotensin II treatment produced a rapid and transient increase in Jak-STAT mediated gene expression that peaked at 4-8 h after angiotensin II treatment (Fig. 6-7). This response was greatly attenuated in cells expressing the Jak2-R1113E plasmid. Thus, these data show that Arg 1113 is also critical for angiotensin II-induced STAT-mediated gene transcription.

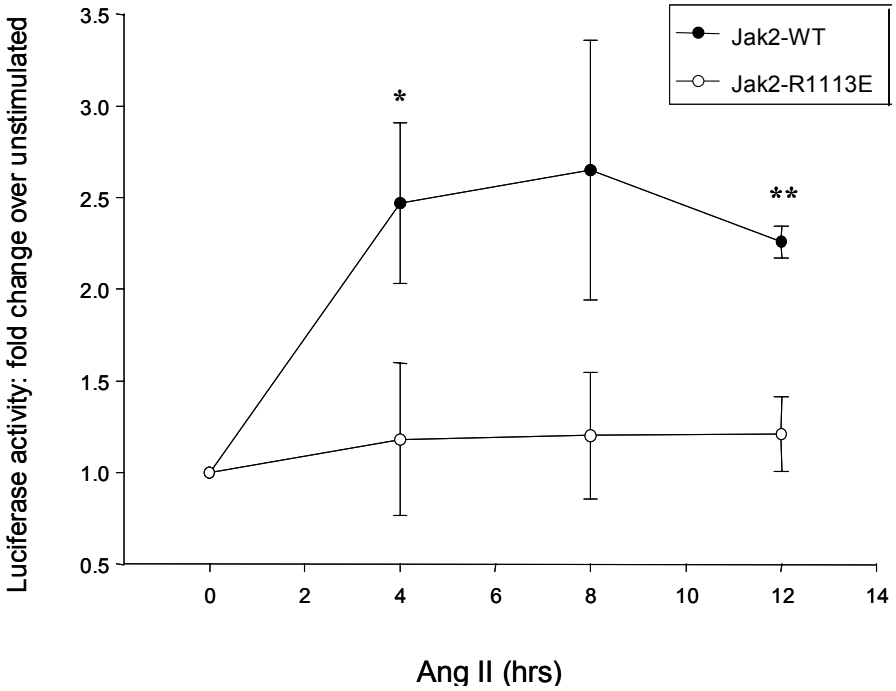


Figure 6-7. Jak2-R1113E is unable to activate STAT-mediated gene transcription in response to angiotensin II. COS-7 cells were transfected with 10 μg of AT_1 receptor, 10 μg of a luciferase reporter plasmid encoding a STAT1-binding sis-inducible element, and either 10 μg of wild type Jak2 or 12.5 μg of Jak2-R1113E cDNA. The cells were treated with 100 nM angiotensin II for the indicated times and detergent extracts were prepared. Luciferase activity was measured using the Reporter Lysis Buffer System (Promega). Shown is one of two independent experiments. Values are expressed as the mean +/- SD. n=4 for each time point, * $p<0.05$, ** $p<0.005$ (Student's *t* test).

Arg 1113 is conserved in different Jak kinase family members and among species expressing Jak2

The data in Figs. 6-4 through 6-7 show that Arg 1113 is essential for ligand dependent Jak2-mediated signaling. We therefore wanted to determine if this amino acid

was highly conserved throughout the evolutionary history of Jak2. Comparison of the amino acid sequence of the different Janus kinase family members, and the amino acid sequence of Jak2 from various species expressing the gene, indicates that Arg 1113 is a highly conserved residue (Fig. 6-8). The highly conserved nature of Arg 1113 further indicates a critical role for this amino acid in Jak2 function.

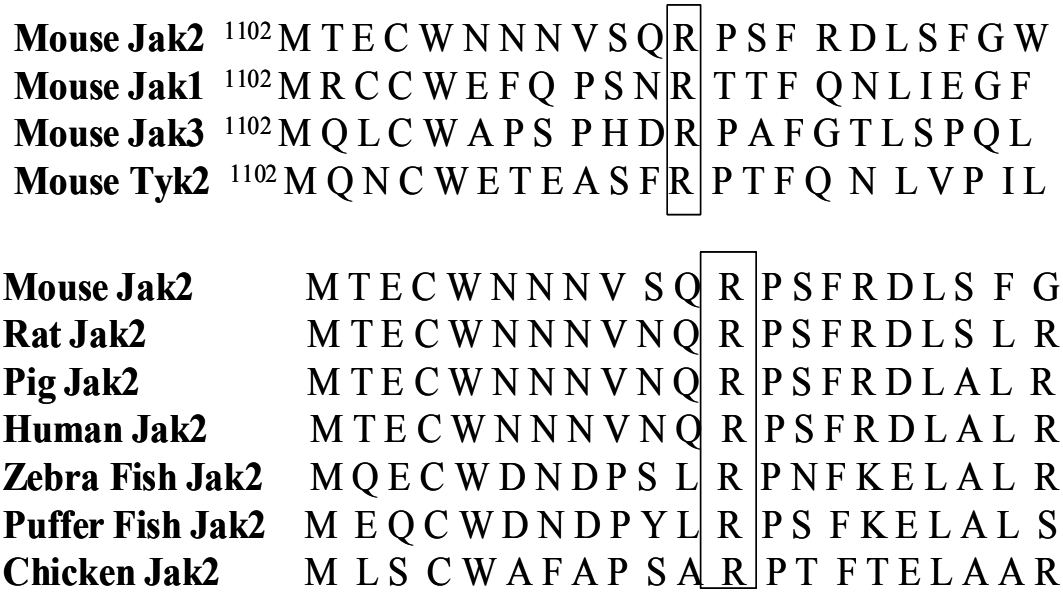


Figure 6-8. Arg 1113 is conserved in Jak2 among species and in different Jak family members Comparison of the amino acid sequence of the different murine Janus kinase family members, and the amino acid sequence of Jak2 from various species, indicates that Arg 1113 is a highly conserved residue throughout evolution.

Discussion

We provided evidence that Glu 1024 forms two distinct hydrogen bonds with Arg 1113 that are critical for Jak2 tyrosine kinase activity. As such, these are the first data describing Arg 1113 as being critical for Jak2 kinase function. Molecular modeling studies identified a putative interaction between Glu 1024 and Arg 1113. Using the program HBPLUS Hydrogen Bond Calculator, we determined that this interaction consisted of two distinct hydrogen bonds between the oxygen groups on the side chain of

Glu 1024 and the terminal amino groups on the side chain of Arg 1113. Using site-directed mutagenesis, we showed that E1024R, E1024D, R1113K, R1113A, and R1113E point mutations all rendered Jak2 catalytically inactive. Converting Glu 1024 to Arg (E1024R), and Arg 1113 to either Ala (R1113A) or Glu (R1113E) changed the charge on the amino acid at those positions and thus likely disrupted the ionic interaction between amino acids 1024 and 1113. While the E1024D and R1113K substitutions maintained the charge at those positions, both of these mutations shortened the side chains of the amino acids at their respective positions. Aspartic acid has a side chain that is one carbon shorter than that of glutamic acid; lysine has a side chain that is one amino group shorter than that of arginine. Thus, despite the conservative nature of the R1113K and E1024D mutations, the kinase function of Jak2 was lost. Consequently, we believe that the shorter side chains were not sufficiently long to maintain the interaction between these two residues, indicating that proper bond length is of critical importance to maintaining this interaction.

Previous studies showed that double mutation of Trp 1020 to Gly and Glu 1024 to Ala (W1020G/E1024A) rendered Jak2 dominant negative (106). Here, we tested whether individual mutation at either Trp 1020 or Glu 1024 alone would not only render Jak2 catalytically inactive, but also dominant negative. We showed that substitution mutations at either position 1020 (W1020G) or position 1024 (E1024A) rendered Jak2 dominant negative. Interestingly, these individual mutants were not able to act as potent a dominant negative as the double Jak2-W1020G/E1024A mutant. We believe, therefore, that the strong dominant negative phenotype displayed by the double mutant is due to an additive dominant negative effect of the two individual mutations. This is a discovery of

two new Jak2 dominant negative molecules, and these mutants may be useful research tools for studying the function of Jak2. In fact, these two dominant negative mutants may offer an advantage over the double W1020G/E1024A dominant negative mutant. Since their dominant negative character is not as strong as that of the double mutant, their expression in cells could be used as a knockdown approach to studying Jak2 function. This could be particularly useful for in vivo models, where Jak2 activation is critical to life.

We next used COS-7 cells to examine the consequences of the Jak2-R1113E mutation in a ligand-dependent signaling system. These cells express very low levels of Jak2 and no AT_1 receptor. When Jak-STAT signaling is reconstituted in these cells they are a reliable, simple-to-use system for determining the functional consequences of Jak2 mutations. Using these cells, we showed that Jak2 containing an R1113E mutation is unable to become activated in response to angiotensin II. Furthermore, we showed that expression of Jak2-R1113E cDNA in cells prevented angiotensin II-dependent Jak2/AT_1 receptor co-association, Jak2/STAT1 co-association, STAT1 tyrosine phosphorylation, and STAT-mediated gene transcription. Together, these data show the critical importance of Arg 1113 to Jak2 function. Further supporting the indispensability of Arg 1113, is the conserved nature of this amino acid amongst Jak family members and throughout Jak2 from various species. We believe that the identification of this and other critical amino acid interactions within the Jak2 kinase domain could provide targets for drug design aimed at disabling Jak2 kinase function.

CHAPTER 7
IDENTIFICATION OF A NOVEL Jak2 INHIBITOR

Introduction

The Jak2 protein is important in both physiology and pathophysiology, as it plays prominent roles in embryonic development, cell signaling, and in cancer and heart disease (61, 92, 102, 103,). Two impediments to better understanding Jak2 function are 1) the lack of an adult knockout animal model and 2) the lack of a Jak2-specific pharmacological inhibitor (102, 103, 130).

