

JAK2 AND SRC FAMILY TYROSINE KINASE SIGNALING VIA THE
ANGIOTENSIN II TYPE 1 RECEPTOR

By

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This dissertation is dedicated to my family, for their constant love, support and guidance

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JAK2 AND SRC FAMILY TYROSINE KINASE SIGNALING VIA THE
ANGIOTENSIN II TYPE 1 RECEPTOR

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The AT₁ receptor is a prototypical G protein-coupled receptor activated through high affinity binding of the hormone, angiotensin II (Ang II). More recent work has demonstrated that the AT₁ receptor can activate tyrosine kinases independent of heterotrimeric G proteins. This dissertation focuses on tyrosine kinase-mediated signaling events downstream of the angiotensin II type 1 (AT₁) receptor. Specifically, the involvement of Src family tyrosine kinases in angiotensin II-induced ERK1/2 activation and cell proliferation is explored. In addition, angiotensin II, Jak2 signaling is explored in detail with respect to Jak2's interaction with the phosphatase, SHP-2. As such, this work provides valuable insight into angiotensin II signaling.

Src family tyrosine kinases mediate as much as 50% of angiotensin II-induced ERK1/2 activation and cell proliferation. The remaining 50% is mediated by heterotrimeric G protein and PKC ζ signaling. In addition, these two signaling cascades activate ERK1/2 and initiate cell proliferation independent of one another. Interestingly,

the cellular consequence associated with ERK1/2 activation by each of these two distinct pathways is different. When ERK1/2 is activated by heterotrimeric G protein and PKC ζ -dependent signaling, it translocates into the nucleus and initiates cellular proliferation through the activation of the transcription factor, elk1. When ERK1/2 is activated by Src kinase-dependent signaling, it remains in the cytoplasm and phosphorylates ribosomal S6 kinase (RSK). Ultimately, RSK translocates into the nucleus and modulates cell proliferation via the activation of the serum response factor (SRF), another known transcription factor. Thus, the cell mediates angiotensin II-induced cell proliferation through the activation of ERK1/2 via two independent signaling pathways.

Jak2 is another tyrosine kinase phosphorylated by angiotensin II. Here, it is demonstrated that angiotensin II, Jak2-dependent signaling requires SHP-2. SHP-2 acts as an adaptor molecule (at the site of the N terminal SH2 domain), serving to recruit Jak2 to the AT₁ receptor via interactions with the Jak2 tyrosine 201 residue. Jak2 then recruits STAT1 and STAT3 proteins, which dimerize and translocate into the nucleus. STAT nuclear translocation initiates the transcription of STAT responsive genes. As such, SHP-2 positively regulates Jak2 signaling in response to Ang II.

Collectively, this work helps to redefine the angiotensin II signaling paradigm, and may aid in the future treatment Ang II-associated diseases.

CHAPTER 1 INTRODUCTION

Overview

Angiotensin II (Ang II) is responsible for a wide array of biological effects which are primarily mediated by the angiotensin II type 1 (AT₁) receptor. The intracellular signaling pathways associated with AT₁ receptor activation relay an Ang II-induced signal from the cell surface to the appropriate intracellular proteins, resulting in a desired cellular outcome. Since the AT₁ receptor is a prototypical G protein-coupled receptor (GPCR), many of these signaling events are dependent upon heterotrimeric G protein-mediated signaling. However, in the 1990's a paradigm shift emerged in the field of angiotensin II signaling when it was discovered that the AT₁ receptor could also activate tyrosine kinases and induce signaling independent of heterotrimeric G proteins. Since then, ongoing work has focused upon the characterization of these tyrosine kinase-mediated signaling events. This dissertation will emphasize two specific tyrosine kinase families activated by the AT₁ receptor: the Janus kinases (JAK) and Src kinases. The following chapter will serve as an introduction to angiotensin II signaling as well as important signaling molecules involved in AT₁ receptor-induced processes, including Janus and Src kinases. The remaining chapters will examine two specific JAK and Src kinase-dependent signaling events. First, the role of Src family tyrosine kinases will be explored in Ang II-induced cell proliferation, and a mechanism will be proposed for this process. Second, the activity of an important Janus kinase family member, Jak2, will be explored with respect to its interactions with a well-known phosphatase, SHP-2. As such,

the following chapters provide information about the signaling mechanisms of two important tyrosine kinase families activated by angiotensin II.

Angiotensin II

History of the Renin Angiotensin System

Angiotensin II was initially identified as a product resulting from the direct cleavage of a plasma substrate (later named angiotensinogen) by the kidney-produced enzyme renin (11, 94). The groups of Braun-Menendez and Page and Helmer independently made this discovery, and named the peptide “hypertensin” and “angiotenin” respectively. Both groups settled on the name “angiotensin” and demonstrated that this peptide was a remarkable inducer of vasoconstriction. Leonard T. Skeggs and colleagues later purified the immediate precursor of Ang II, the 10 amino acid peptide angiotensin I, from hog renin and horse plasma (127). In one study, Skeggs accidentally purified angiotensin I in the presence of 0.15M NaCl, and noticed that an 8 amino acid variant of this protein was formed. This octapeptide turned out to be angiotensin II, which was subsequently shown by Skeggs and colleagues to be produced from the cleavage of angiotensin I by angiotensin-converting enzyme (ACE) (126). Finally, the lung was identified as the tissue-source for ACE (88, 107), and the biochemical pathway known today as the “renin-angiotensin” system was established (Figure 1-1). To this day, angiotensin II remains the primary effector molecule of the renin-angiotensin system.

Physiological Effects of Angiotensin II Associated With Binding of the AT₁ Receptor

The angiotensin II type 1 (AT₁) receptor is regarded as the receptor which mediates the majority of the physiological responses associated with Ang II. It is expressed in a number of tissues, including the vasculature, heart, kidney, lung, adrenal gland, intestine

and brain. Due to the presence of the AT₁ receptor in a number of distinct tissue beds, Ang II is implicated in an array of physiological responses. In the kidney, for example, Ang II increases glomerular filtration rate by stimulating constriction of the efferent arteriole (130). AT₁ receptor stimulation in the adrenal cortex initiates aldosterone synthesis, resulting in the reabsorption of sodium in the distal convoluted tubule of the kidney (92). Ang II also induces sodium reabsorption in the intestine (68), while in the brain Ang II triggers a thirst response by directly stimulating regions of the

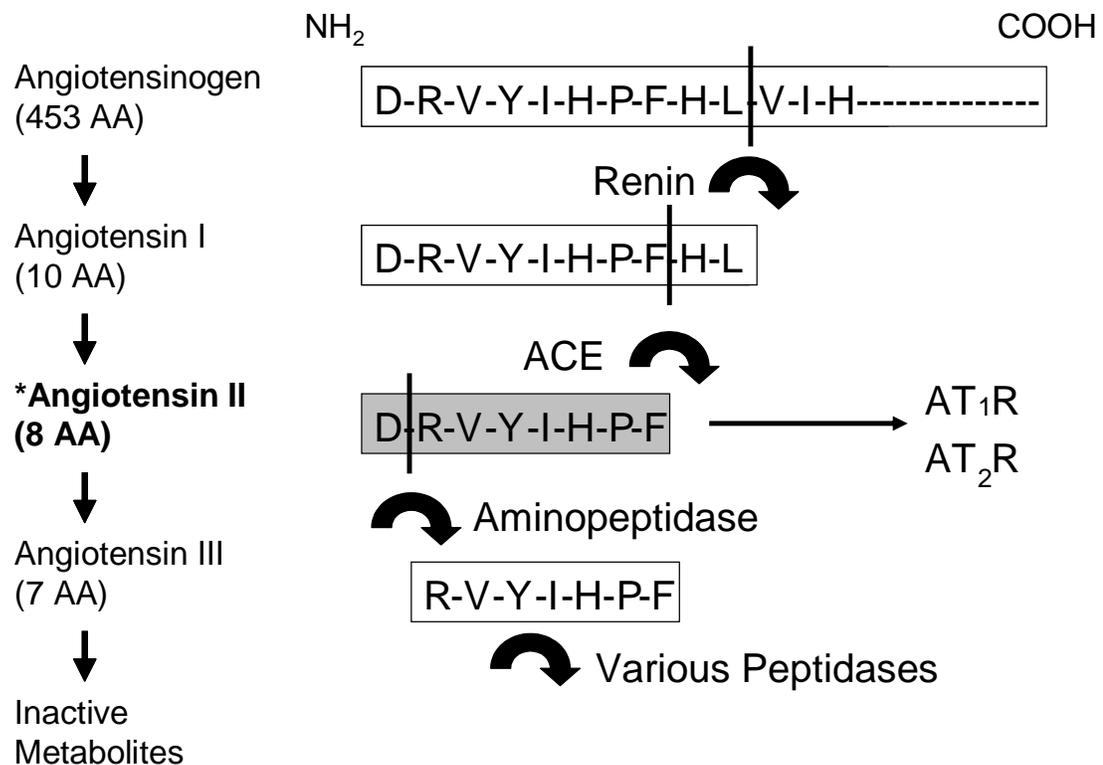


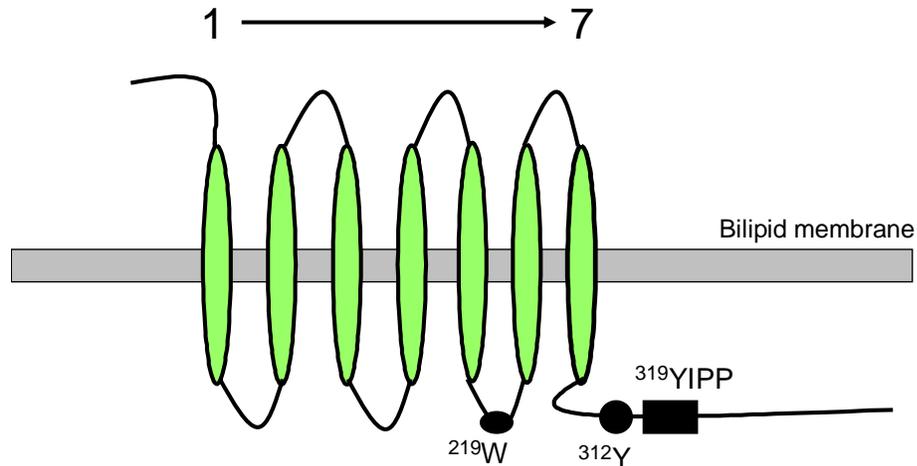
Figure 1-1. Summary of the renin-angiotensin system. Angiotensinogen is cleaved by renin to yield angiotensin I. Angiotensin I is then further cleaved by angiotensin-converting enzyme (ACE) to produce Ang II. Ang II binds to either the AT₁ or AT₂ receptors, or is degraded to angiotensin III and other inactive metabolites by various peptidases.

hypothalamus (99). Finally, Ang II acts as a potent vasoconstrictor within the vasculature (48). These Ang II-induced effects within different tissues collectively help maintain mammalian blood pressure and fluid electrolyte amounts at homeostatic levels. In

addition to its role as a regulator of blood pressure and fluid osmolality, angiotensin II also acts as a potent growth factor. These Ang II-induced cellular growth and proliferative responses are associated with disease states. For example, Ang II has been linked to cardiac hypertrophy. Cardiac hypertrophy is classified as a thickening of the muscles in the wall of the left ventricle to compensate for increases in preload volume, and Ang II has been directly implicated in this condition (25). In addition, Ang II contributes to aberrant vascular smooth muscle cell proliferation during neointimal formation as well as following balloon-injury from angioplasty (55, 93). Finally, Ang II has been linked to angiogenesis during cancer, a process by which new blood vessels form and grow in order to feed rapidly-proliferating tumor cells (24). As such, Ang II is a major contributor to maladaptive growth and proliferative responses associated with cardiovascular diseases and cancer.

Angiotensin II Signaling

The diversity of systemic effects attributed to angiotensin II is in part due to the multitude of intracellular signaling pathways activated by the AT₁ receptor (Figure 1-2). For example, the AT₁ receptor couples to and activates heterotrimeric G proteins, and many downstream signaling events are dependent upon these events. Upon AT₁ receptor activation, the G α subunit binds GTP and dissociates from the G $\beta\gamma$ subunits, allowing each subunit to interact with other signaling molecules. Specifically, angiotensin II can couple to G α_q , which stimulates Phospholipase C beta (PLC β) to convert Phosphatidylinositol 4,5-bisphosphate (PIP₂) to Inositol 1,4,5 Triphosphate (IP₃) and Diacylglycerol (DAG) (113, 151). DAG activates some isoforms of Protein Kinase C (PKC), a serine/threonine kinase that can phosphorylate a number of substrates, while IP₃



<u>Amino Acid Residue</u>	<u>Function</u>
1-309	Src kinase Activation
219-225	Heterotrimeric G protein activation
309-359	IP ₃ release
312-314	Heterotrimeric G protein activation
319-322	Jak2 co-association

Figure 1-2. Diagram of the rat AT_{1a} receptor. The seven transmembrane spanning domains are drawn from left to right as indicated. The positions and functions of amino acid residues important for angiotensin II signaling are also indicated.

binds IP₃ receptors on the endoplasmic reticulum and causes a calcium efflux into the cytoplasm. In addition, the AT₁ receptor can couple to Gβγ, and, in combination with Gα₁₂, activate phospholipase D in vascular smooth muscle cells (147). A final example of AT₁ receptor, heterotrimeric G protein-dependent signaling occurs when Gα_s stimulates adenylate cyclase, which converts ATP to cyclic adenosine monophosphate (cAMP) (135). cAMP then stimulates PKA, an AGC kinase capable of phosphorylating a variety of cellular substrates. Thus, the AT₁ receptor can activate numerous secondary messengers via heterotrimeric G protein-dependent signaling. However, there appears to

be disparity in heterotrimeric G protein-mediated signaling between cell types, which can be explained by cell-specific differences in the expression of α and $\beta\gamma$ subtypes (118).

In addition to being a prototypical GPCR, the AT₁ receptor can also activate a variety of non-receptor associated tyrosine kinases. These include c-Src, Yes, Fyn, Pyk2, Jak2, and FAK (118). The mechanisms of tyrosine kinase activation by the AT₁ receptor are poorly defined in many cases; however, some of the specific amino acid residues on the receptor necessary for tyrosine kinase activation have been identified. Interestingly, many of these residues are different from residues linked to heterotrimeric G protein activation. For example, Jak2 tyrosine kinase has been shown to co-associate with the AT₁ receptor through a specific ³¹⁹YIPP motif on the carboxyl terminal tail (3). In contrast, heterotrimeric G protein activation has been linked to W219-A225 and Y312-L314 motifs on the third intracellular loop and C terminal tail (113, 151). Thus, a number of tyrosine kinases are activated downstream of the AT₁ receptor, and often signal independently of heterotrimeric G proteins.

The JAK Family of Tyrosine Kinases

Structure

The Janus kinase (JAK) family of tyrosine kinases is activated by a number of receptors, including the AT₁ receptor (79). Each member of the Janus family shares the distinct structural feature of having a kinase domain directly adjacent to a pseudokinase domain, and therefore cleverly received their namesake after “Janus,” the Roman god of two opposing faces. Members of the JAK family include Jak1, Jak2, Jak3, and Tyk2. Each of these proteins is approximately 130 kDa in mass, and are structurally similar since they contain seven conserved JAK homology domains. The structure of Jak2 is illustrated as an example of JAK structure (Figure 1-3).

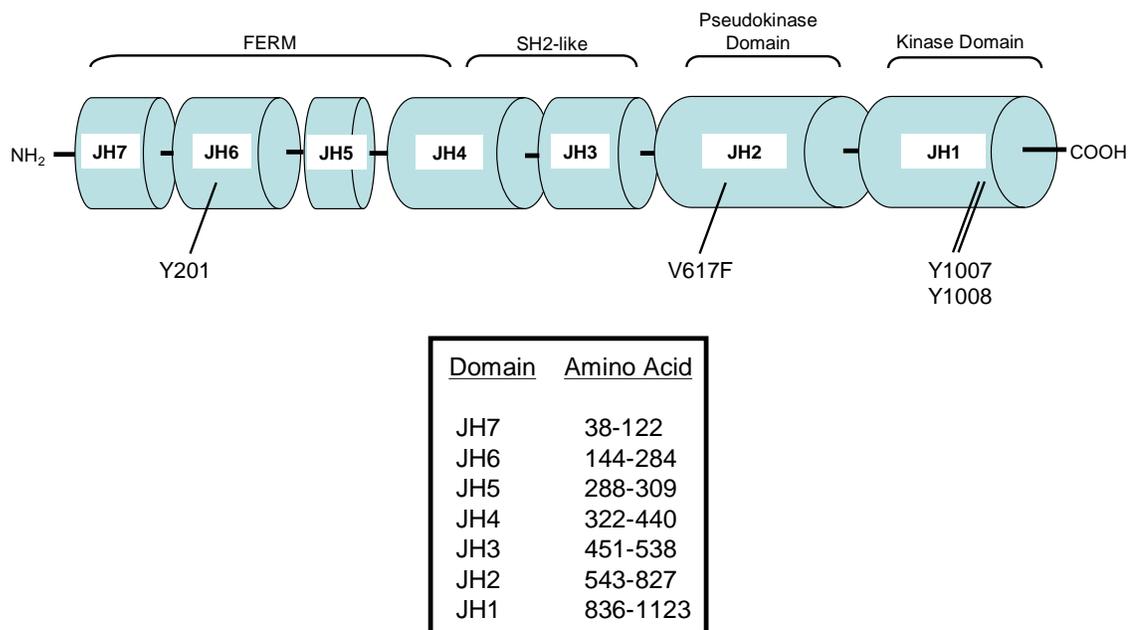


Figure 1-3. Jak2 structural domains. Shown are the positions of the seven-conserved Jak homology (JH) domains as well as the amino acid sequence for each domain within Jak2. Amino acid residues and mutations known to affect Jak2 activity are also indicated. Reproduced with permission from Current Medicinal Chemistry, in press, Copyright 2006 American Chemical Society.

Jak2/STAT Signaling

Of all the JAK family members, Jak2 has been perhaps implicated the most in angiotensin II signaling. Even though Jak2 lacks canonical SH2 and SH3 domains, it is still able to associate with the AT₁ receptor and induce gene transcription. Jak2-dependent gene transcription occurs through the phosphorylation of STAT (signal transducers and activators of transcription) proteins (8, 9, 79). When phosphorylated, STAT proteins form hetero/homodimers and migrate into the nucleus, where they bind STAT recognition sequences within gene promoters. These events trigger STAT-mediated transcription in a variety of early response genes. The Jak2/STAT signaling paradigm was originally identified in the context of cytokine receptors, but more recent

work has established a mechanism by which GPCRs like AT₁ signal through Jak2 (112). The specific events associated with Jak2/STAT signaling are described in detail in Figure 1-4. As such, Jak2 serves as a conduit between angiotensin II binding at the surface of the cell and gene transcription within the nucleus.

The Src Family of Tyrosine Kinases

Structure

The Src family of tyrosine kinases was the first tyrosine kinase family identified. This family of tyrosine kinases is comprised of fourteen different family members, including c-Src, Yes, Fyn, Yrk, Fgr, Lyn, Hck, Lck and Blk. The fourteen different Src kinases are derived from nine separate genes, with alternative splicing accounting for a portion of these gene products. All Src kinase family gene products are similar in size (55 - 62 kDa), and share common structural features, including an SH2 domain, an SH3 domain and a tyrosine kinase domain (Figure 1-5). Three of the Src kinases—c-Src, Yes, Fyn—are ubiquitously expressed, while the expression of other family members is restricted to hematopoietic cells. Due to a common structure and similar expression patterns, there is functional redundancy amongst certain family members, namely c-Src, Yes and Fyn, as demonstrated through the generation of specific knockout mice (62, 131).

c-Src/Yes/Fyn Knockout Mice

Mutations in either the *c-src* or *fyn* genes were shown previously to lead to restricted nonoverlapping phenotypes only in a subset of cells in which these kinases are expressed, while a mutation in the *yes* gene does not lead to an overt phenotype (131). Except for brain, the level or distribution of related Src family kinases is not altered in major tissues, demonstrating that there may be functional redundancy among these Src

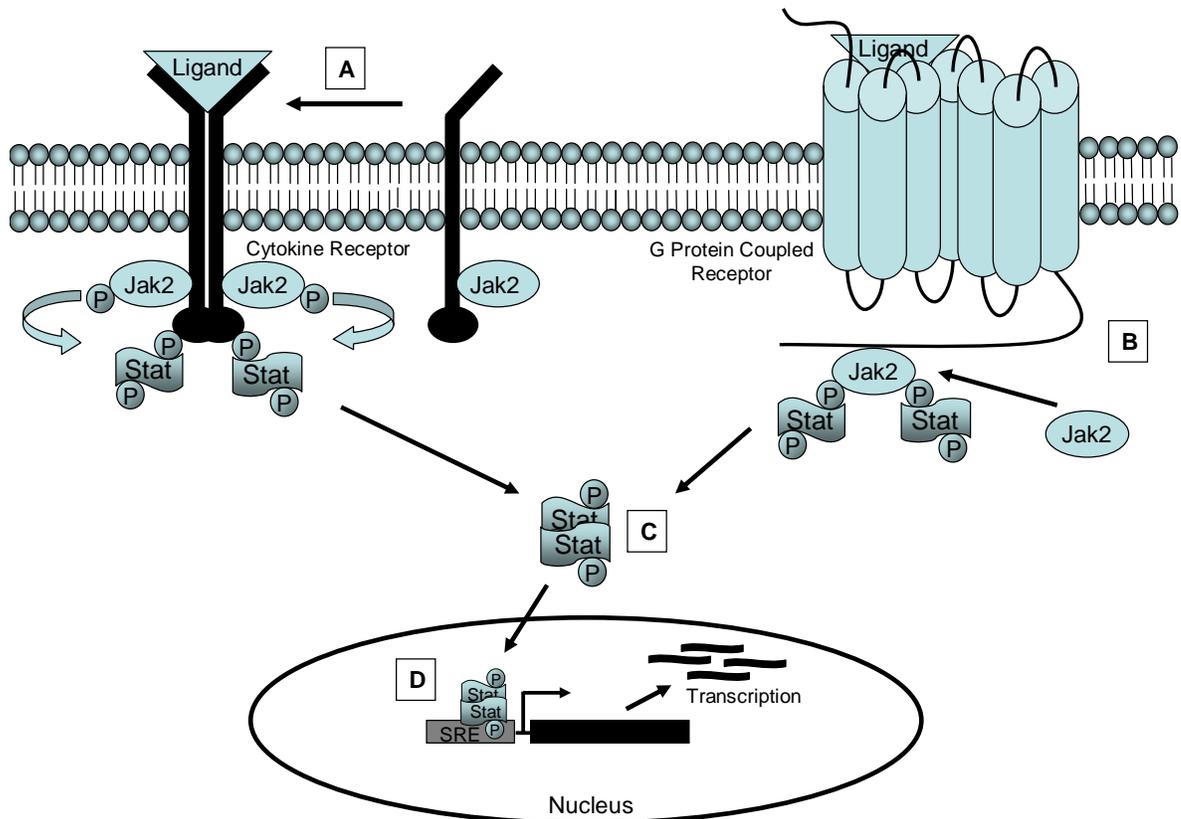
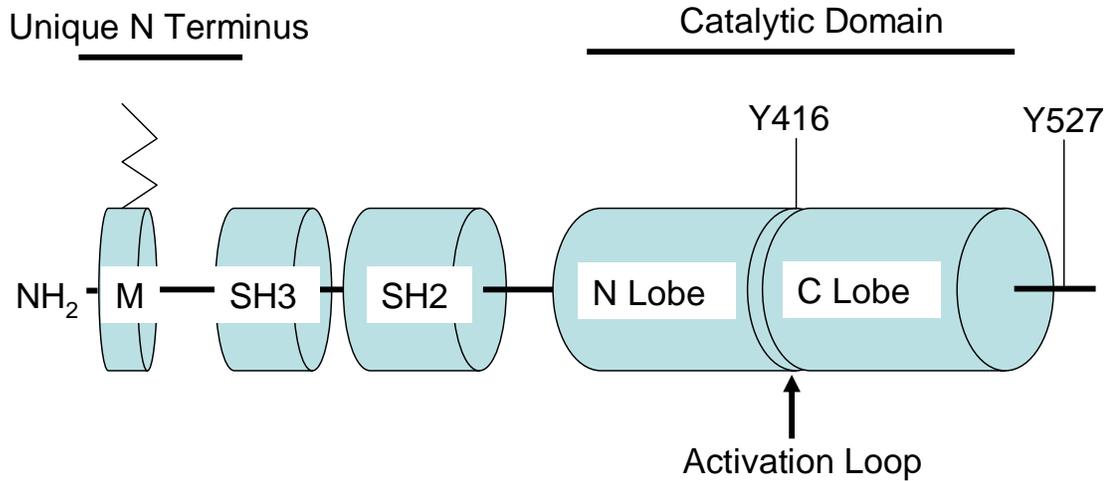


Figure 1-4. The Jak/STAT signaling paradigm. Ligand binding to the receptor initiates receptor activation. For cytokine receptors (A), receptor dimerization triggers Jak2 autophosphorylation, resulting in the Jak2-dependent phosphorylation of STATs. In the case of the AT₁ receptor (B), Jak2 becomes activated in the cytoplasm and is recruited to the receptor, where it phosphorylates the STATs. STAT molecules form homo/hetero dimers upon phosphorylation (C), and translocate into the nucleus. There, STATs bind STAT-responsive elements within gene promoters (D) and initiate transcription. Reproduced with permission from Current Medicinal Chemistry, in press, Copyright 2006 American Chemical Society.

family members. Generation of c-src, yes, or fyn double mutants sought to provide more evidence for this redundancy of function. The src/fyn or src/yes double mutants die perinatally, while a substantial proportion of fyn/yes double mutants are viable but undergo degenerative renal changes leading to diffuse segmental glomerulosclerosis (131). Finally, c-Src/Yes/Fyn triple knockout mice die during development (62). Taken



<u>Region</u>	<u>Function</u>
N terminal myristoylation sequence (M)	Anchors protein to membrane
SH3 domain	Binds proline rich sequences
SH2 domain	Binds phosphorylated tyrosine residues
Catalytic domain	Enzymatic activity; divided into two lobes
Activation loop	Phosphorylation regulated protein activity
C terminal tail	When phosphorylated, binds SH2 domain

Figure 1-5. Structure/function of conserved domains within Src family tyrosine kinases. Shown above are the relative positions of conserved domains and regions characteristic of the Src family members. Conserved phosphorylation sites are also indicated. The putative function of each domain/region is listed in the corresponding table.

together, these data are consistent with the hypothesis that c-Src, Yes, and Fyn tyrosine kinases are able to compensate for the loss of one or more related Src kinases.

AT₁ Receptor-induced Signaling

Like other tyrosine kinases, Src kinases are activated by a wide variety of receptors, including the AT₁ receptor. Work in vascular smooth muscle cells and cardiac myocytes has shown that Src kinases are phosphorylated in response to Ang II treatment (54, 109).

Ang II-induced activation of Src kinases precedes the activation of important downstream signaling events, including the mitogen-activated protein (MAP) kinase (53, 109, 115, 140) and PLC γ 1/IP $_3$ /Ca $^{2+}$ (80) signaling pathways . Work by the Sadoshima laboratory has shown that amino acid residues 1-309 are critical for Src kinase activation and downstream signaling, but not for other Src-independent events such as IP $_3$ release (122). Thus, Src kinases must bind the C-terminal tail of the AT $_1$ receptor in order to become activated. However, the molecular events describing how AT $_1$ receptor activation leads to the activation of Src kinases are not well understood.

ERK1 and ERK2 MAP Kinases

The MAP Kinase Superfamily

MAP kinases are evolutionary conserved enzymes that phosphorylate their substrates on serine/threonine residues. MAP kinase family members are grouped into three sub-families based on their activation sequences: the c-Jun NH $_2$ -terminal kinases (JNKs), the p38 MAP kinases, and the extracellular signal-regulated kinases (ERKs). All of these MAP kinases mediate an intracellular effect in response to extracellular stimuli, although different types of stimuli preferentially activate certain MAP kinases (98). For example, JNKs and p38 MAP kinases are often activated in response to extracellular stress, including UV irradiation, heat shock, osmotic stress and inflammatory kinase stimulation. ERKs are often activated by growth factors and hormones, and mediate different cellular responses, including cell growth, differentiation, proliferation and growth arrest. In addition, the cellular outcome associated with MAP kinase activation will also depend upon the duration and magnitude of activation. For example, transient or sustained patterns of ERK1/2 activation have been shown to differentially affect developmental and adult mammalian cell processes (1, 13, 21, 64, 76, 138).

ERK1/2 Signaling

A general three-tiered signaling cascade describes the activation of all MAP kinases. This starts with the activation of a MAP kinase kinase kinase by a receptor, which phosphorylates a MAP kinase kinase, which in turn phosphorylates a MAP kinase in order to achieve the desired cellular effect. In the case of ERK1/2, the MAP kinase kinase kinase activated is Ras, which phosphorylates Raf (MAP kinase kinase). Raf then phosphorylates MEK1/2, which is the immediate upstream activator of ERK1/2. MEK-induced ERK1/2 phosphorylation occurs via dual threonine and tyrosine phosphorylation at a conserved TEY motif within the activation loop. Activated ERK1/2 then phosphorylates substrates at proline-directed serine and threonine residues. The substrates acted upon by ERK1/2 are often dependent upon the scaffolding proteins bringing the MAP kinase signaling complex together, the ligand-receptor interaction responsible for ERK1/2 activation, the cell type and the presence of substrate within the cell (19, 20). Finally, ERK1/2 signaling is terminated via dephosphorylation by phosphatases. For example, MAP kinase phosphatase 1 dephosphorylates ERK1/2 in a Jak2-dependent manner, effectively shutting off ERK1/2 signaling (111). As such, ERK1/2 signaling occurs in a coordinate manner and is controlled by many proteins. The regulation of ERK1/2 signaling is required in order to control such processes as cell growth, proliferation, differentiation, and growth arrest (19, 105).

Ribosomal S6 kinase

The RSK Family of Proteins

Ribosomal S6 kinase (RSK) is a member of the AGC family of serine/threonine kinases, which also includes protein kinases A, G and C. RSK is the 90 kDa protein within this family, and was originally discovered in *Xenopus laevis* oocytes by Erikson

and Maller in 1985 (35). To date, three isoforms of RSK have been identified (RSK1-3), and each of these proteins are products of separate genes (37). RSK1-3 are approximately 90% identical, and exhibit functional redundancy in tissues where each is expressed, including the brain. Furthermore, RSK homologues are present in a variety of organisms, including human, mouse, rat, chicken, *Drosophila* and *C. elegans*. As such, RSK proteins appear to be physiologically important, which is directly demonstrated by the fact that RSK defects have been linked to human Coffin–Lowry syndrome, a disease characterized by mental retardation, facial and digital dysmorphologies and progressive skeletal malformations (143).

RSK was originally identified for its ability to phosphorylate the 40S ribosomal subunit protein, S6 (36). The phosphorylation of S6 promotes the translation of selected mRNAs important for cell growth. In 1990, however, a 70 kDa S6 kinase (p70^{S6K}) of 60% sequence homology to RSK was identified, and this protein was found to be the primary protein responsible for S6 phosphorylation (6, 7, 17, 63). It is now believed that RSK phosphorylates S6 only under special circumstances, leaving the main function of RSK unknown (37, 38). What is agreed upon is that RSK is a substrate for ERK1/2, although the function of ERK1/2-induced RSK activation is still unknown in most cases, including during AT₁ receptor activation.

Structure and Function of RSK Proteins

The structure of RSK is undoubtedly tied to its function. RSK proteins are unique since they contain both an N terminal and a C terminal kinase domain, connected by a linker region (Figure 1-6). The N terminal kinase domain is responsible for the activation of other kinases, and recognizes the following consensus motifs: Arg/Lys-X-Arg-X-X-

Ser/Thr or Arg-Arg-X-Ser/Thr (66). The C terminal kinase domain is most closely associated with the calcium/calmodulin-dependent group of kinases, and phosphorylation of this domain precedes the activation of the N terminal kinase domain. In addition, phosphorylation of amino acid residues within the linker region has also been shown to play a role in the activation of RSK proteins (37). As such, RSK activation is regulated by ERK1/2 and phosphorylates a variety of substrates containing specific consensus motifs, including transcription factors, cytoplasmic proteins, steroid receptors and ribosomal proteins (Table 1-1).

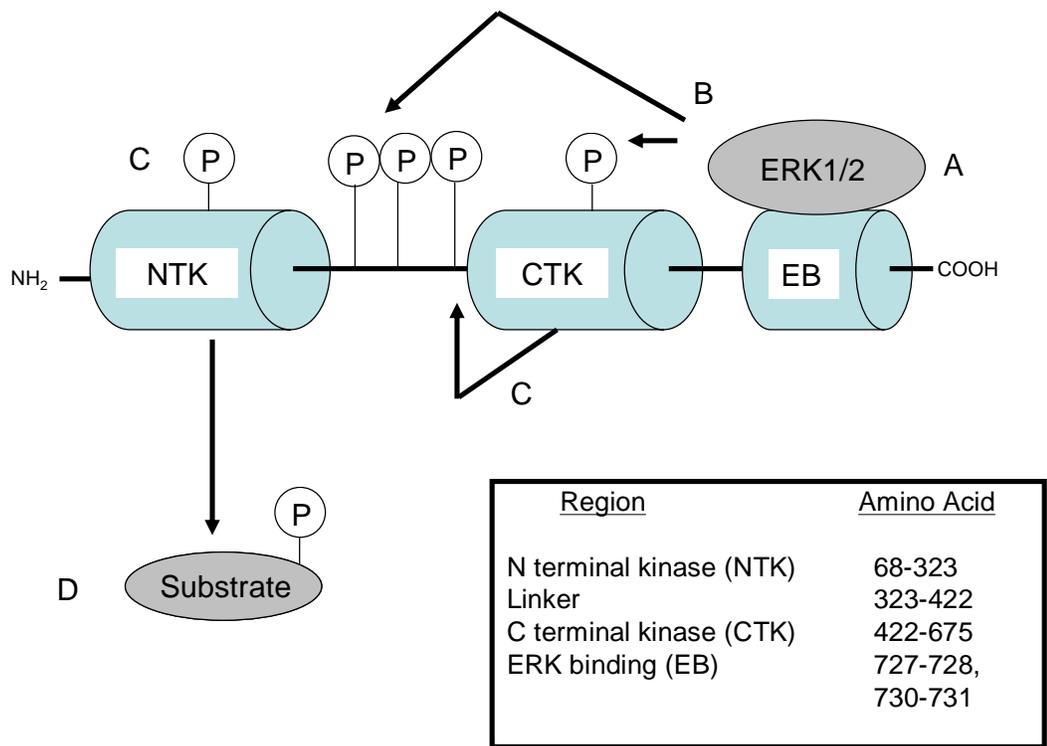


Figure 1-6. Structure and signaling of ribosomal S6 kinase. Shown above are the positions of the N terminal kinase (NTK) domain, the C terminal kinase (CTK) domain, and the ERK-binding (EB) domain. Upon ligand stimulation, ERK1/2 bind to the EB domain (A), which initiates phosphorylation of residues within the CTK and linker region (B). In addition, the CTK phosphorylates the linker region (C). These events are necessary for the activation of the NTK (C), which phosphorylates RSK substrates (D) at specific consensus sequences. Specific amino acid sequences for RSK2 domains and the linker region are indicated.

Table 1-1. Known RSK substrates.

RSK Substrate	Category	Reference
Glycogen synthase kinase 3	Cytoplasmic protein	(34, 139)
Sos	Cytoplasmic protein	(29)
L1 cell adhesion molecule	Cell Adhesion	(155)
Polyribosomes	Translation	(5)
I κ B α /NF κ β	Transcription factor	(39, 119)
Estrogen receptor	Steroid receptor	(59)
CREB	Transcription factor	(23, 40, 156)
c-fos	Transcription factor	(14, 15)
CBP	Transcription co-activator	(87)

The Angiotensin II Signaling Paradigm

A number of proteins, including those just listed, are activated in response to angiotensin II. It was originally thought that the ability of angiotensin II to produce either a pressor response or a growth and proliferative response was dependent upon whether the signaling downstream of the AT₁ receptor was mediated by tyrosine kinases or heterotrimeric G proteins. For example, increases in Ang II-induced vasoconstriction have been shown to be dependent upon Ang II-induced calcium release, which occurs in part through heterotrimeric G protein, PLC β signaling (113, 151). On the other hand, many growth and proliferative responses have been linked to Ang II-induced tyrosine kinase activation, primarily during cardiovascular and cancer disease states (24, 47, 65, 82, 100, 134, 140, 141). As such, heterotrimeric G protein and tyrosine kinase-mediated signaling have often been viewed as independent signaling events, determining the cellular outcome associated with AT₁ receptor activation in a given tissue.