Jak2 knockout mice die embyronically, around E10.5 because of a lack of erythropoesis (102, 103). This work showed the critical role that Jak2 plays in embryonic development and cytokine signal transduction, but also raised a barrier to research on elucidating the mechanisms of Jak2 cellular function. Without an adult Jak2 knockout animal available, studying the function of Jak2 in adult physiology and pathophysiology has been complicated. Furthermore, there is no Jak2-specific pharmacological inhibitor. AG490 is a commercially available Jak2 inhibitor, and while it has been instrumental in elucidating some functions of Jak2 and in identifying Jak2 as a therapeutic target, it suffers from a general lack of specificity. In fact, in Chapter 5, we showed that Jak2 nonspecifically inhibits angiotensin II-mediated ERK2 activation. Because of these problems, there are caveats in all research relying solely on AG490 to study Jak2 kinase function. Clearly, identification of a novel Jak2 inhibitor could aid research efforts.

For these reasons, we sought to identify a potential novel Jak2 inhibitor. We first used homology modeling of the Jak2 kinase domain to identify exposed pockets on the

surface of the protein. We then used a high-throughput program called DOCK, to predict the ability of 6,451 small molecules to interact with a solvent accessible pocket that is adjacent to the activation loop of Jak2, namely, Pocket 36. The compounds were scored *in silico* on their potential ability to interact with Pocket 36. We ordered the top seven scoring compounds, and tested their ability to inhibit Jak2 tyrosine kinase function. One of these, Compound 7, was found to be a potent inhibitor of Jak2.

Results

Homology Modeling and Target Pocket Identification

We used the homology model of the Jak2 kinase domain described in Chapter 6 to identify pockets within the Jak2 kinase domain that could interact with potential small-molecule inhibitors of Jak2. The program SPHGEN identified 49 pockets within the Jak2 kinase domain. The pockets were designated based on their chemical and shape characteristics. We chose the pocket designated as Pocket "36" as a target based on its proximity to the Jak2 activation loop and its large size, which makes it accessible to small-molecules (Fig. 7-1).

Database Screening to Identify Potential Small-Molecule Inhibitors of Jak2

Using Pocket 36 as the target, we used the program DOCK to screen a National Cancer Institute database of known chemical structures for their ability to interact with Pocket 36. We used the program to screen 6,415 compounds of the over 140,000 compounds in the database. The program attempted, *in silico*, to fit each compound into Pocket 36 in 100 different orientations for each compound tested. The compounds were scored on their ability to fit into Pocket 36 and on their ability to interact chemically with Pocket 36. We ordered the seven top-scoring compounds for further testing. These

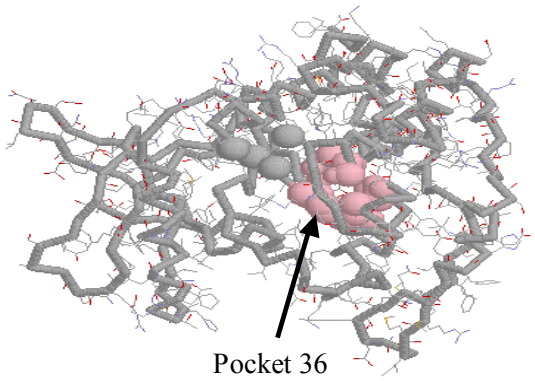


Figure 7-1. SPHGEN identified 49 exposed pockets on the surface of the Jak2 protein Because of its large size, Pocket 36 was chosen as a target pocket for *in silico* compound screening.

compounds were provided to us, free of charge, by the National Cancer Institute, through their Developmental Therapeutics Program. These non-proprietary compounds are offered to the extramural research community for the development of treatments for cancer, AIDS, and opportunistic infections afflicting patients with cancer or AIDS. These were designated Compounds 1-7 (Table 7-1).

Table 7-1. Top 7 scoring compounds

Cmpd #	NSC #	Formula	Name	Mol. Weight
1	7785	C_3H_5NO	2-propenamide	71.0
2	7795	$C_{28}H_{22}N_2O_8S_2\,2Na$	Acid Green 25	625.0
3	7828	$C_{19}H_{12}Cl_2O_5S$	Chlorphenol Red	423.0
4	7830	$C_{36}H_{25}N_5O_6S_2\,2Na$	Acid Black S	734.0
5	7851	$C_4H_3Cl_2N_3$	4,6-Dichloro-5-aminopyrimidine	164.0
6	7893	$C_5H_7N_3O$	Superacil	125.0
7	7908	$C_6H_6Br_6$	Cyclohexane- 1,2,3,4,5,6-hexabromo-	558.0

Compound 7 Inhibits Jak2 Autophosphorylation

To test the ability of each of the seven compounds to inhibit Jak2 tyrosine kinase activity, we used the vaccinia virus transfection/infection protocol. Briefly, BSC-40 cells, a vaccinia virus permissive cell line, are transfected with an expression vector encoding the wild type murine Jak2 cDNA under the control of the T7 promoter. The cells are then infected with a vaccinia virus that produces T7 RNA polymerase. This results in high level Jak2 expression and subsequent tyrosine autophosphorylation independent of exogenous ligand addition. After the initial 1 h vaccinia virus infection, the cells were switched to serum containing media and each compound was added at a final concentration of 100 μM and incubated overnight. Sixteen h later after the addition of the inhibitors, cellular lysates were prepared and immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-phosphotyrosine antibody to detect tyrosine phosphorylated Jak2 (Fig. 7-2A, Top). The results showed that Compound 7 was the only compound to inhibit Jak2 tyrosine autophosphorylation. The membrane was then stripped and re-probed with anti-Jak2 antibody to demonstrate equal protein expression amongst all samples (Fig. 7-2A, Bottom). The identity and structure of compound 7 is shown (Fig. 7-2B). Cyclohexane-1,2,3,4,5,6-hexabromo- is a single aromatic ring structure with a halide on each carbon. It has a molecular weight of 125 daltons.

Compound 7 Inhibits Jak2 Autophosphorylation in a Time-Dependent Manner

We next wanted to determine whether Compound 7 could inhibit Jak2 tyrosine autophosphorylation in a time-dependent manner. For this, BSC-40 cells were transfected/infected as described. Before cell lysis, 100 μM of Compound 7 was applied to the cells for 0, 1, 4, or 16 h. Cellular lysates were then prepared, immunoprecipitated

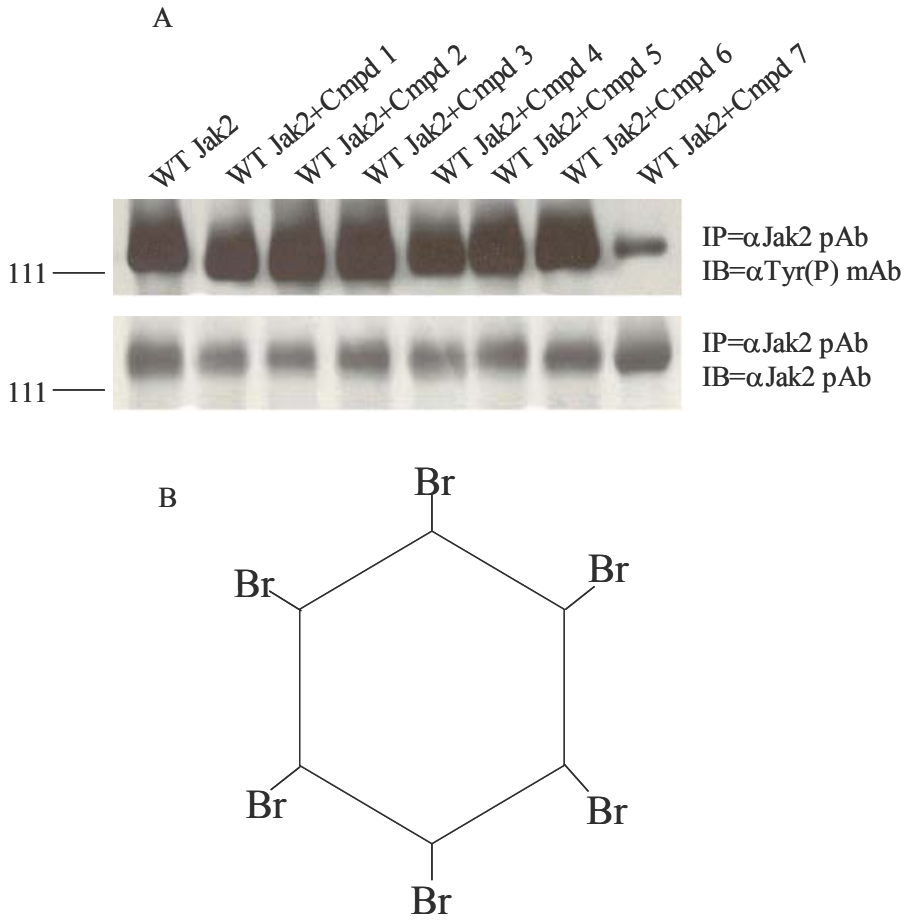


Figure 7-2. Compound 7 inhibits Jak2 autophosphorylation A) The 7 compounds that received the highest score from the DOCK program for their ability to interact with Pocket 36 within the Jak2 kinase domain were tested for their ability to inhibit Jak2 autophosphorylation. BSC-40 cells were transfected with 5 μg of Jak2 cDNA, and then infected with 1 MOI of vaccinia virus for 16 h to drive high-level expression of Jak2 and subsequent Jak2 autophosphorylation. During viral infection, the 7 compounds were incubated with the cells at a concentration of 100 μM each. Cell lysates were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation (Top). The membrane was stripped and re-probed with anti-Jak2 antibody to demonstrate equal Jak2 expression amongst all samples (Bottom). B) Shown is the structure and identity of Compound 7. Shown is one of three independent experiments.

with anti-Jak2 antibody, and immunoblotted with anti-phosphotyrosine antibody to measure tyrosine phosphorylated Jak2 levels (Fig. 7-3, Top). The results showed that 1

or 4 h treatment with Compound 7 was sufficient to block ~75% of the tyrosine autophosphorylation of Jak2. However, 16 h treatment with 50 μM Compound 7 resulted in a virtual elimination of all Jak2 tyrosine autophosphorylation. The membrane was stripped and re-probed with anti-Jak2 antibody to demonstrate equal protein expression amongst all samples (Fig. 7-3, Bottom).