More recent work, however, has shown that heterotrimeric G protein and tyrosine kinase signaling events are often interwoven with respect to the AT₁ receptor. For example, work from our group has shown that Jak2 tyrosine kinase regulates intracellular

calcium release (150), a process which is also highly dependent upon heterotrimeric G proteins (51). Furthermore, Ang II-induced IP₃ production has been shown to be dependent on both heterotrimeric G proteins (through PLCβ1) as well as Src tyrosine kinases (via PLCγ1) (80, 113, 151). Finally, this dissertation will focus extensively on the mechanisms of Ang II-induced cell proliferation, which (as described later) is both heterotrimeric G protein-dependent and tyrosine kinase-dependent. Thus, the portrayal of AT₁ receptor signaling as occurring through two types of linear cascades with no interaction seems oversimplified, since some signaling events are facilitated by interaction between both heterotrimeric G protein and tyrosine kinase signaling. Clearly, more work needs to be done in order to understand the complexity behind angiotensin II signaling.

Summary and Rationale

Angiotensin II signaling occurs in part through heterotrimeric G protein as well as tyrosine kinase-mediated signaling events. Of the kinase-induced signaling pathways, many are reliant upon members of the Janus and Src families of tyrosine kinases. Since Ang II initiates a multitude of tissue-specific effects, it is important to understand how these tyrosine kinases regulate the signaling associated with such processes. In addition, it is also important to understand how JAK and Src kinase activity is regulated since these proteins essentially serve as the “on” switch for many AT₁ receptor-driven signaling processes.

Angiotensin II has already been linked to both cardiovascular disease and cancer, and the AT₁ receptor is currently a target for the treatment of these disease states (24, 31, 49, 60, 137). Not surprisingly, Jak2 and Src kinases have also been implicated in many of these same disease states, and are also promising candidates for therapeutic

intervention (47, 65, 82, 100, 134, 140, 141). The goal of this study is to provide a better understanding of Src family tyrosine kinase and Jak2 tyrosine kinase function with respect to the AT₁ receptor. Specifically, the role of c-Src/Yes/Fyn in angiotensin II-induced cell proliferation will be examined. In addition, the role of SHP-2 in Jak2 signaling will also be explored. Since Src and Janus kinases play such a pivotal role in angiotensin II signaling, it is the hope that the knowledge generated from this work will aid in the treatment of Ang II-associated diseases.

CHAPTER 2
C-SRC/YES/FYN TYROSINE KINASES MEDIATE A PORTION OF ANGIOTENSIN
II-INDUCED ERK1/2 ACTIVATION AND CELLULAR PROLIFERATION

Introduction

Src family tyrosine kinases mediate an array of signaling processes in response to high affinity binding of angiotensin II to the AT₁ receptor (118). Included in this list is the activation of a pro-mitotic MAP kinase signaling cascade, resulting in the phosphorylation of intracellular ERK1/2. Berk *et al* have demonstrated that Ang II-induced ERK1/2 activation is critically mediated by c-Src in vascular smooth muscle cells (53). The authors claim that Ang II-induced ERK1/2 activation was blocked by each of the following: pharmacological inhibition using broad tyrosine kinase inhibitors (CP-188,556 or Genistein), c-Src knockout VSMCs or retroviral transduction of dominant-negative c-Src into rat VSMCs. Thus, they conclude that ERK1/2 activation is completely dependent upon c-Src.

While it is clear from these studies that c-Src is indeed implicated in Ang II-induced ERK1/2 activation, an essential role for c-Src in these signaling events may be over-interpreted. This is demonstrated by the fact that complete inhibition of ERK1/2 activation was only achieved when cells were pretreated with 100 μM of pharmacological inhibitor, while only partial inhibition of ERK1/2 activation occurred in c-Src *-/-* mouse VSMCs or in rat VSMCs transduced with dominant-negative c-Src. These findings also raise speculation as to whether the complete inhibition of Ang II-induced ERK1/2 activation was achieved through the simultaneous, non-specific inhibition of other

signaling proteins via the pharmacological inhibitors utilized in these experiments. In addition, it is not clear if the remaining ERK1/2 activation present in the c-Src dominant-negative transduced VSMCs or c-Src $-/-$ VSMCs was due to functional redundancy by other Src kinases expressed within these cells, incomplete inhibition of c-Src function or activation of ERK1/2 by c-Src-independent signaling. Therefore, the dependency of Ang II-induced ERK1/2 activation on Src family tyrosine kinases is still in question.

Other work by Schiffrin and colleagues has demonstrated a critical role for c-Src in growth signaling by angiotensin II in VSMCs from arteries of hypertensive patients (140). This growth response occurred through the c-Src-dependent activation of ERK1/2 in response to Ang II treatment, and thus implicated c-Src in growth and proliferative effects associated with Ang II. Work done by our group identified a mechanism whereby c-Src activates ERK1/2 via coupling to the Shc/Grb2/Sos signaling cascade, leading to the activation of RAS in response to Ang II (115). However, it should be noted that in each of these reports, ERK1/2 activation was not completely achieved through c-Src inhibition, again raising the question that Ang II-induced ERK1/2 activation is also occurring through other Src kinases or through Src kinase-independent signaling.

Additional work has shown that other Src family tyrosine kinases are also implicated in Ang II-induced ERK1/2 activation. Sadoshima and Izumo demonstrated that in cardiac myocytes, Ang II activates Fyn and stimulates the association of Shc with Fyn and the subsequent formation of a Shc-Grb-Sos complex (109). These authors further demonstrated that Fyn activates Ras through the formation of this complex, thus leading to ERK1/2 activation. As such, Fyn can also activate ERK1/2 in response to angiotensin II. In addition, the Src kinase Yes cannot be ruled out as a mediator of Ang

II-induced ERK1/2 activation due to a similar structure, expression pattern, and already proven ability to compensate for a loss of related Src kinase function (131). Thus, it is apparent that c-Src is not the only Src family tyrosine kinase mediating ERK1/2 activation in response to Ang II, as suggested from previous studies.

Clearly, more work needs to be done in order to determine the contribution that all Src family tyrosine kinases have on Ang II-induced ERK1/2 activation since previous reports have generated conflicting results. This is likely due to the fact that ERK1/2 activation was not examined in a completely Src kinase-deficient background. Here, c-Src/Yes/Fyn-deficient mouse embryonic fibroblast (MEF) cells stably transfected with the AT₁ receptor were utilized. These cells will be described in detail later in this chapter, and provide a powerful system for identifying the dependency of Ang II-induced ERK1/2 activation on Src family tyrosine kinases because they completely lack functional c-Src/Yes/Fyn (62). Therefore, these cells lack the ubiquitously expressed Src kinases present in mammalian cells, and also lack the remainder of Src family kinases since the expression of these proteins is restricted to cells of hematopoietic lineage. As such, the possibility of compensation for the loss of one Src kinase by another is eliminated by the use of the c-Src/Yes/Fyn-deficient cells.

From these studies, it was found that Src kinases mediate approximately 50% of angiotensin II-dependent ERK1/2 activation. It appears that other signaling processes mediate the remainder of ERK1/2 activation independent of Src kinases. Interestingly, a loss of c-Src/Yes/Fyn did not affect the ability of ERK1/2 to translocate into the nucleus, but did cause a reduction in Ang II-induced cell proliferation. As such, c-Src/Yes/Fyn-activated ERK1/2 mediates cell proliferation independent of nuclear translocation. These

findings therefore provide valuable insight into signaling via the AT₁ receptor and the intracellular events associated with Ang II-induced cell proliferation.

Materials and Methods

Creation of WT/AT₁ and SYF/AT₁ Stable Cell Lines

Immortalized SYF and WT MEF cells were a gift from Dr. Philippe Soriano, and were previously isolated from c-Src/Yes/Fyn triple knockout and WT mice respectively at E9.5 (62). Both cell lines lack endogenous AT₁ receptor expression, and were therefore stably transfected by our group with 20 µg of an HA-tagged AT₁ receptor wild type cDNA plasmid as previously described (111). The AT₁ receptor used for transfection has an HA-tag present after the methionine initiation sequence. Two days after transfection, the cells were switched to medium supplemented with 500 µg/ml Zeocin (Invitrogen) to select for stable transfectants. Surviving colonies were ring cloned, and AT₁ receptor binding assays were performed using [¹²⁵I-Sar1,Ile8] angiotensin II (PerkinElmer Life Sciences) as described previously (3). Nonspecific binding was defined as binding in the presence of 1.0 µM unlabelled angiotensin II. Scatchard analysis was used to identify respective WT/AT₁ and SYF/AT₁ clones in which the binding parameters were similar.

Cell Culture and Reagents

WT/AT₁ and SYF/AT₁ cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (Hyclone), 1 mM sodium pyruvate, 10 units/mL penicillin, 10 µg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES and 100 µg/mL Zeocin. WT/AT₁ and SYF/AT₁ cells were growth arrested in serum-free DMEM for 48 hours prior to experiments. PP2 was obtained from Calbiochem and used at concentrations found to have maximum inhibitory

effect (122). Cells were pretreated with inhibitor for the indicated time and stimulated with 100 nM Ang II as described. All cell culture reagents were obtained from Invitrogen. All other reagents were obtained from SIGMA or Fisher.

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blot analysis were performed in order to assess protein expression and phosphorylation in the indicated cells. To prepare whole cell protein lysates, cells were washed in two volumes of ice-cold PBS containing 1 mM Na_3VO_4 and lysed in 0.8 ml ice-cold RIPA buffer (20 mM Tris [pH 7.5], 10% glycerol, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mM EDTA, 50 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 4 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 10 $\mu\text{g}/\text{mL}$ aprotinin). The samples were then sonicated at a medium power setting and incubated on ice for 30 min. Samples were centrifuged at 13,200 rpm for 5 min at 4°C. Supernatants were normalized for protein content using the Bio-Rad Dc assay. Normalized lysates were then either directly resuspended in SDS sample buffer and separated by SDS-PAGE for Western blot analysis or immunoprecipitated.

Immunoprecipitations were performed for 2 hrs at 4°C using 2 μg of the indicated antibody and 20 μL of Protein A/G Plus agarose beads (Santa Cruz Biotechnology). Following immunoprecipitation, samples were centrifuged for 2 min at 7,000 x g. Protein bound A/G beads were then washed in wash buffer (25 mM Tris [pH 7.5], 150 mM NaCl and 0.1% Triton X-100). Samples were washed a total of three times, and resuspended in SDS sample buffer. Protein-A/G bead complexes were boiled for 10 min in order to separate bound proteins. Samples were separated by SDS-PAGE, and transferred onto nitrocellulose membranes.

Whole cell lysates or immunoprecipitates were Western blotted with the indicated antibody for each experiment. Antibodies were used at a final concentration of 1:1000 in 5% milk/TBST plus sodium azide. Membranes were subsequently stripped for 18 min, and then reprobed with the indicated antibody to confirm equal protein loading of all samples. Proteins were detected using enhanced chemiluminescence following the manufacturer's instructions (Amersham).

Antibodies

The cocktail of anti-ERK1/2(P) antibodies were from Promega and Santa Cruz Biotechnology. Note that these antibodies employed in the cocktail recognize the same phospho-tyrosine residues, and were used in order to increase signal:noise ratio. The anti-ERK1/2, the anti-MEK1/2, the anti-MEK1/2(P) and the anti-PKC ζ antibodies were from Santa Cruz Biotechnology. The anti-phosphotyrosine antibody (PY20) was from BD Transduction Laboratories. The immunoprecipitating anti-PLC γ antibody was from Santa Cruz Biotechnology. The immunoprecipitating anti-GFP antibody was from Cell Signaling Technology.

Immunofluorescence

GFP-ERK2 plasmid was kindly provided by Philip J.S. Stork (50). WT/AT₁ and SYF/AT₁ cells were plated onto four-chambered slides (Lab-Tek) and grown to 50% confluency. The cells were washed one time with PBS (pH 7.4) to remove dead cells and debris. Cells were transfected for 5 hrs with 10 μ g of GFP-ERK2 plasmid using Lipofectin (Invitrogen) and following the manufacturer's instructions. The medium was replaced with serum-containing medium and incubated at 37°C for two days. Cells were washed twice in PBS and starved for 48 hr in serum-free medium. Following starvation, all cells were ligand-treated with 100 nM Ang II for 0, 5 or 10 min. Cells were rinsed

once in PBS and fixed in 4% paraformaldehyde for 10 min. Slide chambers were removed and the slides were dipped twice into chilled PBS. Excess PBS was drained from each slide and a coverglass was mounted to each slide using Vectashield + DAPI mounting medium (Vector Labs). The edges of the slide were sealed with nail polish (Maybelline LLC). Slides were viewed on a Zeiss Axioplan II Fluorescence microscope.

Measurement of Cellular ATP Levels

WT/AT₁ and SYF/AT₁ cells were plated onto 100 mm culture dishes and grown to 80% confluency. Cells were serum-starved for 48 hr and then treated with 100 nM Ang II. Cellular ATP levels were assessed using the ViaLight HS proliferation/cytotoxicity kit (Cambrex) following the manufacturer's protocol. A luminometer (Monolight model 2030) was used to measure bioluminescence.

Measurement of Formazan Production

WT/AT₁ and SYF/AT₁ cells were plated onto 96 well plates and grown to 80% confluency. Cells were then starved for 48 hr in serum-free medium and treated with 100 nM Ang II as indicated. Formazan production was measured using the CellTiter 96 Aqueous One Solution Reagent (Promega) following the manufacturer's instructions. Production of formazan was proportional to increased absorbance at 490 nm as measured by spectrophotometry.

Cell Count

WT/AT₁ and SYF/AT₁ cells were plated onto 100 mm culture dishes and grown to 80% confluency. The cells were then serum-starved and treated with 100 nM Ang II as indicated. Both cell types were then counted using a hemacytometer as described (122).

Densitometric Analysis

Western blots were scanned and densitized using UnScanIt Gel Analysis (Silk Scientific). The average pixel value minus background was obtained for each cell type and normalized to the average pixel value for the respective non-Ang II-treated cells.

Statistical Analysis

Data were analyzed by two-way ANOVA. All data passed a Normality Test as well as Equal Variance Test. Pairwise comparisons were made following the Holm-Sidak method. All data are expressed as mean +/- SEM of replicates from three independent experiments. * = $p < 0.05$, ** = $p < 0.01$.

Results

Characterization of WT/AT₁ and SYF/AT₁ Cells

Previous reports have indicated that c-Src is a critical mediator of intracellular ERK1/2 activation (53, 115, 140). The role of Src kinases in intracellular ERK1/2 activation was first explored, specifically in response to angiotensin II using c-Src/Yes/Fyn-deficient (SYF) and wild type (WT) MEF cells. These SYF fibroblasts were previously isolated at E9.5 from a developing c-Src/Yes/Fyn-deficient mouse embryo and have been shown to be completely devoid of these proteins (62). MEF cells containing functional c-Src/Yes/Fyn were also isolated from WT littermates and served as controls (WT cells).

Both the SYF and WT cells do not endogenously express the AT₁ receptor (data not shown). Therefore, the AT₁ receptor was stably transfected into both cell types to constitute angiotensin II signaling. These AT₁ receptor stable cell lines have been named SYF/AT₁ and WT/AT₁ respectively. Saturation binding studies were then performed in order to identify respective SYF/AT₁ and WT/AT₁ clones in which the binding

parameters were similar. SYF/AT₁ (clone #16) and WT/AT₁ (clone #2) both had a K_D of 0.4 nM and a B_{max} of 140-150 fmol/mg protein (Figure 2-1A). To demonstrate that these two cell lines were similar in all aspects other than the levels of c-Src/Yes/Fyn, the expression levels of Jak2 and STAT3, two non-Src kinase dependent genes, were examined. It was found that these two genes were expressed at similar levels in the two cell types (Figures 2-1B and 2-1C). Next, the ability of Ang II to activate PLCγ1 was examined in WT/AT₁ and SYF/AT₁ cells. It was found that both cell types were capable of increasing PLCγ1 tyrosine phosphorylation levels to roughly equal levels, an indication that the AT₁ receptor can signal similarly in both cell types when examining signaling events independent of c-Src/Yes/Fyn (Figure 2-1D). Collectively, these data suggest that the WT/AT₁ and SYF/AT₁ cells are similar in all aspects except for c-Src/Yes/Fyn expression and signaling pathways that are dependent on these three proteins.

Angiotensin II-induced ERK1/2 Activation Is Reduced By About 50% in Src Kinase Deficient Cells

Ang II-induced ERK1/2 activation was next assessed in WT/AT₁ and SYF/AT₁ cells. Cells were stimulated with 100 nM Ang II for 0, 5 and 10 min, and ERK1/2 activity assessed via Western blot. Ang II-dependent ERK1/2 activation was decreased in SYF/AT₁ cells when compared to WT/AT₁ cells after 5 and 10 min of Ang II treatment (Figure 2-2A). Furthermore, Ang II-induced ERK1/2 activation was reduced in WT/AT₁ cells pretreated with the Src family kinase inhibitor, PP2, to levels comparative to Ang II-stimulated SYF/AT₁ cells (Figure 2-2B). Finally, ERK1/2 activation in the SYF/AT₁ cells was partially restored by transiently-transfecting these cells with c-Src (Figure 2-

2C). Taken together, these results demonstrate that Src kinases mediate a portion of Ang II-induced ERK1/2 activation.

It was next determined whether reductions seen in SYF/AT₁ cell ERK1/2 activation were indeed due to the loss of c-Src/Yes/Fyn and not due to clonal artifact. Previous

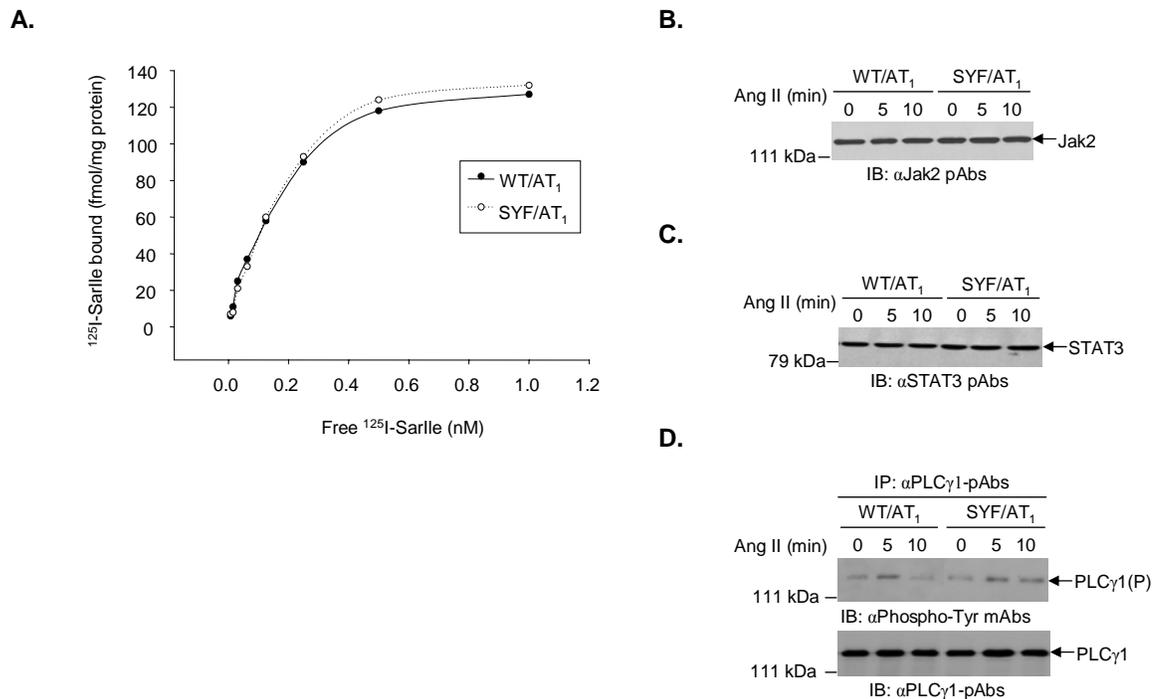


Figure 2-1. Characterization of WT/AT₁ and SYF/AT₁ cells. A: Saturation binding curve for WT/AT₁ and SYF/AT₁ cells. B–D: WT/AT₁ and SYF/AT₁ cells were stimulated with 100 nM Ang II for 0, 5, and 10 min, and whole cell lysates were prepared. B & C: Cell lysates were Western blotted with the indicated antibodies. D: Cell lysates were immunoprecipitated (IP) and then Western blotted with the indicated antibodies (top panel). Western blots were then stripped and reprobed with the indicated antibody to demonstrate equal protein loading. This figure is used with permission from (42).

work in MEF cells showed that PDGF activates ERK1/2 in a c-Src/Yes/Fyn-independent manner (62). Therefore, both WT/AT₁ and SYF/AT₁ cells were stimulated with 30 ng/ml PDGF for 0, 5, and 10 min. All cells were then lysed, and total protein extract was immunoblotted with anti-active ERK1/2 pAbs. Contrary to Ang II treatment, stimulation with PDGF resulted in a similar activation of ERK1/2 in both the WT/AT₁ and SYF/AT₁

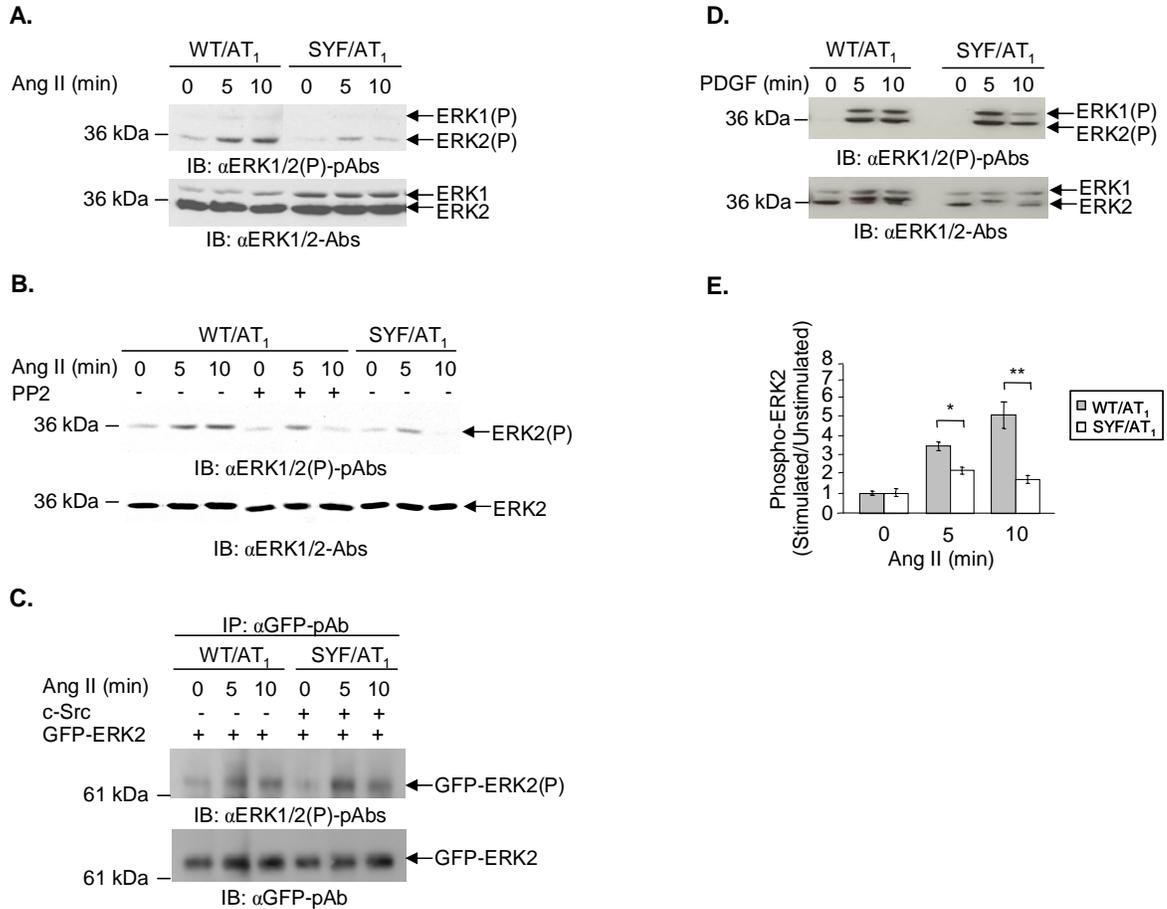


Figure 2-2. Quantification of ERK1/2 activation in response to Ang II in WT/AT₁ and SYF/AT₁ cells. A: WT/AT₁ and SYF/AT₁ cells were treated with 100 nM Ang II, and ERK1/2 activation assessed via Western blot analysis B: WT/AT₁ and SYF/AT₁ cells were pretreated with 30 μM PP2 or DMSO for 60 min, and stimulated with 100 nM Ang II. Active ERK1/2 levels were assessed via Western blot analysis using the indicated antibodies. C: SYF/AT₁ cells were co-transfected with a plasmid encoding GFP-ERK2 and a c-Src-encoding plasmid or empty vector control as indicated. Cells were stimulated with 100 nM Ang II, and whole cell lysates immunoprecipitated with GFP antibody. ERK1/2 activation was assessed via Western blot. D: Cells were pretreated with 30 ng/mL PDGF, and ERK1/2 activation assessed via Western blot. E: Quantification of active ERK2 amounts from A. Fold changes in active ERK2 in response to Ang II treatment were calculated by dividing average ERK2 pixel density in Ang II-treated cells by average pixel density in non-treated controls. This figure is used with permission from (42).

cells (Figure 2-2D). Thus, while SYF/AT₁ cells are capable of activating ERK1/2 to the same degree as WT/AT₁ cells in response to PDGF, the decreased ERK1/2 activation

seen in SYF/AT₁ cells in response to Ang II is in fact due to the specific absence of c-Src/Yes/Fyn-dependent signaling.

Quantification of band densities from 2-2A revealed that decreased ERK1/2 phosphorylation in the SYF/AT₁ cells was statistically significant (Figure 2-2E). Furthermore, maximum Ang II-induced ERK1/2 activation in the SYF/AT₁ cells was observed after 5 min of Ang II treatment, whereas maximum ERK1/2 activation occurred 10 min post-Ang II treatment in the WT/AT₁ cells. Maximum ERK1/2 phosphorylation levels were reduced by about 50% in the SYF/AT₁ cells when compared to peak levels of ERK1/2 phosphorylation in the WT/AT₁ cells. As such, c-Src/Yes/Fyn tyrosine kinases mediate at most 50% of Ang II-induced ERK1/2 activation since roughly half of Ang II-activated ERK1/2 activation persists in their absence.

Angiotensin II-induced ERK1/2 Nuclear Translocation Is Not Dependent Upon Src Kinases

Clearly, Src/Yes/Fyn-dependent signaling is responsible for a portion of Ang II-induced ERK1/2 activation. The effect of a loss of c-Src/Yes/Fyn signaling on Ang II-induced ERK1/2 nuclear translocation was next assessed. Previous reports have shown that ERK1/2 translocates into the nucleus and initiates gene transcription of early response genes via the phosphorylation of specific transcription factor targets (98). Other work has shown that ERK1/2 nuclear translocation is dependent upon heterotrimeric G protein signaling in response to Ang II (122). Therefore, it was next examined whether the elimination of c-Src/Yes/Fyn and therefore a loss of about 50% of Ang II-induced ERK1/2 activation would also affect ERK1/2 nuclear translocation.

WT/AT₁ and SYF/AT₁ cells were transfected with a GFP-ERK2 plasmid in order to track ERK2 movement in response to Ang II treatment. Cells were then stimulated

with 100 nM Ang II, fixed and DAPI stained to visualize the nucleus. In the absence of Ang II, GFP-ERK2 was distributed fairly evenly between both the nucleus and cytoplasm in WT/AT₁ and SYF/AT₁ cells (Figures 2-3A, 2-3B and 2-3G). DAPI counterstain of these images and merging of the GFP and DAPI images confirmed these findings (Figures 2-3D, 2-3E and 2-3J). In contrast, ERK1/2 accumulation was markedly increased in the nucleus of both WT/AT₁ and SYF/AT₁ cells treated with Ang II (Figures 2-3C, 2-3 H and 2-3I), and this finding was confirmed by DAPI counterstain (Figures 2-3F, 2-3K and 2-3L). As such, it appeared that ERK1/2 nuclear translocation was present in both Ang II-stimulated WT/AT₁ and SYF/AT₁ cells. However, there was no statistically significant difference in nuclear fluorescence between Ang II-stimulated SYF/AT₁ cells when compared to WT/AT₁ controls. As such, it appears that c-Src/Yes/Fyn do not influence ERK1/2 nuclear translocation. Quantification of nuclear fluorescence relative to cytoplasmic fluorescence revealed a significant increase in ERK1/2 nuclear fluorescence in both WT/AT₁ and SYF/AT₁ cells stimulated with Ang II, indicative of increased nuclear translocation (Figures 2-3M and 2-3N).

Angiotensin II Induced Cell Proliferation Is Reduced in Src Kinase Deficient Cells

Ang II-induced ERK1/2 activation has been shown to initiate cell proliferation (33, 89, 90, 133). It has primarily been thought that this occurs through the translocation of ERK1/2 into the nucleus and the subsequent initiation of growth response gene transcription (122). Here, it has been demonstrated that Ang II-induced ERK2 nuclear translocation is unaffected by the loss of c-Src/Yes/Fyn-mediated ERK1/2 activation

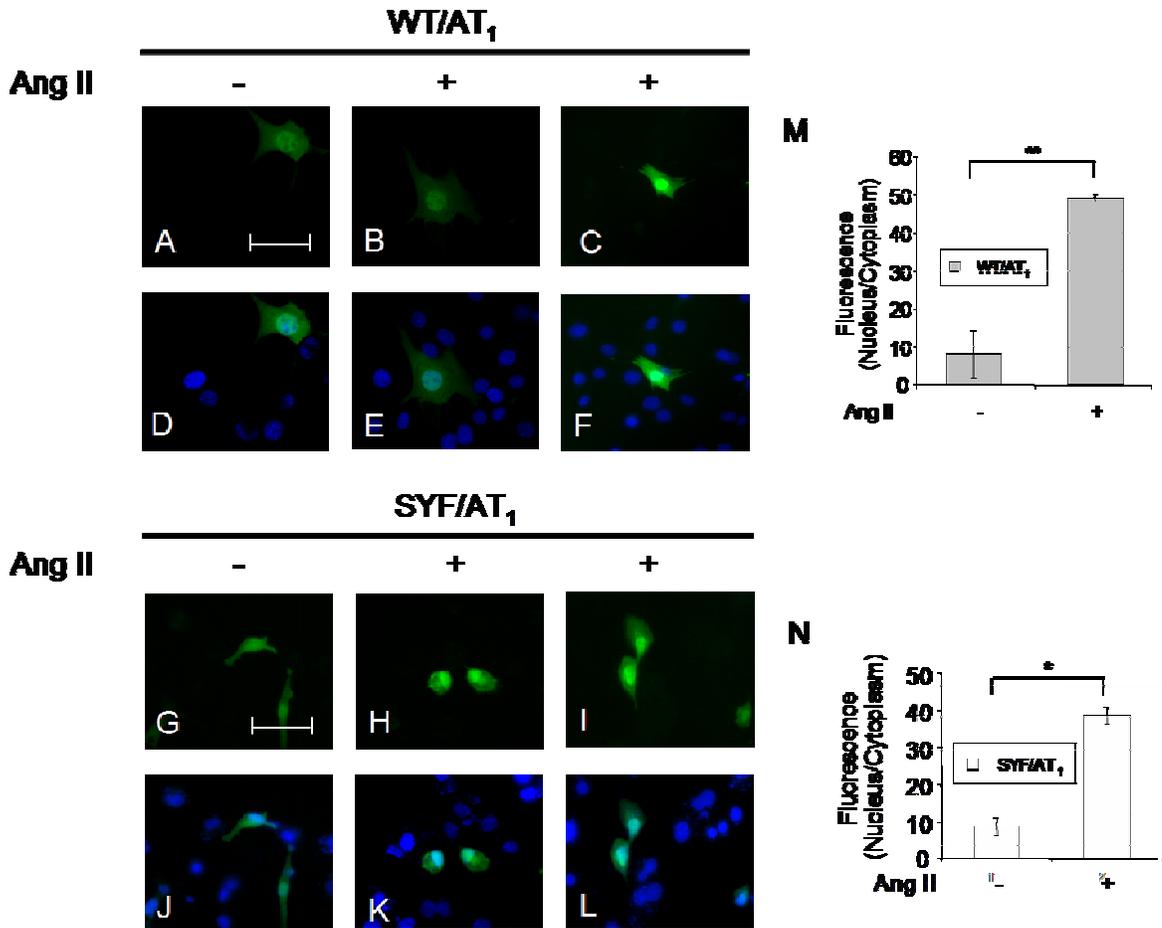


Figure 2-3. Nuclear translocation of active ERK2 is unaffected by the loss of c-Src/Yes/Fyn. A - C, G - I: WT/AT₁ or SYF/AT₁ cells were transfected with a GFP-ERK2 plasmid and then stimulated with Ang II for 0 and 10 min. Nuclear translocation of ERK2 was assessed by fluorescent microscopy. A - C: GFP-ERK2 images in non-treated and Ang II-treated WT/AT₁ cells. G - I: Merging of images A - C respectively with DAPI stained images. G - I: GFP-ERK2 images in non-treated and Ang II-treated SYF/AT₁ cells. J - L: Merging of images G - I respectively with DAPI stained images. M: Nuclear fluorescence from A - C was quantified and normalized to cytoplasmic fluorescence. N: Nuclear fluorescence from G - I was quantified and normalized to cytoplasmic fluorescence. All images are representative of the entire field and were taken at 40X magnification. Bar represents 15 microns. Shown is one of two independent results. This figure is used with permission from (42).

(Figure 2-3). It was next determined whether eliminating c-Src/Yes/Fyn effects Ang II-induced cell proliferation, independent of the ability of ERK1/2 to translocate into the nucleus.

Ang II-induced cell proliferation was assessed via three different methodologies. First, cellular ATP levels were measured since ATP amounts have previously been reported to be excellent indicators of cell number (22). WT/AT₁ and SYF/AT₁ cells were treated with 100 nM Ang II and intracellular ATP levels were measured. After 4 hours of Ang II treatment, ATP levels had already increased over 3 fold in WT/AT₁ cells (Figure 2-4A). ATP levels in SYF/AT₁ cells increased slightly by about 1.75 fold, but were markedly reduced after Ang II treatment compared to WT/AT₁ controls. These data suggest that cell number was decreased in Ang II-stimulated SYF/AT₁ cells when compared to WT/AT₁ controls and therefore that Ang II-induced cell proliferation was significantly reduced by the elimination of c-Src/Yes/Fyn.

Next, Ang II-induced cell proliferation was assessed through the measurement of formazan levels, which has previously been reported to also be an excellent indicator of increased cell number (12). WT/AT₁ and SYF/AT₁ cells were treated with 100 nM Ang II and then formazan production was assessed. Ang II-induced formazan production was significantly increased in WT/AT₁ cells after 5 hours of Ang II treatment (Figure 2-4B). However, formazan production barely increased in SYF/AT₁ cells treated for 5 hours with Ang II. SYF/AT₁ cell formazan production was significantly decreased relative to WT/AT₁ controls.