Collectively, the data show that incubation of Compound 7 does inhibit Jak2 tyrosine autophosphorylation in a time-dependent manner; treatment of cells with 50 μM Compound 7 for 1 or 4 h was sufficient to partially inhibit Jak2 tyrosine kinase autophosphorylation, while treatment of cells for 16 h resulted in near total elimination of Jak2 tyrosine autophosphorylation.

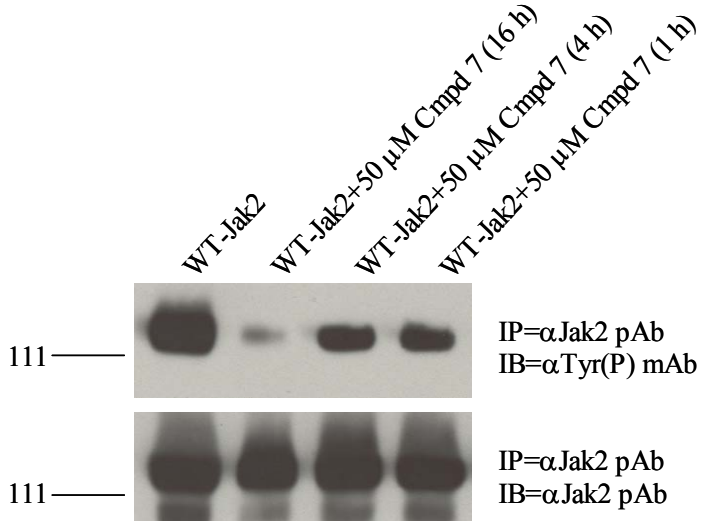


Figure 7-3. Maximal Jak2 inhibition requires 16 h of incubation with Compound 7 Compound 7 was incubated with the cells for 0, 1, 4, or 16 h at a concentration of 50 μM during infection. Cell lysates were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation (Top). The membrane was stripped and re-probed with anti-Jak2 antibody to demonstrate equal Jak2 expression amongst all samples (Bottom). Shown is one of two representative experiments.

Compound 7 Inhibits Jak2 Autophosphorylation in a Dose-Dependent Manner

We next tested the ability of Compound 7 to inhibit Jak2 autophosphorylation in a dose-dependent manner. For this, we again used the BSC-40 cell transfection/infection protocol. The cells were treated for 16 h with Compound 7 at doses of 0, 1, 10, 50, 100, 250, or 500 μM. The next morning, soluble protein lysates were immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-phosphotyrosine antibody to measure the tyrosine phosphorylation levels of Jak2 (Fig. 7-4, Top). The results showed that Compound 7 inhibited Jak2 tyrosine autophosphorylation in a dose-dependent manner with maximal inhibition occurring at 50 μM. The membrane was then stripped and re-probed with anti-Jak2 antibody to demonstrate equal protein expression amongst samples (Fig. 7-4, Bottom).

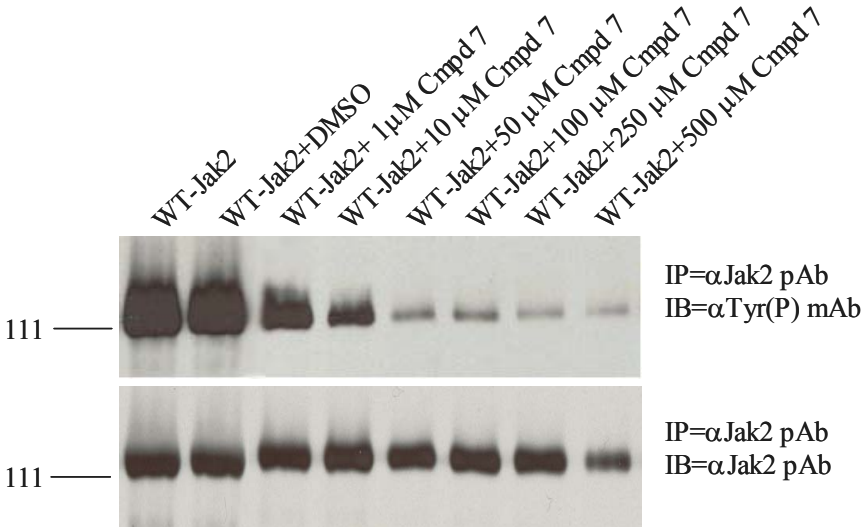


Figure 7-4. Compound 7 inhibits Jak2 in a dose-dependent manner BSC-40 cells were again transfected/infected as described above. Compound 7 was incubated with the cells during infection at a dose of 0, 1, 10, 100, 250, or 500 μM for 16 h. Cell lysates were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation (Top). The membrane was stripped and re-probed with anti-Jak2 antibody to demonstrate equal Jak2 expression amongst all samples (Bottom). Shown is one of two representative experiments.

Collectively, the data in Fig. 4 show that Compound 7 does in fact inhibit Jak2 autophosphorylation in a dose-dependent manner. The amount of material required to inhibit 50% of the Jak2 tyrosine autophosphorylation levels in this assay (IC_{50}) was in the low micromolar range. Additionally, 50 μM Compound 7 was sufficient for maximal Jak2 inhibition.

Compound 7 is Non-Cytotoxic at Concentrations that Maximally Inhibit Jak2 Tyrosine Autophosphorylation

To determine whether Compound 7 was cytotoxic to the cultured cells, we treated BSC-40 cells with Compound 7 at doses of 0, 100, or 500 μM for 16 h. The live cells were then stained with propidium iodide to determine whether Compound 7 was cytotoxic. Propidium iodide selectively stains necrotic cells and fluoresces red, but is excluded by the plasma membranes of healthy, intact cells. The results showed that cells treated with 100 μM Compound 7 showed very little propidium iodide staining, akin to that of untreated cells (Fig. 7-5). In contrast, BSC-40 cells treated with 500 μM Compound 7 did show increased propidium iodide staining, indicating that at a dose of 500 μM Compound 7 is cytotoxic. Since the IC_{50} of Compound 7 is estimated to be in the low micromolar range, and 50 μM Compound 7 maximally inhibits Jak2 tyrosine kinase autophosphorylation, we conclude that the mechanism by which Compound 7 inhibits Jak2 tyrosine kinase autophosphorylation, at these concentrations, is independent of cellular cytotoxicity.

Discussion

Since its discovery in 1992, significant progress has been made in understanding the biochemical and cellular functions of Jak2 tyrosine kinase. Studies have shown essential roles for Jak2 in embryonic development, cell signaling, and the

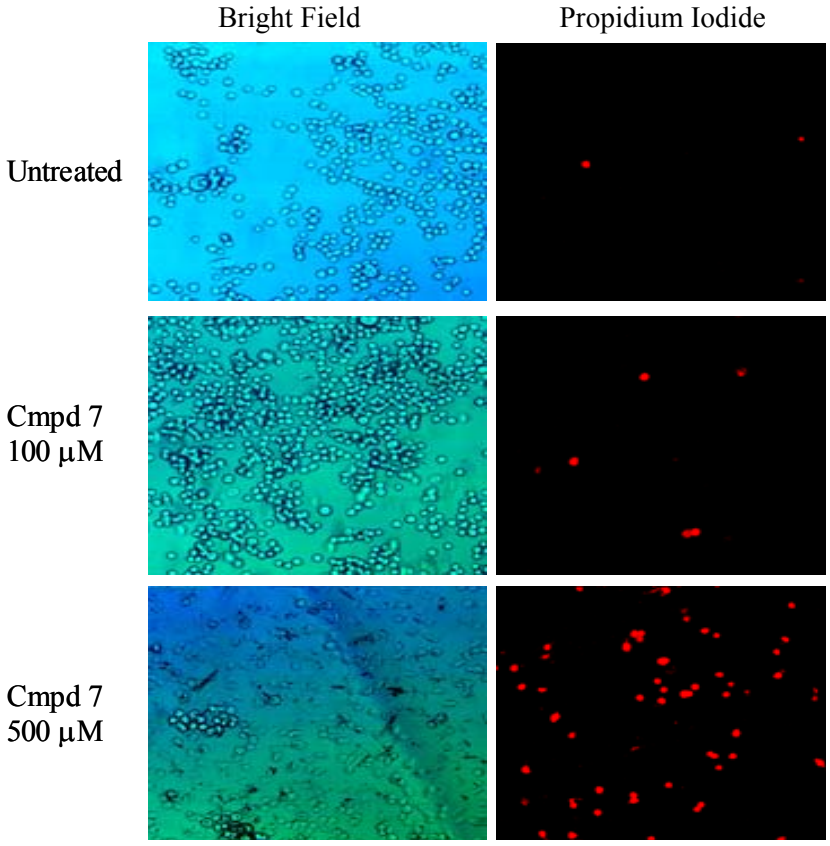


Figure 7-5. Compound 7 is not cytotoxic at a dose of 100 µM BSC-40 cells were grown on microscope slides and treated with 0, 100, or 500 µM Compound 7 for 16 h. The live cells were then stained with 1 µg/mL propidium iodide to determine whether Compound 7 was cytotoxic. The cells were visualized using confocal microscopy. Shown is one of two representative experiments.

pathophysiology of heart disease and cancer (61, 92, 102, 103). Research on this protein, though, has been complicated by the lack of a Jak2-specific pharmacological inhibitor. AG490, a commercially available Jak2 inhibitor, also inhibits several other related tyrosine kinase signaling pathways (98-101).