Lastly, Ang II-induced cell proliferation was analyzed via direct cell count. WT/AT₁ and SYF/AT₁ cells were treated with 100 nM Ang II for 0 and 24 hr. Cells were

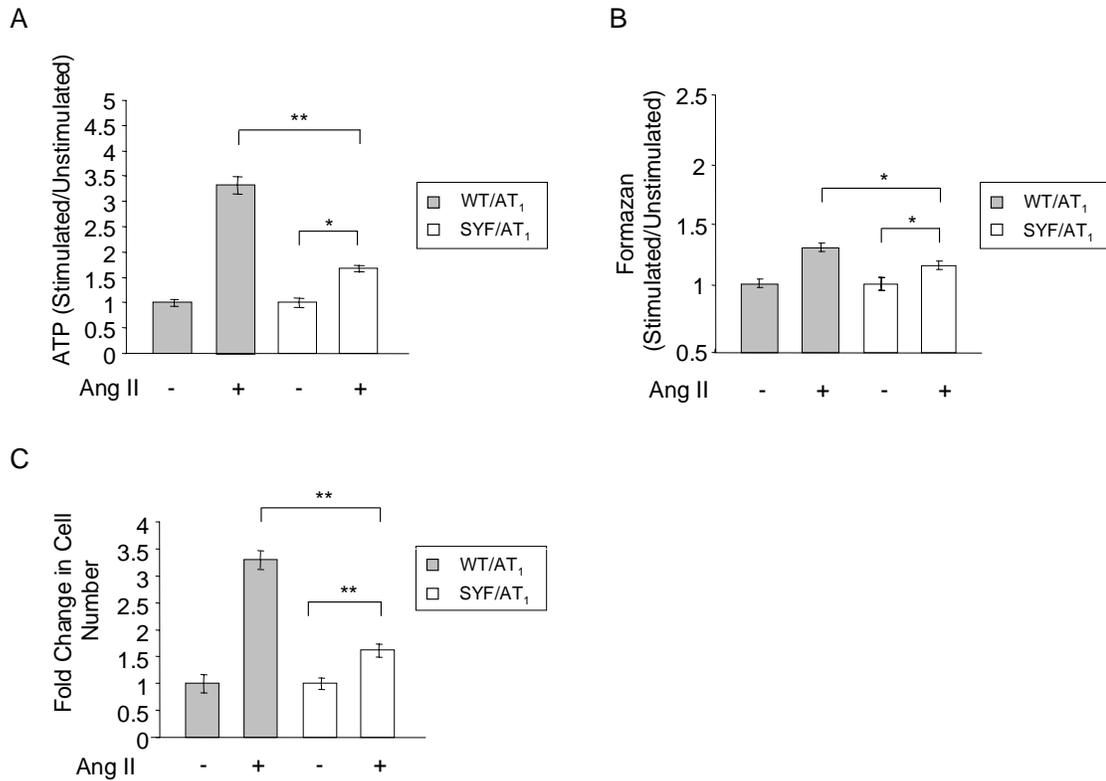


Figure 2-4. Ang II-induced cell proliferation is reduced in Ang II-stimulated SYF/AT₁ cells. A: WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II for 0 and 4 hr. Cellular ATP levels were then measured. Data are expressed as fold change in ATP in Ang II stimulated cells relative to non-treated controls. B: WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II for 0 and 5 hr. Formazan production was then measured. Data are expressed as fold change in formazan in Ang II stimulated cells relative to non-treated controls. C: WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II for 0 and 24 hr. All cells were then detached and counted using a hemacytometer. All data are expressed as a fold change in Ang II-stimulated relative to non-stimulated cells. All data are representative of three independent experiments. WT/AT₁ control cells. Thus, these data also suggest that Ang II-induced cell proliferation is markedly reduced in SYF/AT₁ cells relative to WT controls. This figure is used with permission from (42).

detached and counted using a hemacytometer. WT/AT₁ cell number was increased by over 3 fold when treated with Ang II for 24 hours relative to non-treated controls (Figure 2-4C). SYF/AT₁ cell number also increased, but this increase was significantly reduced when compared to Ang II-treated WT/AT₁ cells. As such, these data show that Ang II-

induced cell proliferation was markedly reduced in SYF/AT₁ cells lacking c-Src/Yes/Fyn-dependent signaling.

In summary, Ang II-induced cell proliferation is reduced when c-Src/Yes/Fyn are eliminated from the cell. Eliminating the ~50% of Ang II-induced ERK1/2 activation dependent upon c-Src/Yes/Fyn therefore alters the ability of these cells to proliferate in response to Ang II. Interestingly, the decrease in Ang II-induced cell proliferation observed in SYF/AT₁ cells occurs independent of the ability of ERK1/2 to translocate into the nucleus. In addition, a portion of cell proliferation is dependent upon c-Src/Yes/Fyn-independent signaling.

Discussion

Here, MEF cells completely devoid of c-Src/Yes/Fyn were utilized. In doing so, all Src kinase function and the possibility that ERK1/2 activation can be mediated via any of these very similar family members has been completely eliminated. It was found that while c-Src/Yes/Fyn tyrosine kinases do play a role in the activation of ERK1/2 as previously reported, ERK1/2 activation is not completely dependent on these proteins and persists at reduced levels in their absence. c-Src/Yes/Fyn are capable of activating only about 50% of intracellular ERK1/2. An explanation for these results is that the remaining 50% of intracellular ERK1/2 are activated by c-Src/Yes/Fyn-independent mechanisms. As such, Ang II-induced ERK1/2 activation occurs through two independent signaling cascades, and is not completely dependent upon Src kinases as previous work has shown (53).

Interestingly, while the Src kinase dependent signaling pathways appears to mediate as much as 50% of Ang II-induced ERK1/2 activation, these signaling events do not influence Ang II-induced ERK1/2 nuclear translocation. It had previously been

thought that ERK1/2 must translocate into the nucleus in order to initiate events necessary for the start of cell proliferation, including the transcription of early response genes such as *c-fos* (14, 15, 109). Interestingly, the loss c-Src/Yes/Fyn had no effect on the ability of ERK1/2 to translocate into the nucleus. ERK1/2 was able to enter the nucleus in the absence of c-Src/Yes/Fyn; however, cell proliferation was still markedly reduced.

Even more striking is the finding that c-Src/Yes/Fyn still influence cell proliferation, independent of ERK1/2 nuclear translocation. An explanation for these findings is that ERK1/2 activated via c-Src/Yes/Fyn-dependent signaling acts upon cytoplasmic proteins to mediate proliferation. Previous work has already shown that ERK1/2 can phosphorylate a number of cytoplasmic substrates, including members of the RSK, MSK and MNK families of proteins (37). Furthermore, many of these proteins have been shown to regulate the activity of transcription factors. For example, RSK has been shown to modulate phosphorylation of both the serum response factor (SRF) as well as CREB, both of which have been shown to be pro-mitotic (37). Therefore, ERK1/2 may also modulate transcription through the phosphorylation of cytoplasmic proteins, which themselves activate transcription factors initiating pro-growth and proliferative events. In addition, ERK1/2 activated via c-Src/Yes/Fyn-independent signaling may translocate into the nucleus to directly mediate transcriptional events (122). These events will be investigated and described in detail in the following chapters.

CHAPTER 3
SRC KINASE-INDEPENDENT ERK1/2 ACTIVATION AND CELL
PROLIFERATION IS MEDIATED BY HETEROTRIMERIC G PROTEINS AND
PKC ζ -DEPENDENT SIGNALING

Introduction

In the previous chapter, it was demonstrated that approximately 50% of Ang II-induced ERK1/2 activation is mediated by Src family tyrosine kinases. In addition, these proteins also mediate a portion of angiotensin II-induced cell proliferation. The remaining 50% of intracellular ERK1/2 activation and portion of Ang II-induced cell proliferation must therefore be occurring through Src kinase-independent signaling. In this chapter, the events underlying Src kinase-independent ERK1/2 activation through the AT₁ receptor will be discussed.

Previous work has implicated numerous proteins other than Src kinases in Ang II-induced ERK1/2 activation. For example, the AT₁ receptor is capable of transactivating other receptors with intrinsic tyrosine kinase activity, including the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), which in turn activate ERK1/2 (32, 74, 85). In addition to receptor transactivation, numerous cytoplasmic kinases have been shown to mediate ERK1/2 activation. Pharmacological inhibition of phosphoinositide 3-kinase (PI3K) blocks ERK1/2 activation in EGF-treated preglomerular smooth muscle cells (4, 45). Furthermore, various isoforms of Protein Kinase C (PKC) have been shown to mediate intracellular ERK1/2 activation in response to different ligands (45, 46, 70, 86). It is not clear from these reports, though, if the mechanism of ERK1/2 activation differs depending upon the receptor activated, or if

ERK1/2 activation occurs simultaneously via multiple independent signaling mechanisms in response to treatment with the same ligand.

Here, we sought to determine whether AT₁ receptor-generated ERK1/2 activation occurs via Src kinase-independent signaling in addition to Src kinase dependent signaling. Specifically, the effect of PDGFR, EGFR, PI3K, PKC, Raf or MEK inhibition on Ang II-induced ERK1/2 activation was first investigated in order to identify a mechanism whereby ERK1/2 activation occurs in a Src kinase-independent manner. In addition, the effect of heterotrimeric G protein and PKC ζ inhibition on Ang II-induced ERK1/2 activation was examined. An attenuation of Src kinase-independent ERK1/2 activation only occurred when heterotrimeric G protein, PKC ζ or MEK activities were inhibited, suggesting that these proteins mediate Ang II-induced ERK1/2 activation independent of Src family tyrosine kinases. In addition, it was found that MEK phosphorylation was dependent upon PKC ζ activity, identifying a mechanism whereby PKC ζ activates ERK1/2 via the upstream activation of MEK. As such, ERK1/2 activation occurs via heterotrimeric G protein/PKC ζ signaling independent of Src family tyrosine kinases in response to AT₁ receptor activation.

It was previously observed that Src kinases mediate about 50% of ERK1/2 activation and a portion of Ang II-induced cell proliferation (Chapter 2). More importantly, Src kinase-activated ERK1/2 mediates cell proliferation without direct translocation of ERK1/2 into the nucleus. Previous work has shown that ERK1/2 translocates into the nucleus to modulate gene transcription in response to Ang II (122). As such, ERK1/2 nuclear translocation must therefore be influenced by Src kinase-independent signaling events. Therefore, whether heterotrimeric G protein/PKC ζ

signaling mediates Ang II-induced ERK1/2 nuclear translocation and cell proliferation was also assessed.

The effect of inhibition of the heterotrimeric G protein/PKC ζ pathway on ERK1/2 nuclear translocation and cell proliferation was determined using a PKC ζ myristoylated pseudosubstrate. In contrast to Src kinases, the nuclear translocation of ERK1/2 is dependent upon PKC ζ activity as pretreatment with the PKC ζ MP abolished Ang II-induced ERK1/2 nuclear translocation. In addition, PKC ζ inhibition also reduced Ang II-induced cell proliferation. Interestingly, PKC ζ inhibition in combination with Src kinase inhibition completely attenuated Ang II-induced cell proliferation. Therefore, ERK1/2 activation and cell proliferation are controlled through both heterotrimeric G protein/PKC ζ -dependent and Src kinase-dependent signaling events in response to angiotensin II. Each of these pathways appears to mediate a portion of this response, but only heterotrimeric G protein/PKC ζ signaling causes ERK1/2 to directly translocate into the nucleus. As such, it appears that Ang II-induced ERK1/2 activation is mediated by two independent signaling cascades operating through distinct mechanisms.

Materials and Methods

Cell Culture

WT/AT₁ and SYF/AT₁ cells were cultured as described in Chapter 2. CHO/AT₁ and CHO/AT₁-M5 cells were cultured in F-12 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 units/mL penicillin, 10 μ g/mL streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES. WT/AT₁ and SYF/AT₁ were growth arrested in serum-free DMEM for 48 h prior to experiments. CHO cells were growth arrested in the same manner for 24 h. All cell culture reagents were obtained from Invitrogen.

Pharmacological Inhibitors

AG1295, AG1478, GDP- β S, Gö6976, Gö6983, LY294002, PD98059, PP2, Raf1 kinase Inhibitor 1 and Rotlerrin were all obtained from Calbiochem and used at concentrations found to have maximum inhibitory effect (32, 46, 74, 85, 89, 117, 122, 140, 148). Sodium fluoride (SIGMA), chelerythrine (LKT Labs), and the PKC ζ myristoylated pseudosubstrate (BioMol) were also used at previously determined concentrations (58, 124, 164). SYF/AT₁ cells were permeabilized with 5 nM saponin (USB) before treatment with GDP- β S (103). All other reagents were obtained from SIGMA or Fisher. Cells were pretreated with inhibitor for the indicated time and stimulated with 100 nM Ang II as described.

siRNA Treatment of WT/AT₁ Cells

siRNA reagents were purchased from Santa Cruz Biotechnology. Cells were grown in 100 mm culture plates (Corning) to 80% confluency. Adherent cells were trypsinized and resuspended in serum-containing medium without antibiotics. Cells and medium were centrifuged at 500 x g for 5 min, and pelleted cells were resuspended in fresh serum-containing medium without antibiotics. Cells were transferred to 6 well culture plates (Corning) and grown to 40–50% confluency. Transfection reagents were prepared as described in the online protocol (<http://www.scbt.com/support/protocols>) with the exception that the concentration of siRNA used was increased four fold. Cells were next transfected for 48 hrs at 37°C with either control siRNA or PKC ζ -specific siRNA in serum-containing medium without antibiotics. Cells were serum-starved for 48 hours and treated with 100 nM Ang II for 0, 5 and 10 min. Cells were lysed and whole cell protein lysates were prepared. Cell lysates were separated on a 10% SDS-PAGE gel,

transferred to a nitrocellulose membrane and Western blotted with the indicated antibodies.

Immunoprecipitation, Western Blotting and Densitometric Analysis

Cells were immunoprecipitated as described in Chapter 2. Proteins were detected using enhanced chemiluminescence as described in Chapter 2. The anti-MEK1/2, the anti-MEK1/2(P) and the anti-PKC ζ antibodies used for Western blotting were from Santa Cruz Biotechnology. Western blots were scanned and densitized using UnScanIt Gel Analysis (Silk Scientific) as described in Chapter 2.

Immunofluorescence and Quantification of Fluorescence

Cells were transfected with GFP-tagged ERK2 and ERK2 movement examined via immunofluorescence as described in Chapter 2. Cells were pretreated with either 1 μ M PKC ζ myristoylated pseudosubstrate or DMSO for 1 hour prior to stimulation with 100 nM Ang II. Nuclear or cytoplasm fluorescence was quantified using the Image J Program (NIH) as described in Chapter 2.

Cell Count

Cell counts were performed using a hemacytometer as described in Chapter 2. Cells were pretreated with either 1 μ M PKC ζ myristoylated pseudosubstrate or DMSO for 1 hour prior to stimulation with 100 nM Ang II.

Statistical Analysis

Data were analyzed by two-way ANOVA. All data passed a Normality Test as well as Equal Variance Test. Pairwise comparisons were made following the Holm-Sidak method. All data are expressed as mean \pm SEM. * = $p < 0.05$, ** = $p < 0.01$.

Results

Src Kinase Independent ERK1/2 Activation Does Not Require EGF Receptor, PDGF Receptor or PI3K Activity

A mechanism for c-Src/Yes/Fyn-independent ERK1/2 activation in response to Ang II was first identified through pharmacological inhibition of candidate proteins. A brief literature search identified proteins which have previously been implicated in ERK1/2 activation in response to various activating ligands (Table 3-1). Pharmacological inhibitors for each of these proteins were then obtained, and SYF/AT₁ cells were treated for the indicated time with concentrations of these inhibitors previously found to suppress protein function, in order to identify proteins that mediate ERK1/2 activation independent of c-Src/Yes/Fyn. Cells were stimulated with 100 nM Ang II and whole cell lysates were Western blotted with phospho-ERK1/2 antibodies to identify changes in active ERK1/2 levels.

Table 3-1. Pharmacological inhibition of Src kinase-independent ERK1/2 inhibition.

Protein	Inhibitor Treatment		
	Inhibitor	Final Concentration	Time (min)
EGFR	AG1478	30 μ M	30
Heterotrimeric G proteins	GDP β S	2 mM	20
MEK	PD98059	50 μ M	60
Raf1	Raf1 kinase 1 inhibitor	30 μ M	60
PDGFR	AG1295	30 μ M	60
PKC	Chelerythrine	30 μ M	60
PI3K	LY294002	30 μ M	60

The involvement of growth factor receptor transactivation on c-Src/Yes/Fyn-independent ERK1/2 activation was first examined. SYF/AT₁ cells were pretreated with either PDGFR (AG1295) or EGFR (AG1478) selective inhibitors, and then stimulated with 100 nM Ang II. No reduction in Ang II-induced ERK1/2 activation was observed

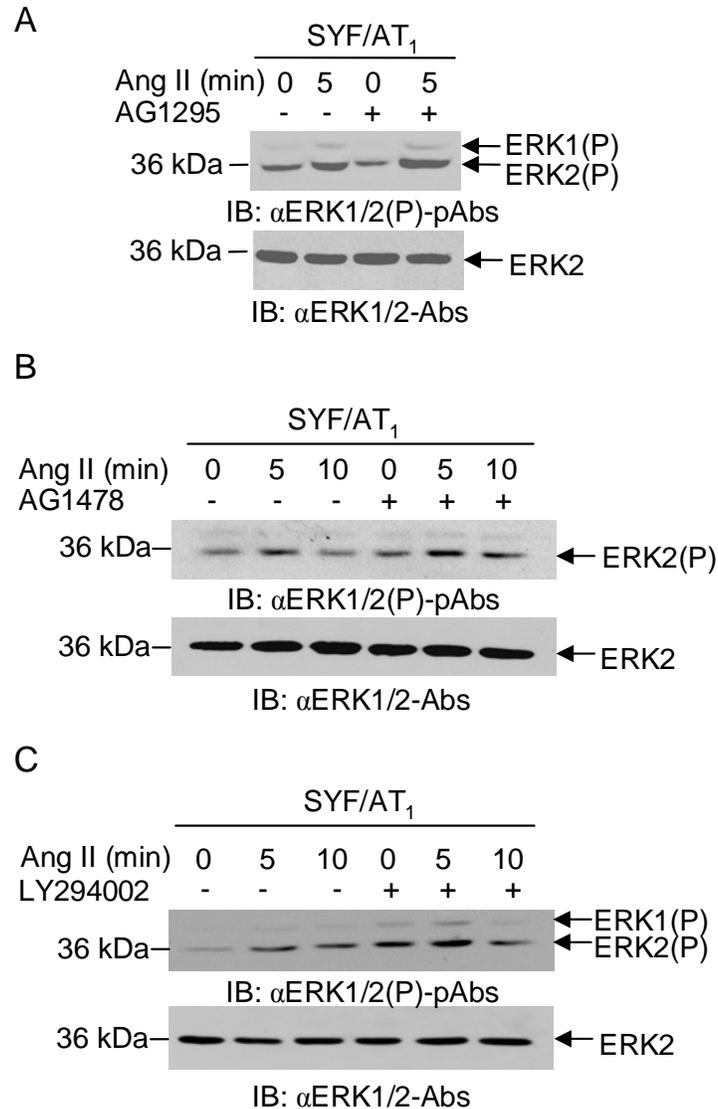


Figure 3-1. ERK1/2 activation in SYF/AT₁ cells does not require transactivation of the PDGFR or the EGFR or activation of PI3K. A – C: SYF/AT₁ cells were pretreated with the indicated pharmacological inhibitors at the concentrations and for the treatment times listed in Table 1. Cells were stimulated with 100 nM Ang II for indicated times, and active ERK1/2 levels were assessed by Western blotting with anti-ERK1/2(P) antibodies (top panels). Total ERK protein loading was demonstrated by stripping the membrane and reprobing with anti-ERK1/2 antibodies (bottom panels). All Westerns are representative of three independent experiments. This figure is used with permission from (42).

in the PDGFR inhibitor-treated SYF/AT₁ cells compared to non-treated controls (3-1A). Similarly, treatment of SYF/AT₁ cells with the EGFR selective inhibitor had no effect on Ang II-induced ERK1/2 activation (Figure 3-1B). Collectively, these data indicate that Ang II-induced ERK1/2 activation occurring through c-Src/Yes/Fyn-independent mechanisms does not require EGFR or PDGFR transactivation.

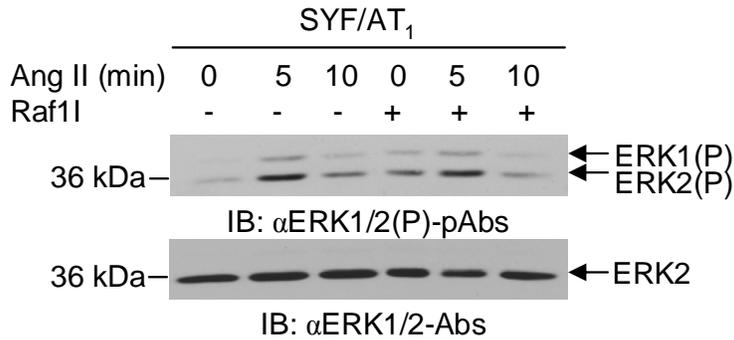
ERK1/2 activation has also previously been reported to occur through PI3K-dependent signaling mechanisms (4, 45, 86). The effect of PI3K inhibition on Ang II-induced ERK1/2 activation was next determined in SYF/AT₁ cells using the PI3K selective inhibitor, LY294002. Control treated SYF/AT₁ cells exhibited the typical ERK1/2 activation response after 5 min of Ang II treatment and no decrease in ERK1/2 activation was observed in LY294002-treated cells (Figure 3-1C). In fact, ERK1/2 activation increased slightly when LY294002 was added to SYF/AT₁ cells. Thus, PI3K does not regulate Ang II-induced ERK1/2 activation independent of c-Src/Yes/Fyn.

Src Kinase Independent ERK1/2 Activation Is Dependent on MEK1/2, But Not Raf1

ERK1/2 activation typically occurs through the activation of a MAPK signaling cascade, in which a MAPK-kinase-kinase (MAPKKK) phosphorylates a MAPK-kinase (MAPKK), which in turn phosphorylate and activate MAPKs such as ERK1/2. The typical MAPKKK involved in ERK1/2 activation is Raf1, while MEK1/2 are the MAPKK which dually phosphorylate ERK1/2. It was next investigated whether pharmacological inhibition of either Raf1 or MEK1/2 would effect Ang II-induced ERK1/2 activation in SYF/AT₁ cells. SYF/AT₁ cells pre-treated with the Raf1 kinase inhibitor exhibited no decrease in ERK1/2 activation when compared to untreated cells (Figure 3-2A). In contrast, Ang II-induced ERK1/2 activation was completely absent from WT/AT₁ cells and SYF/AT₁ treated with the MEK1/2 inhibitor (Figure 3-2B).

Therefore, Ang II-induced ERK1/2 activation occurring via c-Src/Yes/Fyn-independent mechanisms (i.e., in the SYF/AT₁ cells) does not require Raf1. However, all Ang II-induced ERK1/2 activation is dependent upon MEK activation.

A



B

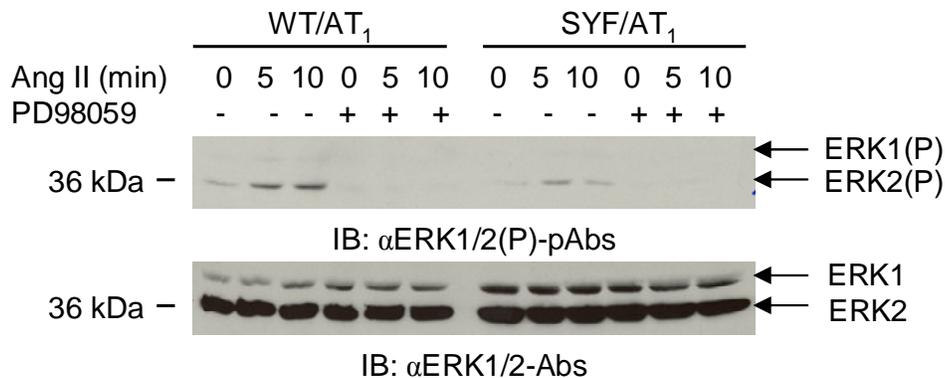


Figure 3-2. ERK1/2 activation in SYF/AT₁ cells requires MEK1/2 activation, but not Raf1 activation. A: WT/AT₁ or SYF/AT₁ cells were pretreated with the indicated pharmacological inhibitors at the concentrations and for the treatment times listed Table 1. Cells were stimulated with 100 nM Ang II for the indicated times, and active ERK1/2 levels were assessed by Western blotting with anti-ERK1/2(P) antibodies (top panels). Total ERK protein loading was demonstrated by stripping the membrane and reprobing with anti-ERK1/2 antibodies (bottom panels). All Westerns are representative of three independent experiments. This figure is used with permission from (42).

In summary, ERK1/2 activation in Ang II-treated SYF/AT₁ cells was not affected when cells were treated with EGFR, PDGFR, PI3K or Raf1 selective pharmacological inhibitors. However, Ang II-induced ERK1/2 activation was attenuated in SYF/AT₁ cells

treated with the MEK1/2 inhibitor. Intracellular ERK1/2 activation occurring independent of c-Src/Yes/Fyn requires upstream MEK1/2 activation in order to dually phosphorylate ERK1/2, but does not depend on AT₁ receptor transactivation of the EGF and PDGF receptors or the phosphorylation of PI3K and Raf1. As such, other proteins must therefore be acting upstream of MEK1/2 in order to activate ERK1/2 in a c-Src/Yes/Fyn-independent manner.

Heterotrimeric G Proteins Mediate A Portion of ERK1/2 Activation In A Src Kinase-independent Manner

Heterotrimeric G proteins have previously been shown to activate ERK1/2 in response to Ang II (122). Whether the remaining 50% of Ang II-induced ERK1/2 activation in the SYF/AT₁ cells was mediated entirely by heterotrimeric G protein signaling was next examined. SYF/AT₁ cells were permeabilized using saponin, and then pretreated with the heterotrimeric G protein inhibitor, GDP-βS (103). The β-phosphate group of this compound has been replaced with a sulfate group, hindering the ability of heterotrimeric G proteins to exchange GDP for GTP and subsequently become activated. Permeabilized SYF/AT₁ cells treated with vehicle control served as controls. Cells were then stimulated with 100 nM Ang II, and ERK1/2 activation assessed via Western blot. SYF/AT₁ cells permeabilized with saponin and treated with vehicle control still demonstrated ERK2 activation in response to Ang II (Figure 3-3A). However, ERK2 activation was attenuated in GDP-βS treated cells since there was no increase in ERK2 phosphorylation levels in Ang II-stimulated cells compared to untreated controls. These data suggest that Ang II-induced activation of ERK1/2 occurring independent of c-Src/Yes/Fyn requires heterotrimeric G protein activation.

The effect of ligand-independent activation of heterotrimeric G proteins on ERK1/2 activation in the SYF/AT₁ cells was next tested. SYF/AT₁ cells were pretreated with NaF, which causes heterotrimeric G proteins to become constitutively activated independent of exogenous ligand addition (58). Cells were then stimulated with 100 nM Ang II, and ERK1/2 activation assessed via Western blot. Basal ERK1/2 activation was significantly increased in SYF/AT₁ cells treated with NaF when compared to untreated cells (Figure 3-3B). Furthermore, the addition of Ang II did not further increase ERK1/2 activation levels, suggesting that Ang II activates ERK1/2 in SYF/AT₁ cells via a mechanism that is dependent upon heterotrimeric G proteins. The addition of EGF, which activates ERK1/2 in a c-Src/Yes/Fyn dependent manner (10), further increased ERK1/2 activation in NaF treated cells (data not shown). These data therefore suggest that Ang II-induced ERK1/2 activation occurring independent of c-Src/Yes/Fyn requires heterotrimeric G protein activation.

To further demonstrate that heterotrimeric G proteins mediate all c-Src/Yes/Fyn-independent ERK1/2 activation in response to Ang II, a previously generated CHO cell line stably transfected with a mutant AT₁ receptor was utilized. This mutant AT₁ receptor contains specific mutations in the carboxyl terminus preventing heterotrimeric G proteins from coupling to the receptor and becoming activated (28). These cells are denoted as CHO/AT₁-M5 cells here, and retain their ability to activate tyrosine kinases such as c-Src/Yes/Fyn. CHO cells stably expressing wild type AT₁ receptor with similar affinity and abundance (denoted as CHO/AT₁) were used as controls. Both cell types were stimulated with 100 nM Ang II, and ERK1/2 activation assessed via Western Blot. Ang II-induced ERK1/2 activation in CHO/AT₁ cells reached maximum levels after 5

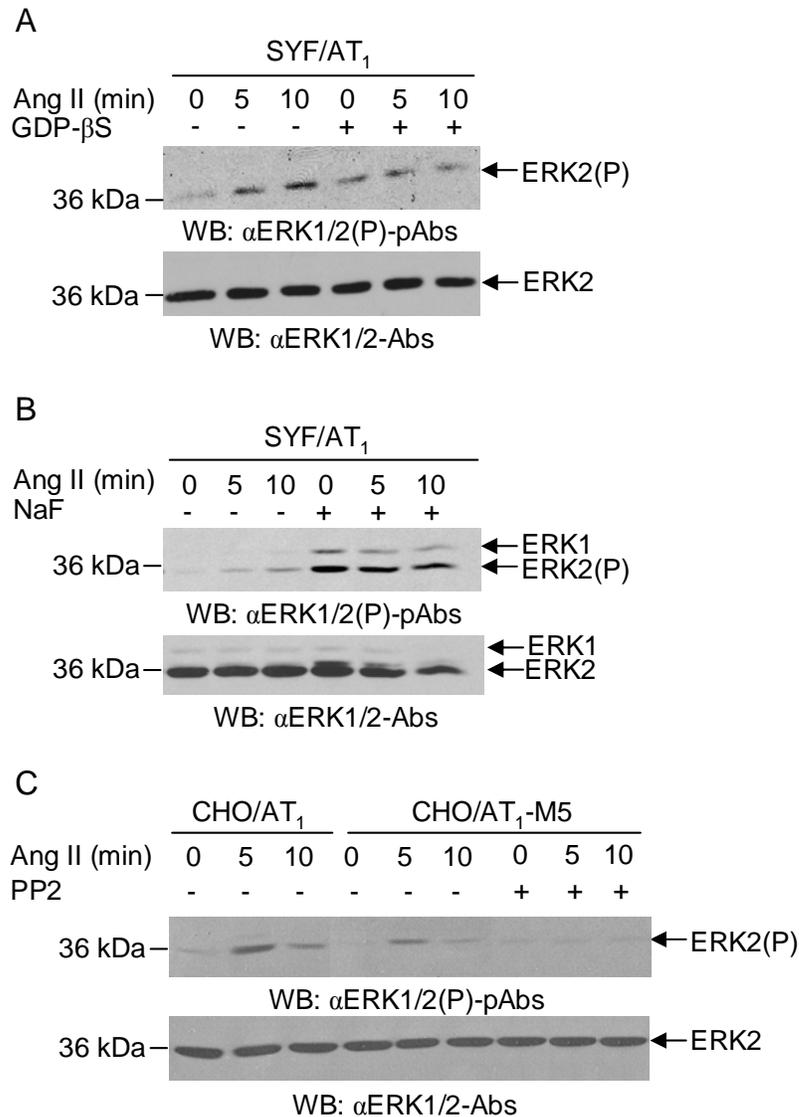


Figure 3-3. Ang II-induced ERK1/2 activation is partially dependent upon heterotrimeric G proteins. A and B: SYF/AT₁ cells were pretreated with either 2 mM GDP-βS for 20 min or 2 mM NaF for 1 hr, respectively, and stimulated with 100 nM Ang II for 0, 5, and 10 min. C: CHO/AT₁ and CHO/AT₁-M5 cells were first pretreated with either 30 μM PP2 or DMSO for 1 hour before Ang II treatment. ERK2 phosphorylation was determined via Western blot using the indicated antibodies. A-C: Total ERK protein loading was demonstrated in each case by stripping the membrane and reprobing with the anti-ERK1/2 antibodies (bottom panels). All Westerns are representative of three independent experiments. This figure is used with permission from (42).

min of Ang II treatment (Figure 3-3C). ERK1/2 activation also occurred in CHO/AT₁-M5 cells in response to Ang II, but maximum ERK1/2 activation was significantly reduced compared to CHO/AT₁ cells. Therefore, Ang II-induced ERK1/2 activation is partially dependent upon heterotrimeric G protein activation. Furthermore, pretreatment of CHO/AT₁-M5 cells with PP2 completely blocked Ang II-induced ERK1/2 activation, indicating that the remaining 50% of ERK1/2 activation is dependent upon Src kinases.

Protein Kinase C ζ Mediates ERK1/2 Activation In A Src Kinase-independent Manner

Protein kinase C isoforms have been shown to be activated downstream of heterotrimeric G proteins and mediate ERK1/2 activation (45, 46, 70, 86). The specific PKC isoforms responsible for activating ERK1/2 in a c-Src/Yes/Fyn independent manner were next identified. SYF/AT₁ cells were first treated with the broad range PKC inhibitor, chelerythrine. Cells were then stimulated with 100 nM Ang II, and ERK1/2 activation assessed by Western blot. ERK1/2 activation was eliminated in SYF/AT₁ cells treated with chelerythrine relative to DMSO-treated controls (Figure 3-4A). As such, these data confirm that Src kinase-independent ERK1/2 activation in response to Ang II specifically requires PKC.

Over twelve different isotypes of PKC have been identified to date, and a number of PKC isoforms have already been linked to ERK1/2 activation in response to stimulation by various ligands (46, 70, 86). Therefore, the specific PKC isoforms mediating Ang II-induced ERK1/2 activation in the SYF/AT₁ cells were next identified. SYF/AT₁ cells were pretreated with pharmacological inhibitors specific for a number of different PKC isoforms (Figures 3-4B, 3-4C and 3-4D). Cells were then stimulated with

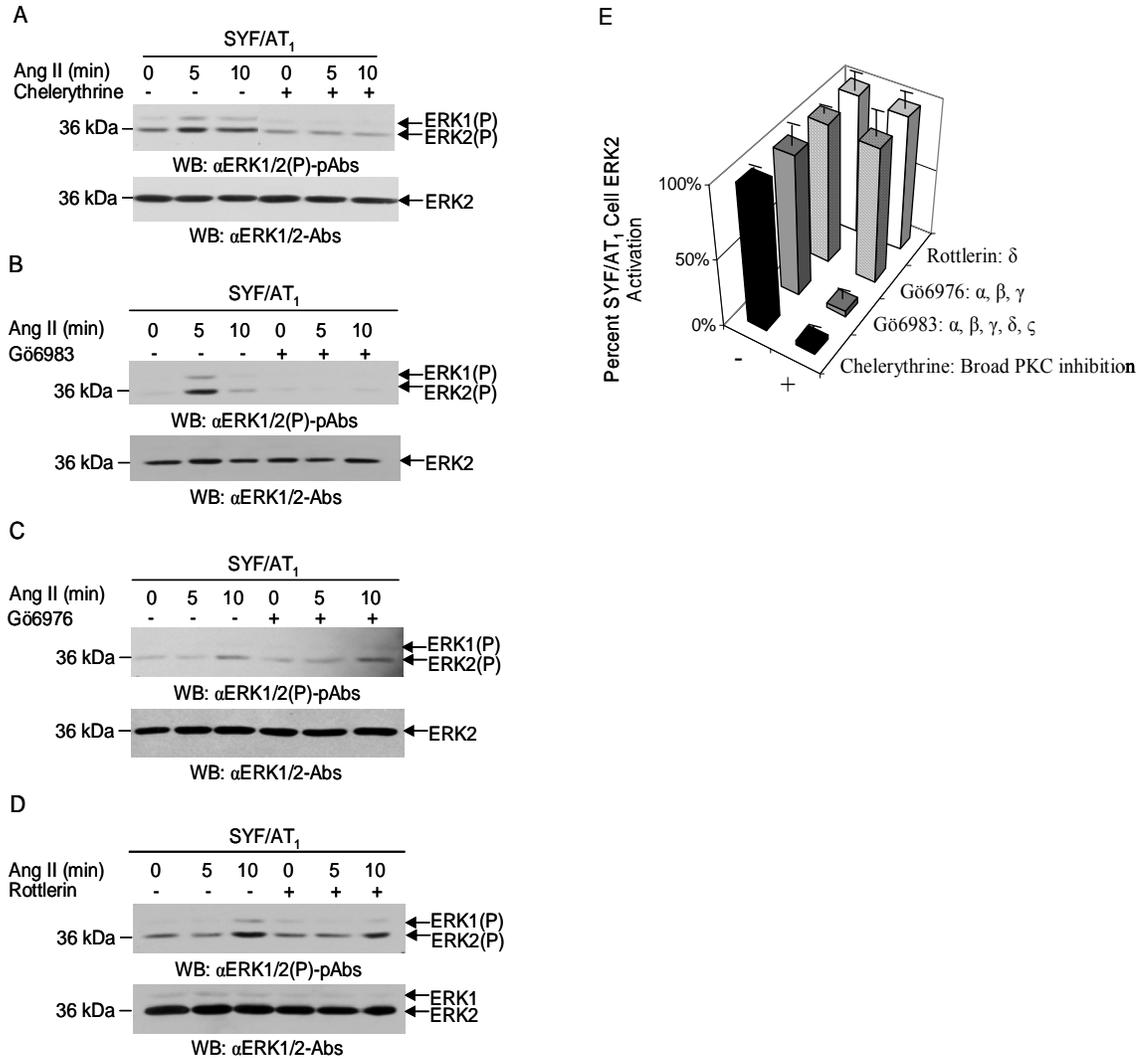


Figure 3-4. Ang II-induced ERK1/2 activation is partially dependent upon PKC. A: SYF/AT₁ cells were pretreated with either DMSO or 30 μM Chelerythrine (A), 30 μM Gö6983 (B), 30 μM Gö6979 (C) or 30 μM Rottlerin (D) for 60 min, and then stimulated with 100 nM Ang II for 0, 5, and 10 min. ERK1/2 activation was assessed via Western blotted using the indicated antibodies (top panel). Total ERK1/2 protein loading was demonstrated by stripping the membrane and reprobing with the indicated antibodies (bottom panels). These data are representative of three independent experiments. E: The effect of inhibitor treatment on the percent maximum Ang II-induced ERK2 activation. Maximum Ang II-induced ERK2 activation was quantified from A – D in the presence (+) or absence (-) of inhibitor. Phospho-ERK2 bands were densitized. Values were then normalized to ERK2 activation in Ang II-stimulated cells not pretreated with inhibitor, and multiplied by 100. Gö6983 and chelerythrine pretreatment significantly reduced ERK1/2 activation compared to vehicle-treated controls. This figure is used with permission from (42).