This work is significant for three fundamental reasons. First, we used homology modeling of the Jak2 kinase domain and high-throughput compound docking *in silico* to identify potential Jak2 inhibitors. We found that cyclohexane-1,2,3,4,5,6-hexabromo-, designated as Compound 7, potently inhibited Jak2 tyrosine autophosphorylation in cultured BSC-40 cells. Compound 7 inhibited Jak2 autophosphorylation in both a dose-

and time-dependent manner. Based on these autophosphorylation assays, it appears that a 16 h treatment with 1 μM Compound 7 is sufficient to reduce Jak2 tyrosine autophosphorylation levels by about 50%, while 50 μM Compound 7 eliminates virtually all detectable Jak2 tyrosine autophosphorylation. Furthermore, even at doses as high as 100 μM, Compound 7 is not cytotoxic to cultured cells. As such, it inhibits Jak2 tyrosine autophosphorylation at concentrations that are well below its cytopathic threshold.

Second, the results shown here using the DOCK program, demonstrate proof-of-principle in using *in silico*-based strategies for identifying biological interactions. Here, screening just 6,451 compounds for their ability to interact with one target pocket on the Jak2 kinase domain, we successfully used the DOCK program to identify a novel Jak2 inhibitor. We will therefore use the DOCK program for screening additional compounds for their ability to bind multiple targets within the Jak2 kinase domain. In fact, the library that we have available for screening contains over 140,000 compounds of known chemical structure. Furthermore, we identified 49 exposed pockets on the Jak2 kinase domain. By screening the entire library of compounds using multiple target pockets, we expect to identify several additional small molecule inhibitors of Jak2.

Third, AG490 falls within the general class of tyrosine kinase inhibitors known as tyrphostins. The molecular structure of AG490 is known; it contains two aromatic ring structures linked by a spacer containing four carbons and an amide group. Compound 7 is noticeably different from AG490 in that it contains only a single aromatic ring without any spacers. As such, our work here suggests that Compound 7, with its single aromatic ring, could serve as a potential lead compound for future synthesis reactions with the hopes of identifying a specific Jak2 inhibitor.

A final possibility for identifying additional small molecule inhibitors of Jak2 using database screening is to model the site where AG490 binds to the Jak2 kinase domain. We could then use this binding site as a target for database screening. This has the advantage of using an area that is known to bind a small molecule inhibitor of Jak2 as a target. This may allow us to identify Jak2 inhibitors that have the same site of action as AG490, but are more specific for Jak2 than AG490.

Collectively, the work shown here has identified cyclohexane-1,2,3,4,5,6-hexabromo- as a small molecule inhibitor of Jak2 tyrosine kinase. Because of the universal importance of Jak2 in mediating both the physiological and pathophysiological actions within animals, this compound, and potential derivatives of it, may have important therapeutic value.

CHAPTER 8
CONCLUSIONS AND PERSPECTIVES

The Jak2 tyrosine kinase protein was discovered in 1991. It was quickly identified as a key mediator of cytokine signaling. Since then, roles for Jak2 in mediating signaling through GPCRs and during cellular stress, including oxidative stress, have been identified. Despite this, study of Jak2 has been complicated for two reasons: 1) lack of an adult knockout animal and 2) lack of a specific Jak2 inhibitor. In these studies we used several strategies to circumvent these problems, and we significantly improved our understanding of Jak2 structure, function, and pharmacology. We used Jak2 -/- cells to identify a novel role for Jak2 in angiotensin II-dependent inactivation of ERK2. We used cells expressing a Jak2 dominant negative mutant to identify Jak2 as an essential mediator of oxidative stress-induced apoptosis in vascular smooth muscle cells. Finally, we used homology modeling of the Jak2 kinase domain to identify an amino acid interaction within Jak2 that is critical for Jak2 function, and to identify a novel small molecule inhibitor of Jak2.

Role of Jak2 in Angiotensin II-Dependent ERK2 Signaling

Previous studies suggested that Jak2 was required for angiotensin II-dependent activation of ERK2. These studies, though, relied solely on the Jak2 inhibitor AG490 to determine this role for Jak2. While AG490 potently inhibits Jak2, it nonspecifically inhibits several other signaling pathways. For this reason, we used Jak2 -/- cells to specifically study the role that Jak2 plays in angiotensin II-dependent ERK2 signaling.

In Chapter 4, we showed that Jak2 is essential for inactivation of ERK2 after angiotensin II treatment. Moreover, we showed that the previously published results demonstrating that Jak2 is required for angiotensin II-dependent activation of ERK2, may be an artifact caused by using AG490 to study Jak2 function.

These studies may open a new area of research that will further explore crosstalk between the Jak2 signaling pathway and the ERK signaling pathway. Future studies should further elucidate the role that Jak2 plays in the regulation of angiontensin II signaling. Ultimately, it will be interesting to determine the physiological importance of this novel role for Jak2 *in vivo*, where angiotensin II plays critical roles during cardiovascular disease.

Role of Jak2 during Oxidative Stress

It was discovered in 1998, that Jak2 is strongly activated in vascular smooth muscle cells by oxidative stress in the form of hydrogen peroxide. Since then, there has been little research into the physiological role that Jak2 plays during oxidative stress. In Chapter 5, we used expression of a Jak2 dominant negative mutant to show, for the first time, a physiological endpoint of Jak2 activation by hydrogen peroxide in vascular smooth muscle cells. We found that hydrogen peroxide resulted in apoptosis of control vascular smooth muscle cells, but failed to induce apoptosis in cells expressing a dominant negative Jak2, indicating that Jak2 activation is essential for oxidative stress-induced apoptosis of vascular smooth muscle cells.

These results could have profound consequences on diseases where oxidative stress-induced apoptosis contributes to pathology. Atherosclerosis is one such disease. During atherosclerosis, circulating macrophages release high amounts of hydrogen peroxide on vascular smooth muscle cells within the fibrous cap of the atherosclerotic

plaque. This can lead to apoptosis of the cells, and subsequent weakening of the fibrous cap. When the cap weakens, plaque rupture often occurs, which can result in thrombus formation, and subsequent heart attack or stroke. First, the role that Jak2 plays in oxidative stress *in vivo* must be determined.

Jak2 Structure-Function

Point mutations at both Trp 1020 and Glu 1024 render Jak2 dominant negative. As discussed above, we used this dominant negative mutant to determine the role of Jak2 during oxidative stress. We also used this dominant negative mutant to better understand the structure of Jak2. Previously, we showed that mutation of either Trp 1020 or Glu 1024 individually rendered Jak2 catalytically inactive. In Chapter 6, we showed that these individual point mutations also render Jak2 dominant negative. Moreover, we determined the reason that Glu 1024 is critical for Jak2 function. This amino acid forms two distinct hydrogen bonds Arg 1113.

Critical amino acid interactions within the Jak2 kinase domain could be targets for drug design aimed at disabling Jak2 kinase function. Novel Jak2 inhibitors would be useful research tools and could possibly hold therapeutic potential. For this reason, the structure of the Jak2 protein should continue to be explored. Hopefully, in the future, the crystal structure of Jak2 will be determined. This will provide further insight into the Jak2 structure-function relationship, and could lead to design of Jak2 inhibitors.

Identification of a Novel Jak2 Inhibitor

A better understanding of Jak2 structure could lead to design of novel Jak2 inhibitors. In addition to pursuing a better understanding of Jak2 structure, we took a direct approach to identifying novel Jak2 inhibitors. We used homology modeling of the Jak2 kinase domain to identify target pockets for *in silico* compound docking. Using the

DOCK program, we identified cyclohexane-1,2,3,4,5,6-hexabromo-, designated as Compound 7, as a novel Jak2 inhibitor.

Compound 7 may prove to be a useful research tool for studying Jak2 function. With modification, Compound 7 could also be improved as a Jak2 inhibitor. Importantly, the identification of Compound 7 provides proof-of-principle that high throughput compound docking using a homology model of the Jak2 kinase domain can lead to the identification of novel Jak2 inhibitors. For this reason, additional compounds should be screened for their ability to interact with binding targets within the Jak2 kinase domain.

LIST OF REFERENCES

1. Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J,J. (1990) Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene* **5,** 1329-1336.

2. Wilks, A.F., Harpur, A.G., Kurban, R.R., Ralph, S.J., Zurcher, G., and Ziemiecki, A. (1991) Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. *Mol. Cell. Biol.* **11,** 2057-2065.

3. Harpur, A.G., Andres, A.C., Ziemiecki, A., Aston, R.R., and Wilks, A.F. (1992) JAK2, a third member of the JAK family of protein tyrosine kinases. *Oncogene*. **7,** 1347-1353.

4. Duhe, R.J., Rui, H., Greenwood, J.D., Garvey, K., and Farrar, W.L. (1995) Cloning of the gene encoding rat JAK2, a protein tyrosine kinase. *Gene* **158,** 281-285.

5. Kawamura, M., McVicar, D.W., Johnston, J.A., Blake, T.B., Chen, Y.Q., Lal, B.K., Lloyd, A.R., Kelvin, D.J., Staples, J.E., Ortaldo, J.R., and O'Shea, J.J. (1994) Molecular cloning of L-JAK, a Janus family protein-tyrosine kinase expressed in natural killer cells and activated leukocytes. *Proc. Natl. Acad. Sci. (USA)* **91,** 6374-6378.

6. Takahashi, T. and Shirasawa, T. (1994) Molecular cloning of rat JAK3, a novel member of the JAK family of protein tyrosine kinases. *FEBS Letters* **342,** 124-128.

7. Rane, S.G. and Reddy, E.P. (1994) JAK3: a novel JAK kinase associated with terminal differentiation of hematopoietic cells. *Oncogene*. **9,** 2415-2423.

8. Oates, A.C., Brownlie, A., Pratt, S.J., Irvine, D.V., Liao, E.C., Paw, B.H., Dorian, K.J., Johnson, S.L., Postlethwait, J.H., Zon, L.I., and Wilks, A.F. (1999) Gene duplication of zebrafish JAK2 homologs is accompanied by divergent embryonic expression patterns: only jak2a is expressed during erythropoiesis. *Blood* **94,** 2622-2636.

9. Binari, R., and Perrimon, N. (1994) Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes Dev.* **8,** 300-312.

10. Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle JN. (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* **74,** 227-236.

11. Argetsinger, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvennoinen, O., Ihle, J.N., and Carter-Su, C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell* **74,** 237-244.

12. Silvennoinen, O., Witthuhn, B.A., Quelle, F.W., Cleveland, J.L., Yi,T., and Ihle, J.N. (1993) Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. *Proc. Natl. Acad. Sci. (USA)* **90,** 8429-8433.

13. Rui, H., Kirken, R.A., and Farrar, W.L. (1994) Activation of receptor-associated tyrosine kinase JAK2 by prolactin. *J. Biol. Chem.* **269,** 5364-5368.

14. Narazaki, M., Witthuhn, B.A., Yoshida, K., Silvennoinen, O., Yasukawa, K., Ihle, J.N., Kishimoto, T., and Taga, T. (1994) Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. *Proc. Natl. Acad. Sci. (USA)* **91,** 2285-2289.

15. Watling, D., Guschin, D., Muller, M., Silvennoinen, O., Witthuhn, B.A., Quelle, F.W., Rogers, N.C., Schindler, C., Stark, G.R., Ihle, J.N., and Kerr, I.M. (1993) Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature* **366,** 166-170.

16. Schindler, C., Shuai, K., Prezioso, V.R., and Darnell, J.E., Jr. (1992) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* **257,** 809-813.

17. Shuai, K., Schindler, C., Prezioso, V.R., and Darnell, J.E., Jr. (1992) Activation of transcription by IFN-gamma: tyrosine phosphorylation of a 91-kD DNA binding protein. *Science* **258,** 1808-1812.

18. Marrero, M.B., Schieffer, B., Paxton, W.G., Heerdt, L., Berk, B.C., Delafontaine, P., and Bernstein, K.E. (1995) Direct stimulation of Jak-STAT pathway by the angiotensin II AT1 receptor. *Nature* **375,** 247-250.

19. Tanaka, N., Asao, H., Ohbo, K., Ishii, N., Takeshita, T., Nakamura, M., Sasaki, H., and Sugamura, K. (1994) Physical association of JAK1 and JAK2 tyrosine kinases with the interleukin 2 receptor beta and gamma chains. *Proc. Natl. Acad. Sci. U S A*. **91,** 7271-7275.

20. Takaki, S., Kanazawa, H., Shiiba, M., and Takatsu, K. (1994) A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor alpha chain and its function in IL-5-mediated growth signal transduction. *Mol. Cell. Biol*. **14,** 7404-7413.

21. Yin, T., Yasukawa, K., Taga, T., Kishimoto, T., and Yang, Y.C. (1994) Identification of a 130-kilodalton tyrosine-phosphorylated protein induced by interleukin-11 as JAK2 tyrosine kinase, which associates with gp130 signal transducer. *Exp. Hematol*. **22,** 467-472.

22. Bacon, C.M., McVicar, D.W., Ortaldo, J.R., Rees, R.C., O'Shea, J.J., and Johnston, J.A. (1995) Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. *J. Exp. Med*. **181,** 399-404.

23. Quelle, F.W., Sato, N., Witthuhn, B.A., Inhorn, R.C., Eder, M., Miyajima, A., Griffin, J.D., and Ihle, J.N. (1994) JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. *Mol. Cell. Biol.* **14,** 4335-4341.

24. Wang, Y. and Fuller, G.M. (1995) Interleukin-6 and ciliary neurotrophic factor trigger janus kinase activation and early gene response in rat hepatocytes. *Gene* **162,** 285-289.

25. Shimoda, K., Iwasaki, H., Okamura, S., Ohno, Y., Kubota, A., Arima, F., Otsuka, T., and Niho, Y. (1994) G-CSF induces tyrosine phosphorylation of the JAK2 protein in the human myeloid G-CSF responsive and proliferative cells, but not in mature neutrophils. *Biochem Biophys Res Commun*. **203,** 922-928.

26. Drachman, J.G., Griffin, J.D., and Kaushansky K. (1995) The c-Mpl ligand (thrombopoietin) stimulates tyrosine phosphorylation of Jak2, Shc, and c-Mpl. *J Biol Chem*. **270,** 4979-4982.

27. Ghilardi, N. and Skoda, R.C. (1997) The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. *Mol. Endocrinol*. **11,** 393-399.

28. Ju, H., Venema, V.J., Liang, H., Harris, M.B., Zou, R., and Venema, R.C. (2000) Bradykinin activates the Janus-activated kinase/signal transducers and activators of transcription (JAK-STAT) pathway in vascular endothelial cells: localization of JAK-STAT signalling proteins in plasmalemmal caveolae. *Biochem. J*. **351,** 257-264.

29. Peeler, T.C., Conrad, K.M., and Baker, K.M. (1996) Endothelin stimulates *sis*-inducing factor-like DNA binding activity in CHO-K1 cells expressing ET_A receptors. *Biochem. Biophys. Res. Comm.* **221,** 62-66.

30. Lukashova, V., Chen, Z., Duhe, R.J., Rola-Pleszczynski, M., and Stankova, J. (2003) Janus kinase 2 activation by the platelet-activating factor receptor (PAFR): roles of Tyk2 and PAFR C terminus. *J. Immunol*. **171,** 3794-3800.

31. Buggy, J.J. (1998) Binding of α-melanocyte-stimulating hormone to its G protein-coupled receptor on B-lymphocytes activates the Jak-STAT pathway. *Biochem. J.* **331,** 211-216.

32. Sasaguri, T., Teruya, H., Ishida, A., Abumiya, T., and Ogata, J. (2000) Linkage between α_1 adrenergic receptor and the Jak-STAT signaling pathway in vascular smooth muscle cells. *Biochem. Biophys. Res. Comm.* **268,** 25-30.

33. Pan, J, Fukudo, K., Saito, M., Matsuzaki, J., Kodama, H., Sano, M., Takahashi, T, Kato, T, and Ogawa, S. (1999) Mechanical Stretch activates the JAK-STAT pathway in rat cardiomyocytes. *Circ. Res.* **84,** 1127-1136.

34. Mascareno, E., El-Shafei, M., Maulik, N., Sato, M., Guo, Y., Das, D.K., and Siddiqui, M.A. (2001) JAK-STAT signaling is associated with cardiac dysfunction during ischemia and reperfusion. *Circulation* **104,** 325-329.

35. Madamanchi, N.R., Li, S., Patterson, C., and Runge, M.S. (2001) Reactive oxygen species regulate heat-shock protein 70 via the JAK-STAT pathway. *Arteriocler Thromb Vasc Biol*. **3,** 321-326.

36. Simon, A.R., Rai, U., Fanburg, B.L., and Cochran, B.H. (1998) Activation of the JAK-STAT pathway by reactive oxygen species. *Am J Physiol*. **275,** C1640-1652.

37. Jenab, S. and Morris, P.L. (1996) Differential activation of signal transducer and activator of transcription (STAT)-3 and STAT-1 transcription factors and c-fos messenger ribonucleic acid by interleukin-6 and interferon-gamma in Sertoli cells. *Endocrinology* **137,** 4738-4743.

38. Ali, M.S., Sayeski, P.P., and Bernstein, K.E. (2000) Jak2 acts as both a STAT1 kinase and as a molecular bridge linking STAT1 to the angiotensin II AT1 receptor. *J. Biol. Chem.* **275,** 15586-15593.

39. Sayeski, P.P., Ali, M.S., Safavi, A., Lyles, M., Kim, S.O., Frank, S.J., and Bernstein, K.E. (1999) A catalytically active Jak2 is required for the angiotensin II-dependent activation of Fyn. *J. Biol. Chem.* **274,** 33131-33142.

40. Marrero, M.B., Schieffer, B., Li, B., Sun, J., Harp, J.B., and Ling, B.N. (1997) Role of Janus Kinase/Signal Transducer and Activator of Transcription and Mitogen-activated Protein Kinase cascades in angiotensin II- and platelet-derived growth factor-induced vascular smooth muscle cell proliferation. *J. Biol. Chem.* **272,** 24684-24690.

41. Sayeski, P.P., Ali, M.S., Hawks, K., Frank, S.J., and Bernstein, K.E. (1999) The angiotensin II-dependent association of Jak2 and c-Src requires the N-terminus of Jak2 and the SH2 domain of c-Src. *Circ Res*. **84,** 1332-1338.

42. Chauhan, D., Kharbanda, S.M., Ogata, A., Urashima, M., Frank, D., Malik, N., Kufe, D.W., and Anderson, K.C. (1995) Oncostatin M induces association of Grb2 with Janus kinase JAK2 in multiple myeloma cells. *J. Exp. Med*. **182,** 1801-1806.

43. Fuhrer, D.K. and Yang, Y.C. (1996) Complex formation of JAK2 with PP2A, P13K, and Yes in response to the hematopoietic cytokine interleukin-11. *Biochem. Biophys. Res. Comm*. **224,** 289-296.

44. Xia, K., Mukhopadhyay, N.K., Inhorn, R.C., Barber, D.L., Rose, P.E., Lee, R.S., Narsimhan, R.P., D'Andrea, A.D., Griffin, J.D., and Roberts, T.M. (1996) The cytokine-activated tyrosine kinase JAK2 activates Raf-1 in a p21ras-dependent manner. *Proc Natl Acad Sci U S A*. **93,** 11681-11686.

45. VanderKuur, J., Allevato, G., Billestrup, N., Norstedt, G., and Carter-Su, C. (1995) Growth hormone-promoted tyrosyl phosphorylation of SHC proteins and SHC association with Grb2. *J. Biol. Chem*. **270,** 7587-7593.

46. Fuhrer, D.K., Feng, G.S., and Yang, Y.C. (1995) Syp associates with gp130 and Janus kinase 2 in response to interleukin-11 in 3T3-L1 mouse preadipocytes. *J. Biol. Chem*. **270,** 24826-24830.