100 nM Ang II, and ERK1/2 activation assessed via Western blot. A common trend was observed in that Ang II-induced ERK1/2 activation was only reduced by pretreatment with compounds that inhibited a subset of PKC isoforms that included PKC ζ (Figures 3-4E).

The effect of PKC ζ inhibition on Ang II-induced ERK1/2 activation was next assessed using a PKC ζ myristoylated pseudosubstrate (MP), a potent and specific inhibitor for PKC ζ (164). WT/AT₁ and SYF/AT₁ cells were first pretreated with either PKC ζ MP or vehicle control. Both cell types were then stimulated with 100 nM Ang II, and ERK1/2 activation assessed via Western blot. ERK1/2 activation in WT/AT₁ cells was significantly reduced with the addition of the PKC ζ MP, while ERK1/2 activation in SYF/AT₁ cells treated with PKC ζ MP was significantly reduced to levels found in non-ligand treated cells (Figures 3-5A and 3-5B). Interestingly, treatment of WT/AT₁ cells with PKC ζ MP reduced Ang II-induced ERK1/2 activation to levels present in Ang II-stimulated SYF/AT₁ cells. As such, PKC ζ appears to mediate Ang II-induced ERK1/2 activation independent of Src kinases.

In order to reconfirm that roughly half of Ang II-induced ERK1/2 activation is mediated by PKC ζ , PKC ζ -specific siRNA was utilized. WT/AT₁ cells were transfected with either a scrambled siRNA control or a PKC ζ -specific siRNA. The cells were then stimulated with 100 nM Ang II, and ERK1/2 activation was then assessed via Western blot. It was found that ERK1/2 activation occurred normally in response to Ang II stimulation in WT/AT₁ cells transfected with control siRNA (Figure 3-6A). ERK1/2 activation was significantly reduced in PKC ζ -siRNA transfected cells when compared to

cells transfected with control siRNA. This reduction equated to about a 50% decrease in ERK1/2 activity (Figure 3-6B), and was the maximum reduction in active ERK1/2

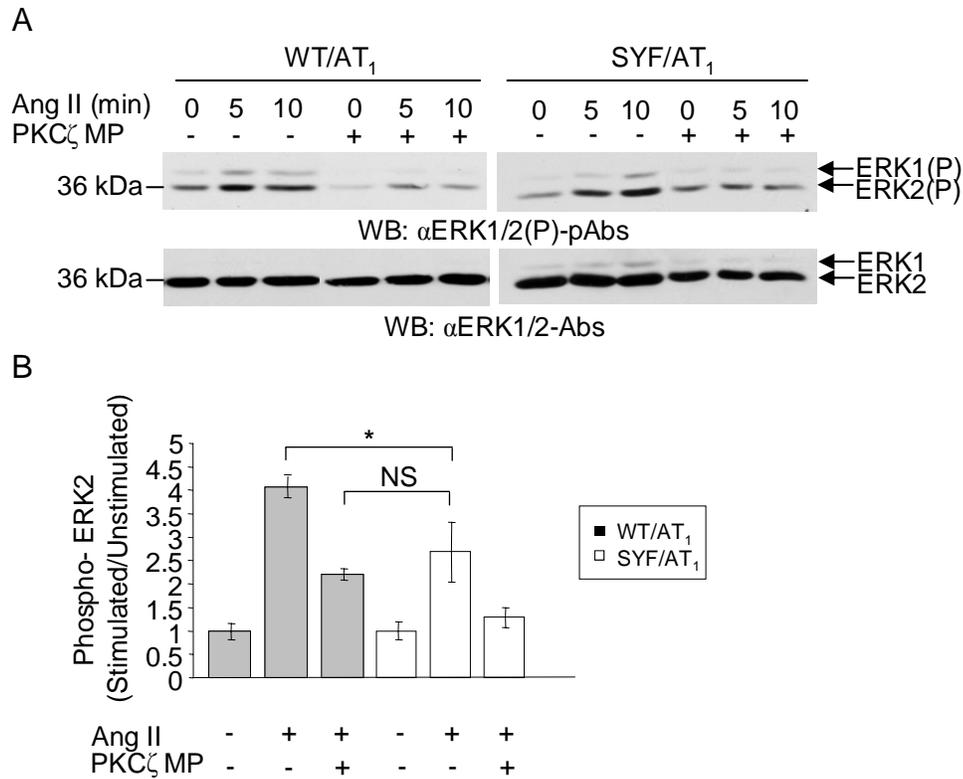


Figure 3-5. PKC ζ mediates Ang II-induced ERK1/2 activation independent of c-Src/Yes/Fyn. A: WT/AT₁ and SYF/AT₁ cells were pretreated with 1 μ M PKC ζ MP for 1 hr, and then stimulated with Ang II for 0, 5 and 10 min. ERK1/2 activation was then assessed via Western blot analysis with the indicated antibodies (top panel). Total ERK1/2 protein loading was demonstrated by stripping the membrane and reprobing with the indicated antibodies (bottom panel). B: Phosphorylated ERK2 amounts from A (5 min Ang II treatment) were quantified via densitometric analysis and expressed as a fold change relative to unstimulated controls. This figure is used with permission from (42).

capable of being observed without causing cell lethality (data not shown). Reprobing 3-6A with anti-ERK1/2-Abs confirmed that total ERK1/2 protein levels were similar in control siRNA and PKC ζ siRNA transfected cells. Additionally, a knockdown of PKC ζ was only observed in cells transfected with PKC ζ -specific siRNA, and did not affect other PKC isoforms including PKC α . These data further demonstrate that PKC ζ partially

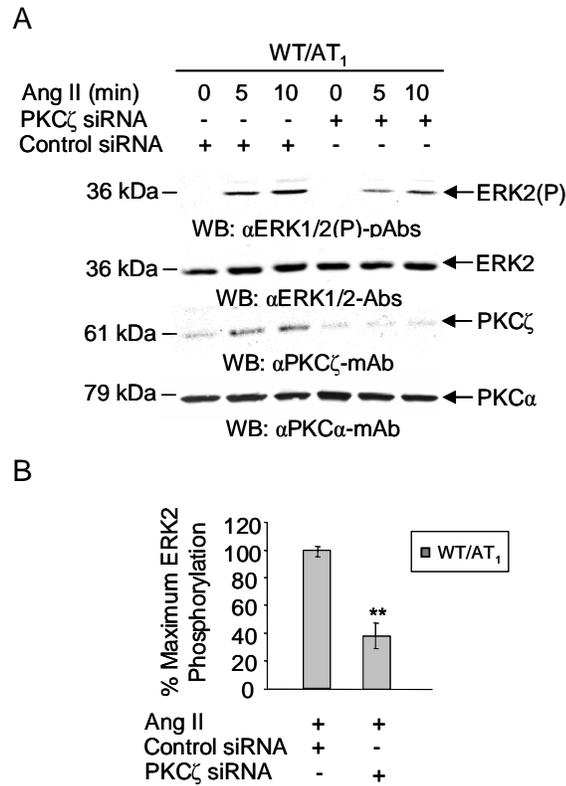


Figure 3-6. PKC ζ -specific siRNA attenuated Ang II-induced ERK1/2 activation in WT/AT₁ cells. A: WT/AT₁ cells were transfected with either PKC ζ siRNA or control siRNA. All cells were then treated with 100 nM Ang II for 0, 5, 10 min, and ERK1/2 activation assessed via Western blot analysis with the indicated antibodies (first panel). Total ERK1/2 protein loading was demonstrated by stripping the membrane and reprobing with the indicated antibodies (second panel). PKC ζ protein knockdown was confirmed by Western blotting with the indicated antibody (third panel). PKC α protein levels were demonstrated by reprobing with the indicated antibody (fourth panel). D: The percentage of maximum ERK2 phosphorylation from C was determined by dividing by ERK2 activation in Ang II and control siRNA treated cells, and multiplying by 100. This figure is used with permission from (42).

mediates Ang II-induced ERK1/2 activation.

PKC ζ Mediates MEK1/2 Activation Independent of Src Kinases

Finally, whether PKC ζ is acting upstream of MEK1/2 in order to activate ERK1/2 independent of c-Src/Yes/Fyn was investigated. SYF/AT₁ cells were pretreated with PKC ζ MP and then stimulated with Ang II for 0, 5 and 10 min. Whole cell lysates were

then Western blotted with anti-phosphospecific MEK1/2 antibodies in order to assess MEK1/2 activation. SYF/AT₁ cells not pre-treated with the PKC ζ MP exhibited an Ang II-dependent increase in MEK2 activation (Figure 3-7). However, Ang II-dependent MEK2 activation was absent from SYF/AT₁ cells treated with PKC ζ pseudosubstrate. Thus, PKC ζ appears to be acting upstream of MEK in order to activate ERK1/2 in SYF/AT₁ cells.

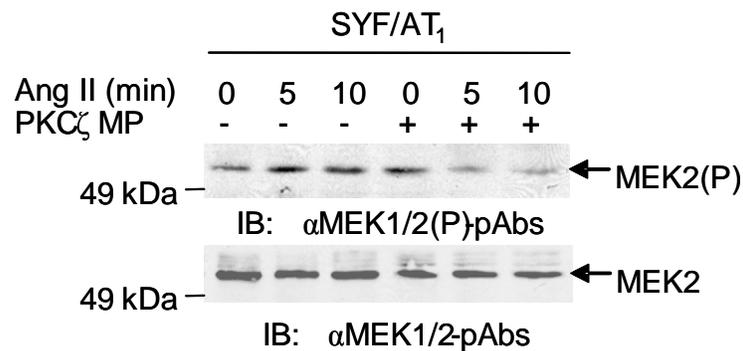


Figure 3-7. MEK phosphorylation is dependent upon PKC ζ . Cells were pretreated with either 1 μ M PKC ζ MP or DMSO for 1 hr, and then stimulated with 100 nM Ang II. Whole cell lysates were Western blotted with anti-phospho-MEK1/2 antibodies. The membrane was then stripped and reprobed with anti-MEK1/2 antibodies in order to demonstrate equal protein loading. This Western is representative of three independent experiments. This figure is used with permission from (42).

ERK1/2 Nuclear Translocation Is Dependent Upon PKC ζ In Response to Angiotensin II

Collectively, these data thus far indicate that Ang II-induced ERK1/2 activation occurs via c-Src/Yes/Fyn-dependent and heterotrimeric G protein/PKC ζ -dependent signaling. Currently, the functional consequence of having two independent mechanisms of ERK1/2 activation in response to Ang II-induced activation of the AT₁ receptor is not well understood. Previous reports have shown that ERK1/2 translocates into the nucleus and initiates gene transcription of early response genes via the phosphorylation of specific transcription factor targets (98). Other work has shown that ERK1/2 nuclear

translocation is dependent upon heterotrimeric G protein signaling in response to Ang II (122). In Chapter 2, it is demonstrated that a loss of c-Src/Yes/Fyn does not affect Ang II-induced ERK1/2 nuclear translocation, but results in a reduction in Ang II-induced cell proliferation. ERK1/2 nuclear translocation may therefore be controlled by heterotrimeric G protein/PKC ζ signaling, leading to the induction of cell proliferation. The effect of blocking heterotrimeric G protein/PKC ζ signaling on both Ang II-induced ERK1/2 nuclear translocation and cell proliferation was next examined through inhibition of the PKC ζ -dependent signaling pathway using the PKC ζ MP.

Ang II-dependent ERK1/2 nuclear translocation was examined in SYF/AT₁ cells in the presence or absence of PKC ζ MP. These cells are only able to activate ERK1/2 and induce cell proliferation in a Src kinase independent manner (Chapter 2), allowing one to test the effect of PKC ζ inhibition on Src kinase-independent ERK1/2 nuclear translocation and Ang II-induced cell proliferation. SYF/AT₁ cells were transfected with a GFP-ERK2 plasmid in order to track ERK2 movement in response to Ang II treatment. Cells were pretreated with either DMSO or PKC ζ MP for 1 hour, and then stimulated with 100 nM Ang II. Cells were then fixed, and DAPI stained to visualize the nucleus. In the absence of Ang II, GFP-ERK2 was distributed fairly evenly between the nucleus and cytoplasm of the SYF/AT₁ cells (Figure 3-8A). DAPI counterstain of figures 3-8A and merging of the GFP and DAPI images confirmed these findings (Figure 3-8F). In contrast, ERK1/2 accumulation was markedly increased in the nucleus of SYF/AT₁ cells treated with Ang II (Figure 3-8B & 3-8C), and this finding was confirmed by DAPI counterstain (Figure 3-8G and 3-8H). ERK2 nuclear translocation was blocked in SYF/AT₁ cells pretreated with PKC ζ MP (Figure 3-8D and 3-8E), and merging of GFP

and DAPI images confirmed this reduction in Ang II-induced ERK2 nuclear translocation (Figure 3-8I and 3-8J). Quantification of nuclear fluorescence relative to cytoplasmic fluorescence revealed that Ang II-induced GFP-ERK2 nuclear translocation was blocked in the presence of PKC ζ MP (Figure 3-8K). Thus, ERK1/2 nuclear translocation in response to Ang II is influenced by heterotrimeric G protein/PKC ζ signaling, while c-Src/Yes/Fyn also mediate ERK1/2 activation but do not influence ERK1/2 nuclear translocation.

Cell Proliferation Is Attenuated Through Inhibition of PKC ζ Signaling In Response to Angiotensin II

Ang II-induced ERK1/2 activation has been shown to initiate cell proliferation (33, 89, 90, 133). It has primarily been thought that this occurs through the translocation of ERK1/2 into the nucleus and the subsequent initiation of growth response gene transcription (122). It has been demonstrated that Ang II-induced ERK2 nuclear translocation is unaffected by the loss of c-Src/Yes/Fyn-mediated ERK1/2 activation (Chapter 2). The effect of eliminating heterotrimeric G protein/PKC ζ signaling on Ang II-induced cell proliferation was next investigated, independent of the ability of ERK1/2 to translocate into the nucleus.

Both WT/AT₁ and SYF/AT₁ cells were pretreated with PKC ζ MP and then stimulated with 100 nM Ang II. Changes in cell number were assessed via a direct cell count. Ang II-induced cell proliferation was reduced in WT/AT₁ cells pretreated with PKC ζ MP when compared to DMSO-treated controls (Figure 3-9). Interestingly, Ang II-induced cell proliferation was not significantly different in PKC ζ MP pretreated WT/AT₁ cells and SYF/AT₁ cells stimulated with Ang II, suggesting that PKC ζ -dependent signaling mediates the portion of Ang II-induced cell proliferation not controlled by Src

kinases. Finally, Ang II-induced cell proliferation was completely blocked in SYF/AT₁ cells pretreated with PKC ζ MP. These data collectively suggest that both PKC ζ and c-Src/Yes/Fyn mediate Ang II-induced cell proliferation through the activation of ERK1/2, though the mechanisms by which this occurs appear to be different since ERK1/2

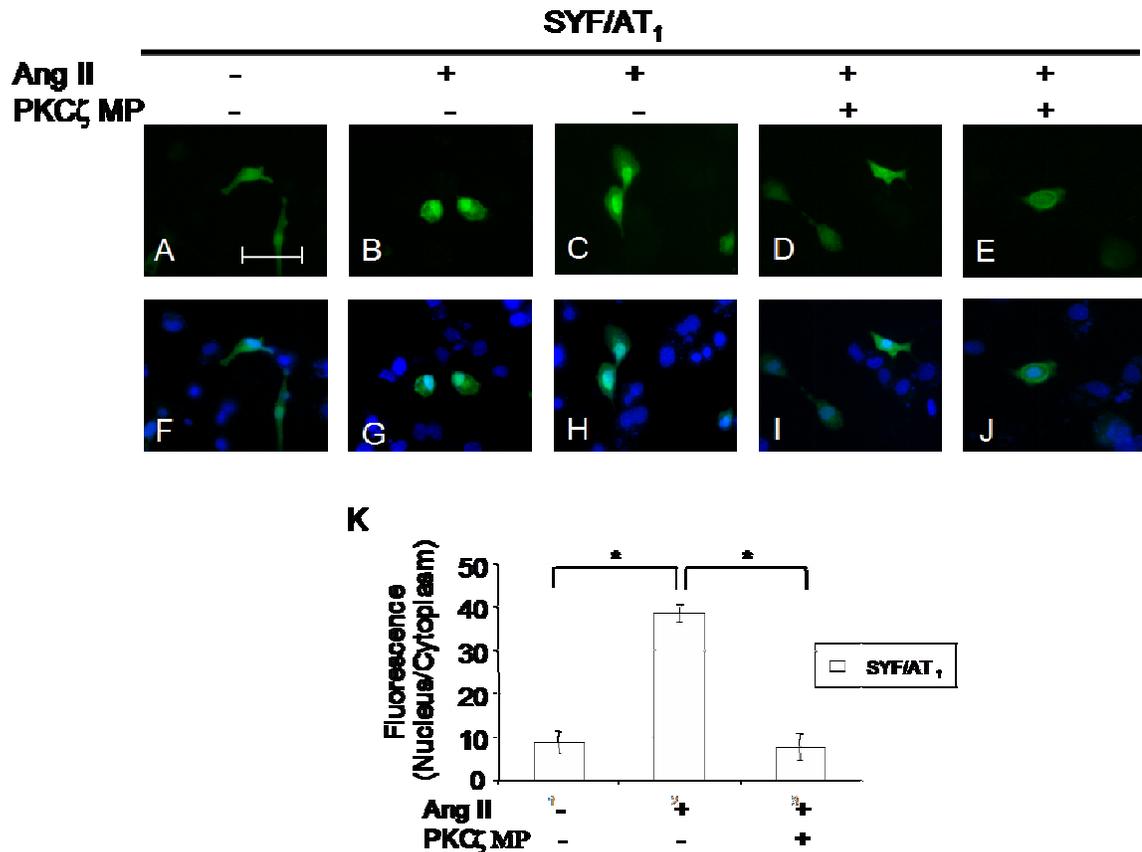


Figure 3-8. Nuclear translocation of active ERK2 is controlled by PKC ζ -dependent signaling. A - F: SYF/AT₁ cells were transfected with a GFP-ERK2 plasmid and then stimulated with 100 nM Ang II for 0 and 10 min. Nuclear translocation of ERK2 was assessed by fluorescent microscopy. A - C: GFP-ERK2 images in cells treated with 100 nM Ang II for 0 and 10 min. D & E: GFP-ERK2 images in cells pretreated with 1 μ M PKC ζ MP or DMSO (1 hr), then stimulated with 100 nM Ang II. F - J: Merging of images A - E respectively with DAPI stained images. K: Nuclear fluorescence from A - E was quantified and normalized to cytoplasmic fluorescence. All images are representative of the entire field and were taken at 40X magnification. Bar represents 15 microns. Shown is one of two independent results. This figure is used with permission from (42).