47. Zhu, T., Goh, E.L., and Lobie, P.E. (1998) Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2. Fak is not required for stat-mediated transcription. *J. Biol. Chem*. **273,** 10682-10689.

48. Feng, J., Witthuhn, B.A., Matsuda, T., Kohlhuber, F., Kerr, I.M., and Ihle, J.N. (1997) Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. *Mol. Cell. Biol.* **17,** 2497-2501.

49. Rui, L., and Carter-Su, C. (1999) Identification of SH2-Bβ as a potent cytoplasmic activator of the tyrosine kinase Janus kinase 2. *Proc. Natl. Acad. Sci. (USA)* **96,** 7172-7177.

50. Kim, S.O., Jiang, J., Yi, W., Feng, G.S., and Frank, S.J. (1998) Involvement of the Src homology 2-containing tyrosine phosphatase SHP-2 in growth hormone signaling. *J. Biol. Chem.* **273,** 2344-2354.

51. You, M., Yu, D.H., and Feng, G.S. (1999) Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak-STAT pathway. *Mol. Cell. Biol.* **19,** 2416-2424.

52. Jiao, H., Berrada, K., Yang, W., Tabrizi, M., Platanias, L.C., and Yi, T. (1996) Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol. Cell. Biol.* **16,** 6985-6992.

53. Shultz, L.D., Schweitzer, P.A., Rajan, T.V., Yi, T., Ihle, J.N., Matthews, R.J., Thomas, M.L., and Beier, D.R. (1993) Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. *Cell* **73,** 1445-1454.

54. Tsui, H.W., Siminovitch, K.A., de Souza, L., and Tsui, F.W. (1993) Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. *Nature Genet*. **4,** 124-129.

55. Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., Hara, T., and Miyajima, A. (1995) A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J.* **14,** 2816-2826.

56. Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J.N., and Yoshimura, A. (1999) The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *EMBO J.* **18,** 1309-1320.

57. Chung, C.D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., and Shuai, K. (1997) Specific inhibition of Stat3 signal transduction by PIAS3. *Science* **278,** 1803-1805.

58. Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D., and Shuai, K. (1998) Inhibition of Stat1-mediated gene activation by PIAS1. *Proc. Natl. Acad. Sci. (USA*) **95,** 10626-10631.

59. Harrison, D.A., Binari, R., Nahreini, T.S., Gilman, M., and Perrimon, N. (1995) Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *EMBO J.* **14,** 2857-2865.

60. Luo, H., Rose, P., Barber, D., Hanratty, W.P., Lee, S., Roberts, T.M., D'Andrea, A.D., and Dearolf, C.R. (1997) Mutation in the Jak kinase JH2 domain hyperactivates Drosophila and mammalian Jak-Stat pathways. *Mol. Cell. Biol.* **17,** 1562-1571.

61. Meydan, N., Grunberger, T., Dadi, H., Shahar, M., Arpaia, E., Lapidot, Z., Leeder, J.S., Freedman, M., Cohen, A., Gazit, A., Levitzki, A., and Roifman, C.M. (1996) Inhibition of acute lymphoblastic leukemia by a Jak-2 inhibitor. *Nature* **379,** 645-648.

62. Lacronique, V., Boureux, A., Valle, V.D., Poirel, H., Quang, C.T., Mauchauffe, M., Berthou, C., Lassard, M., Berger, R., Ghysdael, J., and Bernard, O.A. (1997) A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science* **278**, 1309-1312.

63. Peeters, P., Raynaud, S.D., Cools, J., Wlodarska, I., Grosgeorge, J., Philip, P., Monpoux, F., Van Rompaey, L., Baens, M., Van der Berghe, H., and Marynen, P. (1997) Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. *Blood* **90**, 2535-2540.

64. Ho, J.M., Beattie, B.K., Squire, J.A., Frank, D.A., and Barber, D.L. (1999) Fusion of the ets transcription factor TEL to Jak2 results in constitutive Jak-Stat signaling. *Blood* **93**, 4354-4364.

65. Lacronique, V., Boureux, A., Monni, R., Dumon, S., Mauchauffe, M., Mayeux, P., Gouilleux., F., Berger, R., Gisselbrecht, S., Ghysdael, J., and Bernard, O.A. (2000) Transforming properties of chimeric TEL-JAK proteins in BA/F3 cells. *Blood* **95**, 2076-2083.

66. Carron, C., Cormier, F., Janin, A., Lacronique, V., Giovannini, M., Daniel, M.T., Bernard, O., and Ghysdael, J. (2000) TEL-JAK2 transgenic mice develop T-cell leukemia. *Blood* **95**, 3891-3899.

67. Yoshikawa, H., Matsubara, K., Qian, G.S., Jackson, P., Groopman, J.D., Mann, J.E., Harris, C.C., and Herman, J.G. (2001) SOCS-1, a negative regulator of the JAK-STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nature Genet.* **1,** 29-35.

68. Real, P.J., Sierra, A., De Juan, A., Segovia, J.C., Lopez-Vega, J.M., and Fernandez-Luna, J.L. (2002) Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. *Oncogene.* **50,** 7611-7618.

69. Yamauchi, T., Yamauchi, N., Ueki, K., Sugiyama, T., Waki, H., Miki, H., Tobe, K., Matsuda, S., Tsushima**,** T., Yamamoto, T., Fujita, T., Taketani, Y., Fukayama, M., Kimura, S., Yazaki, Y., Nagai, R., and Kadowaki, T. (2000) Constitutive tyrosine phosphorylation of ErbB-2 via Jak2 autocrine secretion of prolactin in human breast cancer. *J. Biol. Chem.* **275,** 33937-33944.

70. Kim, S.O., Houtman, J.C., Jiang, J., Ruppert, J.M., Bertics, P.J., and Frank, S.J. (1999) Growth hormone-induced alteration in ErbB-2 phosphorylation status in 3T3-F442a fibroblasts. *J. Biol. Chem.* **274**, 36015-36024.

71. Gao, B., Shen, X., Kunos, G., Meng, Q., Goldberg., I.D., Rosen, E.M., and Fan, S. (2001) Constitutive activation of JAK-STAT3 signaling by BRCA1 in human prostate cancer cells. *FEBS Letters.* **488**, 179-184.

72. Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B., and Castelli, W.P. (1990) Prognostic implications of echocardiographically determined left ventricular mass in the Framingham heart study. *N. Engl. J. Med.* **322**, 1561-1566.

73. Zak, R. (1984) Factors controlling cardiac growth. In Zak R, ed, *Growth of the Heart in Health and Disease.* 165-185. New York, NY; Raven Press.

74. Casale, P.N., Devereux, R.B., Milner, M., Zullo, G., Harshfield, G.A., Pickering, T.G., and Laragh, J.H. (1986) Value of echocardiographic measurement of left ventricular mass in predicting cardiovascular morbid events in hypertensive men. *Ann. Intern. Med.* **105**, 173-178.

75. Zimmer, H.G., Kolbeck-Ruhmkorff, C., and Zierhut, W. (1995) Cardiac hypertrophy induced by alpha- and beta-adrenergic receptor stimulation. *Cardioscience* **6**, 47-57.

76. Komuro, I., Kaida, T., Shibazaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F., and Yazaki, Y. (1990) Stretching cardiac myocytes stimulates protooncogene expression. *J. Biol. Chem.* **265**, 3595-3598.

77. Sadoshima, J. and Izumo, S. (1993) Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of autocrine/paracrine mechanism. *EMBO J.***12**, 1681-1692.

78. Rozich, J.D., Barnes, M.A., Schmid, P.G., Zile, M.R., McDermott, P.J., and Cooper, G 4th. (1995) Load effects on gene expression during cardiac hypertrophy. *J. Mol. Cell. Cardiol.* **27**, 485-499.

79. Komuro, I., Kudo, S., Yamazaki, T., Zou, Y., Shiojima, I., and Yazaki, Y. (1996) Mechanical stretch activates the stress-activated protein kinases in cardiac myocytes. *FASEB J.* **10**, 631-636.

80. Yamaziki, T., Tobe, K., Hoh, E., Maemura, K., Kaida, T., Komuro, I., Tamemoto, H., Kadowaki, T., Nagai, R., and Yazaki, Y. (1993) Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. *J. Biol. Chem.* **268**, 12069-12076.

81. Sadoshima, J., Xu, Y., Slayter, H.S., and Izumo, S. (1993) Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes *in vitro*. *Cell* **75**, 977-984.

82. Ito, H., Hirata, Y., Adachi, S., Tanaka, M., Tsujino, M., Koike, A., Nogami, A., Murumo, F., and Hiroe, M. (1993) Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes. *Circ. Res.* **81**, 656-663.

83. Kodama, H., Fukada, K., Pan, J., Makino, S., Baba, A., Hori, S., and Ogawa, S. (1997) Leukemia inhibitory factor, a potent cardiac hypertrophic cytokine, activates the JAK-STAT pathway in cardiomyocytes. *Circ. Res.* **81**, 656-663.

84. Kunisada, K., Hirota, H., Fujio, Y., Matsui, H., Tani, Y., Yamauchi-Takihara, K., and Kishimoto, T. (1996) Activation of JAK-STAT and MAP kinases by leukemia inhibitory factor through gp130 in cardiac myocytes. *Circulation* **94**, 2626-2632.

85. Pennica, D., Wood, W.I., and Chien, K.R. (1996) Cardiotrophin-1: a multifunctional cytokine that signals via LIF receptor-gp130 dependent pathways. *Cytokine Growth Factor Rev*. **7**, 81-91.

86. Pan, J., Keiichi, F., Hiroaki, K., Shinji, M., Toshiyuki, T., Motoaki, S., Shingo, H., and Satoshi, O. (1997) Role of angiotensin II in activation of the JAK-STAT pathway induced by acute pressure overload in the rat heart. *Circ. Res.* **81**, 611-617.

87. Pan, J., Fukuda, K., Kodama, H., Sano, M., Takahashi, T., Makino, S., Kato, T., Manabe, T., Hori, S., and Ogawa, S. (1998) Involvement of gp130-mediated signaling in pressure overload-induced activation of the JAK-STAT pathway in rodent heart. *Heart Vessels* **13**, 199-208.

88. Pennica, D., King, K.L., Shaw, K.J., Luis, E., Rullamas, J., Luoh, S.M., Darbonne, W.C., Knutzon, D.S., Yen, R., Chien, K.R., Baker, J.B., and Wood, W.I. (1995) Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc. Natl. Acad. Sci. (USA*) **92**, 1142-1146.

89. Pennica, D., Shaw, K.J., Swanson, T.A., Moore, M.W., Shelton, D.L., Zioncheck, K.A., Rosenthal, A., Taga, T., Paioni, N.F., and Wood, W.I. (1995) Cardiotrophin-1: biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J. Biol. Chem.* **270**, 10915-10922.

90. Fukuzawa, J., Booz, G.W., Hunt, R.A., Shimizu, N., Karoor, V., Baker, K.M., and Dostal, D.E. (2000) Cardiotrophin-1 increases angiotensinogen mRNA in rat cardiac myocytes through STAT3: an autocrine loop for hypertrophy *Hypertension* **35**, 1191-1196.

91. Hunter, J.J. and Chien, K.R. (1999) Signaling pathways for cardiac hypertrophy and failure. *N. Engl. J. Med.* **341**, 1276-1283.

92. Podewski, E.K., Hilfiker-Kleiner, D., Hilfiker, A., Moraweitz, H., Lichtenberg, A., Wollert, K.C., and Drexler, H. (2003) Alterations in janus kinase (JAK)-signal transducers and activators of transcription (STAT) signalling in patients with end-stage dilated cardiomyopathy. *Circulation* **107**, 798-802.

93. Seki, Y., Kai, H. Shibata, R., Nagata, T., Yasukawa, H., Yosimura, A., and Imaizumi, T. (2000) Role of the JAK-STAT pathway in rat carotid artery remodeling after vascular injury. *Circ. Res.* **87,** 12-18.

94. Xie, S., Lin, H., Sun, T., and Arlinghaus, R.B. (2002) Jak2 is involved in c-Myc induction by Bcr-Abl. *Oncogene* **47,** 7137-7146.

95. Miyamoto, N., Sugita, K., Goi, K., Inukai, T., Lijima, K., Tezuka, T., Kojika, S., Nakamura, M., Kagami, K., and Nakazawa, S. (2001) The Jak2 inhibitor AG490 predominantly abrogates the growth of human B-precursor leukemic cells with 11q23 translocation or Philadelphia chromosome. *Leukemia* **11,** 1758-1768.

96. Kodama, H., Fukuda, K., Pan, J., Sano, M., Takahashi, T., Kato, T., Makino, S., Manabe, T., Murata, M., and Ogawa, S. (2000) Significance of ERK cascade compared with JAK-STAT and PI3-K pathway in gp-130-mediated cardiac hypertrophy. *Am. J. Physiol. Heart Circ. Physiol.* **279,** H1635-644.

97. El-Adawi, H., Deng, L., Tramontano, A., Smith, S., Mascareno, E., Ganguly, K., Castillo, R., and El-Sherif, N. (2003) The functional role of the JAK-STAT pathway in post-infarction remodeling. *Cardiovasc. Res.* **57,** 129-138.

98. Kleinberger-Doron, N., Shelah, N., Capone, R., Gazit, A., and Levitzki, A. (1998) Inhibition of Cdk2 activation by selected tyrphostins causes cell cycle arrest at late G1 and S phase. *Exp. Cell Res.* **241,** 340-351.

99. Oda, Y., Renaux, B., Bjorge, J., Saifeddine, M., Fujita, D.J., and Hollenberg, M.D. (1999) cSrc is a major cytosolic tryrosine kinase in vascular tissue. *Can. J. Physiol. Pharmacol.* **77**, 606-617.

100. Osherov, N.A., Gazit, C., Gilon, and Levitzki, A. (1993) Selective inhibition of the EGF and HER2/Neu receptors by Tyrphostins. *J. Biol. Chem*. **268,** 11134–11142.

101. Gu, Y., Zou, Y., Aikawa, R., Hayashi, D., Kudoh, S., Yamauchi, T., Uozumi, H., Zhu, W., Kadowaki, T., Yazaki, Y., and Komuro, I. (2001) Growth hormone signalling and apoptosis in neonatal rat cardiomyocytes. *Mol. Cell. Biochem.* **223,** 35-46.

102. Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M., Grosveld, G., and Ihle, J.N. (1998) Jak2 is essential for signaling through a variety of cytokine receptors. *Cell* **93,** 385-395.

103. Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1998) Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* **93,** 397-409.

104. Duhe, R.J. and Farrar, W.L. (1995) Characterization of active and inactive forms of the JAK2 protein-tyrosine kinase produced via the baculovirus expression vector system. *J. Biol. Chem.* **270,** 23084-23089.

105. Rui, L., Mathews, L.S., Hotta, K., Gustafson, T.A., and Carter-Su, C. (1997) Identification of SH2-Bβ as a substrate of the tyrosine kinase JAK2 involved in growth hormone signaling. *Mol. Cell. Biol.* **17,** 6633-6644.

106. Zhuang, H., Patel, S.V., He, T.C., Sonsteby, S.K., Niu, Z., and Wojchowski, D.M. (1994) Inhibition of erythropoietin-induced mitogenesis by a kinase-deficient form of Jak2. *J. Biol. Chem.* **269,** 21411-21414.

107. VonDerLinden, D., Ma, X., Sandberg, E.M., Gernert, K., Bernstein, K.E., and Sayeski, P.P. (2002) Mutation of glutamic acid residue 1046 abolishes Jak2 tyrosine kinase activity. *Mol. Cell. Biochem.* **241,** 87-94.

108. Frank, S.J., Yi, W., Zhao, Y., Goldsmith, J.F., Gilliland, G., Jiang, J., Sakai, I., and Kraft, A.S. (1995) Regions of the Jak2 tyrosine kinase required for coupling to the growth hormone receptor. *J. Biol. Chem.* **270,** 14776-14785.

109. Saharinen, P., Takaluoma, K., and Silvennoinen, O. (2000) Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. *Mol. Cell. Biol.* **20,** 3387-3395.

110. Lindauer, K., Loerting, T., Liedl, K.R., and Kroemer, R.T. (2001) Prediction of the structure of human Janus kinase 2 (JAK2) comprising the two carboxy-terminal domains reveals a mechanism for autoregulation. *Protein Engineering* **14,** 27-37.

111. Chen, M., Cheng, A., Candotti, F., Zhou, Y-J., Hymel, A., Fasth, A., Notarangelo, L.D., and O'Shea, J.J. (2000) Complex effects of naturally occurring mutations in the JAK3 psuedokinase domain: evidence for interactions between the kinase and psuedokinase domains. *Mol. Cell. Biol.* **20,** 947-956.

112. Saharinen, P. and Silvennoinen, O. (2002) The psuedokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. *J. Biol. Chem.* **277,** 47954-47963.

113. Saharinen, P., Vihinen, M., and Silvennoinen, O. (2003) Autoinhibition of Jak2 tyrosine kinase is dependent on specific regions in its psuedokinase domain. *Mol. Biol. Cell* **14,** 1448-1459.

114. Bernards, A. (1991) Predicted Tyk2 protein contains two tandem protein kinase domains. *Oncogene* **6,** 1185-1187.

115. Higgins, D.G. Thompson, J.D., and Gibson, T.J. (1996) Using CLUSTAL for multiple sequence alignments. *Methods in Enzymology* **266,** 383-402.

116. Kampa, D. and Burnside, J. (2000) Computational and functional analysis of the putative SH2 domain in Janus kinases. *Biochem. Biophys. Res. Comm.* **278,** 175-182.

117. Giordanetto, F. and Kroemer, R.T. (2002) Prediction of the structure of human Janus kinase 2 (JAK2) comprising JAK homology domains 1 through 7. *Protein Engineering* **15,** 727-737.

118. Girault, J.A., Labesse, G., Mornon, J., and Callebaut, I. (1998) Janus kinases and focal adhesion kinases play in the 4.1 band: a superfamily of band 4.1 domains important for cell structure and signal transduction. *Mol. Med.* **4,** 751-769.

119. Leto, T.L. and Marchesi, V.T. (1984) A structural model of human erythrocyte protein 4.1. *J. Biol. Chem.* **259,** 4603-4608.

120. Banville, D., Ahmad, S., Stocco, R., and Shen, S.H. (1994) A novel protein-tyrosine phosphatase with homology to both the cytoskeletal proteins of the band 4.1 family and junction-associated guanylate kinases. *J. Biol. Chem.* **269,** 22320-22327.

121. Schaller, M.D., Borgman, C.A., Cobb, B.S., Vines, R.R., Reynolds, A.B., and Parsons, J.T. (1992) pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. (USA)* **89,** 5192-5196.

122. Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T., Tsukita, S., and Tsukita, S. (1998) Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. *J. Cell. Biol.* **140,** 885-895.

123. Frank, S.J., Gilliland, G., Kraft, A.S., and Arnold, C.S. (1994) Interaction of the growth hormone receptor cytoplasmic domain with the JAK2 tyrosine kinase. *Endocrinology* **135,** 2228-2239.

124. Tanner, J.W., Chen, W., Young, R.L., Longmore, G.D., and Shaw, A.S. (1995) The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. *J. Biol. Chem.* **270,** 6523-6530.

125. Zhao, Y.M., Wagner, F., Frank, S.J., and Kraft, A.S. (1995) The amino-terminal portion of the JAK2 protein kinase is necessary for binding and phosphorylation of the granulocyte-macrophage colony-stimulating factor receptor beta c chain. *J. Biol. Chem.* **270,** 13814-13818.

126. Sayeski, P.P., Ali, M.S., Frank, S.J., and Bernstein KE (2001) The angiotensin II-dependent nuclear translocation of Stat1 is mediated by the Jak2 protein motif 231YRFRR. *J. Biol. Chem.* **276,** 10556-10563.

127. Chen, M., Cheng, A., Chen, Y.Q., Hymel, A. Hanson, E.P., Kimmel, L., Minami, Y. Taniguchi, T., Changelian, P.S., and O'Shea, J.J. (1997) The amino terminus of Jak3 is necessary and sufficient for binding to the common gamma chain and confers the ability to transmit interleukin 2-mediated signals. *Proc. Natl. Acad. Sci. (USA)* **94,** 6910-6915.

128. Kohlhuber, F., Rogers, N.C., Watling, D., Feng, J., Guschin, D., Briscoe, J., Witthuhn, B.A., Kotenko, S.V., Pestka, S., Stark, G.R., Ihle, J.N., and Kerr, I.M. (1997) A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol. Cell. Biol.* **17,** 695-706.

129. VanderKuur, J.A., Wang, X., Zhang, L., Campbell, G.S., Allevato, G., Billestrup, N., Norstedt, G., and Carter-Su, C. (1994) Domains of the growth hormone receptor required for association and activation of JAK2 tyrosine kinase. *J. Biol. Chem.* **269,** 21709-21717.

130. Sandberg, E.M., Ma, X., Vonderlinden, D., Godeny, M.D., and Sayeski, P.P. (2004) Jak2 tyrosine kinase mediates angiotensin II-dependent inactivation of ERK2 via induction of mitogen-activated protein kinase phosphatase 1. *J. Biol. Chem.* **279,** 1956-1967.

131. Anderson, N.G., Maller, J., Tonks, N.K., and Sturgill, T.W. (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature.* **343**, 651-653.

132. Crews, C., Alessandrini, A., and Erikson, R.L. (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* **258**, 478-480.

133. Kosako, H., Gotoh, Y., Matsuda, S., Ishikawa, M., Nishida, E. (1992) Xenopus MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. *EMBO*. **11**, 2903-2908.

134. Wu, J., Harrison, J.K., Vincent, L.A., Haystead, C., Haystead, T.A.J., Michel, H., Hunt, D.F., Lynch, K.R., and Sturgill, T.W. (1993) Molecular structure of a protein-tyrosine/threonine kinase activating p42 mitogen-activated protein (MAP) kinase: MAP kinase kinase. *PNAS*. **90**, 173-177.

135. Takahasi, T., Kawahara, Y., Okuda, M., Ueno, H., Takeshita, Y., and Yokoyama, M. (1997) Angiotensin II stimulates mitogen-activated protein kinases and protein synthesis by a Ras-independent pathway in vascular smooth muscle cells. *J. Biol. Chem.* **272**, 16018-16022.

136. Pearson, G., Robinson, T.B.G., Xu, B., Karandikar, M., Berman, K., and Cobb, M.H. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine Reviews*. **22**(2), 153-183.

137. Keyse, S.M. (2000) Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Curr. Opin. In Cell Biol*. **12**, 186-192

138. Brondello, J.M., Pouyssegur, J., and Mckenzie, F.R. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. (1999) *Science*. **286**, 2514-2517.

139. Wang, X-J., Fuhrer, D.K., Marshall, M.S., and Yang, Y-C. (1995) Interleukin-11 induces complex formation of Grb2, Fyn, and JAK2 in 3T3L1 cells. *J. Biol. Chem.* **47**, 27999-28002.

140. Winston, L.A., and Hunter, T. (1995) JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. *J. Biol. Chem.* **52**, 30837-30840.

141. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884-888.

142. Voisin, L., Foisy, S., Giasson, E., Lambert, C., Moreau, P., and Meloche, S. (2002) EGF receptor transactivation is obligatory for protein synthesis stimulation by G protein-coupled receptors. *Amer. J. Physiol.; Cell Physiol*. **283**, C446-455.

143. Gschwind, A., Prenzel, N., and Ullrich, A. (2002) Lysophosphatidic acid-induced squamous cell carcinoma cell proliferation and motility involves epidermal growth factor receptor signal transactivation. *Cancer Res*. **62**, 6329-6336.

144. Salazar, E.P., Hunger-Glaser, I., and Rozengurt, E. (2003) Dissociation of focal adhesion kinase and paxillin tyrosine phosphorylation induced by bombesin and lysophosphatidic acid from epidermal growth factor receptor transactivation in Swiss 3T3 cells. *J. Cell Physiol*. **194,** 314-324.

145. Zhong, M., Yang, M., and Sanborn, B.M. (2003) Extracellular signal-regulated kinase 1/2 activation by myometrial oxytocin receptor involves G alpha (q) G beta gamma and epidermal growth factor receptor tyrosine kinase activation. *Endocrinology* **144**, 2947-2956.

146. Sano, H., Zhu, X., Sano, A., Boetticher, E.E., Shioya, T., Jacobs, B., Munoz, N.M., and Leff, A.R. (2001) Extracellular signal-regulated kinase 1/2-mediated phosphorylation of cytosolic phospholipase A2 is essential for human eosinophil adhesion to fibronectin. *J. Immunol*. **166**, 3515-3521.

147. Zhen, X., Torres, C., Cai, G., and Friedman E. (2002) Inhibition of protein tyrosine/mitogen-activated protein kinase phosphatase activity is associated with D2 dopamine receptor supersensitivity in a rat model of Parkinson's disease. *Mol. Pharm*. **62**, 1356-1363.

148. Marshall, C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80,** 179-185.

149. Agell, N., Bachs, O., Rocamora, N., and Villalonga, P. (2002) Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin. *Cell Signal*. **14,** 649-654.

150. Adachi, T., Kar, S., Wang, M., and Carr, B.I. (2002) Transient and sustained ERK phosphorylation and nuclear translocation in growth control. *J. Cell. Physiol*. **192,** 151-159.

151. Ruchatz, H., Coluccia, A.M., Stano, P., Marchesi, E., and Gambacorti-Passerini, C. (2003) Constitutive activation of Jak2 contributes to proliferation and resistance to apoptosis in NPM/ALK-transformed cells. *Exp. Hematol*. **31,** 309-315.

152. De Vos, J., Jourdan, M., Tarte, K., Jasmin, C., and Klein, B. (2000) JAK2 tyrosine kinase inhibitor tyrphostin AG490 downregulates the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways and induces apoptosis in myeloma cells. *Br. J. Haematol*. **109,** 823-828.

153. Rao, G.N., and Berk, B.C. (1992) Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ. Res.* **70**, 593-599.

154. Sotnikova, R. (1998) Investigation of the mechanisms underlying H2O2-evoked contraction in the isolated rat aorta. *Gen. Pharm.* **31**, 115-119.

155. Li, P.F., Dietz, R., and von Harsdorf, R. (1997) Differential effect of hydrogen peroxide and superoxide anion on apoptosis and proliferation of vascular smooth muscle cells. *Circulation* **96**, 3602-3609.

156. Geng, Y.J., and Libby, P. (1995) Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme. *Am. J. Pathol.* **147**, 251-266.

157. Isner, J.M., Kearney, M., Bortman, S., and Passeri, J. (1995) Apoptosis in human atherosclerosis and restenosis. *Circulation* **91**, 2703-2711.

158. Hattori, R., Maulik, N., Otani, H., Zhu, L., Cordis, G., Engleman, R.M., Siddiqui, M.A., and Das, D.K. (2001) Role of STAT3 in ischemic preconditioning. *J. Mol. Cell. Cardiol.* **33**, 1929-1936.

159. Naderi, J., Hung, M., Pandey, S. (2003) Oxidative stress-induced apoptosis in dividing fibroblasts involves activation of p38 MAP kinase and over-expression of Bax: resistance of quiescent cells to oxidative stress. *Apoptosis* **8**, 91-100.

160. Sarosi, L.A., Rieber, M.S., and Rieber, M. (2003) Hydrogen peroxide increases a 55-kDa tyrosinase concomitantly with induction of p53-dependent p21 waf1 expression and a greater Bax/Bcl-2 ratio in pigmented melanoma. *Biochem. Biophys. Res. Commun.* **312**, 355-359.

161. Desagher, S., and Martinou, J-C. (2000) Mitochondria as the central control point of apoptosis *Trends in Cell Biology* **10**, 369-377.

162. Katoh, I., Tomimori, Y., Ikawa, Y., and Kurata, S.I. (2004) Dimerization and processing of procaspase-9 by redox stress in mitochondria. *J. Biol. Chem.* **279,** 15515-15523.

163. Yamakawa, H., Ito, Y., Naganawa, T., Banno, Y., Nakashima, S., Yoshimura, S., Sawada, M., Nishimura, Y., Nozawa, Y., and Sakai, N. (2000) Activation of caspase-9 and -3 during H2O2-induced apoptosis of PC12 cells independent of ceramide formation. *Neurol. Res.* **22**, 556-562.

164. Li, J., Li, W., Su, J., Liu, W., Altura, B.T., and Altura, B.M. (2003) Hydrogen peroxide induces apoptosis in cerebral vascular smooth muscle cells: possible relation to neurodegenerative diseases and strokes. *Brain Res. Bull.* **62**, 101-106.

BIOGRAPHICAL SKETCH

Eric Sandberg was born on May 12, 1978 in Ft. Lauderdale, FL. He grew up in South Florida and attended Chaminade-Madonna College Preparatory, where he was first inspired to pursue a career in science by his high school Chemistry teacher, Marcia Colon. He earned his Bachelor of Science degree in microbiology and cell science at the University of Florida, in May 2000. During his undergraduate years, Eric conducted research in plant molecular biology, under the supervision of Dr. William B. Gurley and his graduate student, Shai Lawit. Eric began pursuing his Ph.D. in biomedical science at the University of Florida College of Medicine in August 2000, under the mentorship of Dr. Peter P. Sayeski. Eric plans to pursue postdoctoral work in cancer biology.