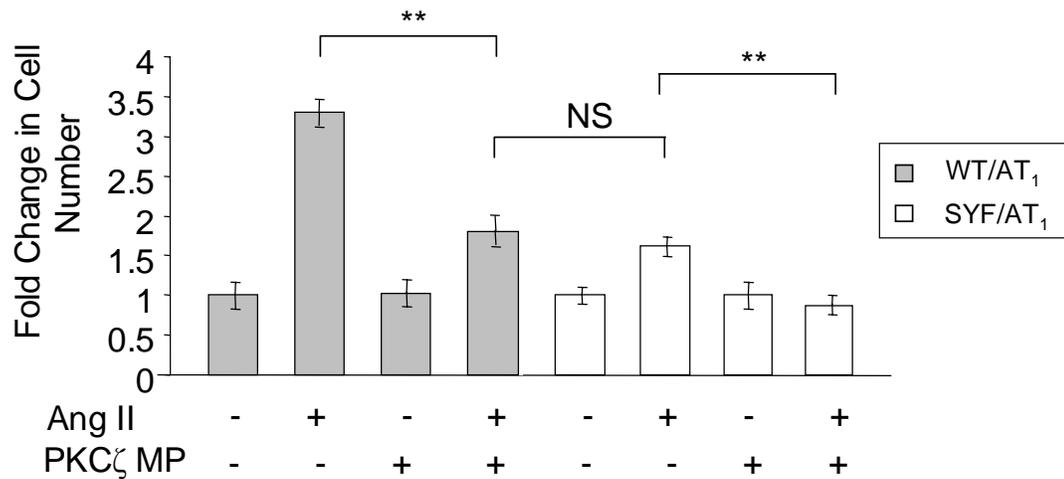


Figure 3-9. Ang II-induced cell proliferation is completely attenuated by blocking c-Src/Yes/Fyn and PKC ζ -dependent signaling. WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II for 0 and 24 hr. Some cells were pretreated with 1 μ M PKC ζ MP for 1 hr. All cells were then detached and counted using a hemacytometer. All data are the mean of three independent experiments. This figure is used with permission from (42).

translocates into the nucleus in response to activation by PKC ζ and remains in the cytoplasm when activated by c-Src/Yes/Fyn.

Discussion

A diverse set of signaling pathways have been implicated in ERK1/2 activation, but the precise mechanisms of ERK1/2 activation in response to Ang II are not fully understood (4, 45, 69, 70, 85, 86, 89, 109, 115, 117, 140, 153). It appears that ERK1/2 activation occurs via multiple signaling mechanisms. However, the cellular outcome associated with the activation of ERK1/2 via different signaling cascades is in question. Are these signaling pathways functionally redundant, or does the activation of ERK1/2 by one pathway result in a different cellular outcome than when ERK1/2 is activated by another signaling cascade?

Here, SYF/AT₁ MEF cells were utilized in order to identify the mechanism underlying Src kinase-independent ERK1/2 activation in response to Ang II as well as a functional consequence for activating ERK1/2 in this manner. The advantage of utilizing these cells is that ERK1/2 activation was examined in a Src kinase-deficient background, eliminating all Src kinase function and the possibility that ERK1/2 activation can be mediated via any of these very similar family members. It was found that while c-Src/Yes/Fyn tyrosine kinases do play a role in the activation of ERK1/2 as previously reported (54, 115, 140), ERK1/2 activation is not completely dependent on these proteins and persists at reduced levels in their absence. Interestingly, c-Src/Yes/Fyn are capable of activating only about 50% of intracellular ERK1/2. This seems to be a generalized phenomenon in other cells types as well, including CHO and RASM cells. An explanation for these results is that the remaining 50% of intracellular ERK1/2 are activated by c-Src/Yes/Fyn-independent mechanisms. This was subsequently confirmed since it was found that about 50% of Ang II-induced ERK1/2 activation involved heterotrimeric G protein and PKC ζ signaling. In summary, Ang II-induced ERK1/2 activation occurs via two specific mechanisms that work independent of one another.

While both pathways activate an equal portion of ERK1/2 and contribute to cell proliferation, the mechanism whereby each pathway independently mediates this effect appears to be different. It had previously been thought that ERK1/2 must translocate into the nucleus in order to initiate events necessary for cell proliferation to occur, including the transcription of early response genes such as *c-fos* (14, 15, 109). Interestingly, the results in this Chapter indicate that the loss c-Src/Yes/Fyn had no effect on the ability of ERK1/2 to translocate into the nucleus. ERK1/2 was able to enter the nucleus in the

absence of c-Src/Yes/Fyn; however, cell proliferation was still markedly reduced. An explanation for these findings is that ERK1/2 activated via c-Src/Yes/Fyn-dependent signaling acts upon cytoplasmic proteins to mediate proliferation, while ERK1/2 activated via PKC ζ -dependent signaling translocates into the nucleus to directly mediate transcriptional events (14). A mechanism describing how c-Src/Yes/Fyn-activated ERK1/2 initiates Ang II-induced cell proliferation is described in the next chapter.

The utility of having two mechanisms that dually activate ERK1/2 in response to stimulation of the AT₁ receptor by Ang II is nonetheless intriguing. Both c-Src/Yes/Fyn and heterotrimeric G protein-dependent signaling appear to occur simultaneously in response to Ang II, and exhibit an additive effect on ERK1/2 activation and subsequent cell proliferation in response to Ang II. Within adult mammalian systems, Ang II-induced cell proliferation is associated with abnormal cell proliferation during cardiovascular diseases and cancer, and to date has not been implicated in cell growth and proliferation during a non-disease state (24, 140, 145, 157-159). As such, this study may have therapeutic merit since local inhibition of both heterotrimeric G protein/PKC ζ signaling as well as c-Src/Yes/Fyn-dependent signaling may be necessary in order to completely block Ang II-induced cell proliferation during disease states.

CHAPTER 4
ERK1/2 REGULATES ANGIOTENSIN II-DEPENDENT CELL PROLIFERATION
VIA THE CYTOPLASMIC ACTIVATION OF RSK2 AND NUCLEAR ACTIVATION
OF ELK1

Introduction

In the previous chapters, ERK1/2 activation is shown to be mediated independently by either Src family tyrosine kinase-dependent signaling or heterotrimeric G protein/PKC ζ -dependent signaling in two different cell types. Both of these signaling mechanisms accounted for roughly 50% of Ang II-induced ERK1/2 activation. Interestingly, heterotrimeric G protein/PKC ζ signaling regulates ERK1/2 nuclear translocation, while c-Src/Yes/Fyn-dependent signaling influences cytoplasmic ERK1/2 activation in response to Ang II. Furthermore, these two pathways were both implicated in Ang II-induced cell proliferation, with inhibition of both signaling cascades necessary in order to achieve complete attenuation of Ang II-induced cell proliferation. These data were very striking since previous reports had shown that ERK1/2 nuclear translocation was a critical step in initiating the transcription of early response genes such as *c-fos*, which promotes cell proliferation (109). However, it appears here that cytoplasmic ERK1/2, under the control of c-Src/Yes/Fyn, also mediates Ang II-induced cell proliferation independent of nuclear ERK1/2.

One explanation for how cytoplasmic ERK1/2 can influence early response gene transcription is that it phosphorylates cytoplasmic substrates, which in turn translocate into the nucleus and regulate transcriptional activity. Members of the Ribosomal S6 kinase (RSK) family of proteins are well-known cytoplasmic targets of ERK1/2, and

have been shown to promote the transcription and translation of selected mRNAs important for cell growth (57). Previous reports have shown that ERK1/2 activates RSK family proteins, however it is not clear if these proteins represent a possible pathway by which Ang II-activated ERK can initiate the events leading to cell proliferation (110, 128, 132).

In this chapter, the mechanisms whereby ERK1/2 activated by either c-Src/Yes/Fyn or heterotrimeric G proteins/PKC ζ -dependent signaling generates the proliferative response associated with AT₁ receptor activation will be defined. It was hypothesized that c-Src/Yes/Fyn-activated ERK1/2 mediates Ang II-induced cell proliferation through RSK, whereas heterotrimeric G protein/PKC ζ signaling regulates cell proliferation through control of ERK1/2 nuclear translocation and subsequent elk1 activation. To examine this, WT/AT₁ and SYF/AT₁ cells or CHO/AT₁ and CHO/AT₁-M5 cells were utilized. Each of these cell lines have been stably transfected with the AT₁ receptor and were the same as those utilized in previous chapters. It was found that ERK1/2, activated via c-Src/Yes/Fyn-dependent signaling, phosphorylates ribosomal S6 kinase 2 (RSK2), which subsequently translocates into the nucleus and modulates *c-fos* activity at the transcriptional and post-translational levels. These events partially mediate cell proliferation since pretreatment with SL0101, a potent and specific inhibitor of RSK, significantly attenuated Ang II-induced cell proliferation. ERK1/2 activated by heterotrimeric G protein/PKC ζ signaling localizes to the nucleus, where it phosphorylates the transcription factor elk1 and regulates *c-fos* transcription. Together with ERK1/2-RSK signaling, these events mediate Ang II-induced cell proliferation. As such, this study demonstrates that the AT₁ receptor coordinately utilizes both heterotrimeric G

protein and Src family tyrosine kinase signaling to achieve a common cellular outcome via two different mechanisms acting in distinct cellular compartments.

Materials and Methods

Antibodies and Pharmacological Inhibitors

A cocktail of phospho-specific ERK1/2 antibodies (Promega and Santa Cruz Biotechnologies) were used in order to increase signal to noise ratio. Both of these antibodies are specific for phospho-threonine 202 and phospho-tyrosine 204 within the conserved TEY motif. The phospho-specific RSK polyclonal antibody (α RSK(P)-pAb) was purchased from Cell Signaling Technologies and recognizes the phospho-threonine 356/phospho-serine 360 motif. The phospho-specific SRF antibody [SRF(P)] recognizes the phosphorylated Ser 103 residue; the phospho-specific elk1 [elk1(P)] monoclonal antibody recognizes phospho-serine 383. Both of these antibodies were purchased from Cell Signaling Technologies. The RSK1 antibody (α RSK1-pAb) and the RSK2 antibody (α RSK2-pAb) were obtained from Santa Cruz Biotechnology. A cocktail of ERK1/2 antibodies (α ERK1/2-Abs) were used to measure total ERK1/2 protein levels. This cocktail consisted of ERK1/2 monoclonal and polyclonal antibodies from Santa Cruz Biotechnology. The ERK1/2 monoclonal antibody was used separately for immunofluorescence. The α Tyr(P) mAb (PY20) was from BD Transduction Laboratories. The c-fos polyclonal antibody (α cfos-pAb) was from Santa Cruz Biotechnology. The phospho-serine polyclonal antibody [α Ser(P)-pAb)] was purchased from AnaSpec, Inc. The SL0101 compound was purchased from Toronto Research Pharmaceuticals. The PKC ζ myristoylated pseudosubstrate (PKC ζ MP) was purchased from Biomol Laboratories. PP2 and PD98059 compounds were obtained from Calbiochem. Leptomycin B (LMB) was purchased from Sigma.

Cell lines and Cell Culture

The Chinese hamster ovary cell lines (CHO/AT₁-WT and CHO/AT₁-M5 cells) were a gift from Dr. Kenneth Bernstein (Emory University), and have an equal abundance of AT₁ receptor as well as affinity for Ang II (28). The WT/AT₁ and SYF/AT₁ mouse embryonic fibroblast cells are described in Chapter 2. VSMC cells were cultured in the same media, but without Zeocin. WT/AT₁, SYF/AT₁, and VSMC were growth arrested in serum-free DMEM for 48 h prior to experiments.

Cell Lysate Preparation, Immunoprecipitation and Western Blotting

For Westerns, cells were lysed in radioimmune precipitation assay (RIPA) buffer containing protease inhibitors as described in Chapter 2. Cell lysates were immunoprecipitated where indicated exactly as described in Chapter 2. Proteins were detected using enhanced chemiluminescence exactly as described in Chapter 2 (117).

Densitometric Analysis

Western blots were scanned and densitized using UnScanIt Gel Analysis (Silk Scientific) as described in Chapter 2. The average pixel value minus background was obtained for each cell type and normalized to the average pixel value for the respective non-Ang II-treated cells.

Immunofluorescence

WT/AT₁ and SYF/AT₁ cells were plated onto four-chambered slides (Lab-Tek) and grown to 70% confluency. Cells were serum-starved in serum free DMEM supplemented with BSA for 48 hr. Following starvation, all cells were ligand-treated with 100 nM Ang II. Slide chambers were removed from slides, and cells were washed one time with PBS (pH 7.4). Cells were fixed in 4% paraformaldehyde (Fisher) for 10 min at room temperature, rinsed in PBS and permeabilized for 3 min in acetone (Fisher) at -20°C.

Cells were rinsed three times in PBS, and blocked for 15 min in 3% BSA/PBS at room temperature in a homemade hydration chamber to prevent evaporation. Cells were incubated with the primary antibody indicated for each experiment (1:500 in 3% BSA/PBS) for 1 hr. All cells were next rinsed three times in PBS. Cells were incubated with the appropriate fluorochrome-conjugated secondary antibody (1:100 in 3% BSA/PBS) for 1 hour at room temperature in a hydration chamber. In the case of the α RSK2-pAb, the α rabbit IgG-FITC secondary antibody (Sigma) was used. For the α ERK2-mAb and α elk1(P)-mAb, the α mouse IgG-Texas Red secondary antibody (Sigma) was used, while α Goat IgG-FITC secondary antibody (Santa Cruz) was utilized in conjunction with the α SRF(P)-pAb. Upon completion of incubation with the appropriate secondary antibody, cells were washed three times in PBS. A coverglass was mounted to each slide using Vectashield + DAPI mounting medium (Vector Labs). The edges of the slides were sealed with nail polish sealant (Maybelline LLC) and allowed to dry. All dry slides were stored at -20°C until viewed. Slides were viewed on a Zeiss Axioplan II Fluorescence microscope.

Quantification of nuclear and cytoplasmic fluorescence

Fluorescence in the nucleus or cytoplasm was quantified using the Image J Program (NIH) as described in Chapter 2.

***c-fos* transcriptional activity**

SREw/Luc, mSRF/Luc, mTCF/Luc and TK/Luc plasmids were kindly provided by Dr. Jessica Schwartz (University of Michigan) (71). WT/AT₁ and SYF/AT₁ cells were plated onto four-chambered slides (LabTek) and transiently-transfected with each individual plasmid using 8 μL lipofectin (Invitrogen). All transfected cells were incubated for 5 hr at 37°C . The transfection was stopped by washing cells in PBS, and

incubating the cells in serum-containing DMEM for 12-16 hrs. Cells were serum-starved in DMEM + BSA (0.5% wt/vol) for 12-16 hrs, and treated with 100 nM Ang II for the amount of time indicated in each experiment. Cells were placed in 1X Reporter Lysis Buffer (Promega), and exposed to one -80°C freeze/thaw cycle (30 min each) to aid in the disruption of cell membrane integrity. Cells were placed on a shaker at room temperature for one additional hour to ensure complete lysis, and then transferred to a microcentrifuge tube. Cells were centrifuged at 12,000 x g for 2 min at 4°C, and 20 µL of cell lysate was combined with 100 µL luciferin substrate (Promega). Luciferase activity was measured in a Monolight 3010 Luminometer (PharMingen) at 10 sec intervals.

Cell Migration Assay

Cell migration assays were performed using a commercially available Cell Migration Kit (BioLabs). VSMCs were grown in 6 well cell culture plates. Cells were detached, and placed in the upper chamber of a migration apparatus in serum-free DMEM. The lower chamber of the apparatus was filled with serum-free DMEM. Cells were then stimulated with 100 nM Ang II in the presence of SL0101 or vehicle for 24 hr, and allowed to migrate from the upper chamber through a nylon membrane and into the lower chamber. The membrane was washed, stained and photographed to visualize migratory cells. The membrane was then placed in destaining solution and migratory cell number was quantified indirectly via spectrophotometry at absorption 595 nm.

Cell Count

WT/AT₁ and SYF/AT₁ cells were plated onto 100 mm culture dishes and grown to 80% confluency. Cells were serum-starved and treated with 100 nM Ang II as indicated. Both cell types were then counted using a hemacytometer as described (122).

Statistical Analysis

Data were analyzed by two-way ANOVA. All data passed a Normality Test as well as Equal Variance Test. Pairwise comparisons were made following the Holm-Sidak method. All data are expressed as mean +/- SEM. * = $p < 0.05$, ** = $p < 0.01$.

Results

RSK Phosphorylation and ERK1/2-RSK Co-association Are Dependent Upon Src Kinases in Response to Angiotensin II

The data in Chapter 2 suggest that ERK1/2 activated by c-Src/Yes/Fyn acts upon cytosolic proteins to promote Ang II-dependent cell proliferation. It was first examined whether or not the phosphorylation of RSK, a well known cytoplasmic substrate of ERK1/2 (109, 128, 132), was dependent upon c-Src/Yes/Fyn and ERK1/2 signaling.

WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II, and RSK activation assessed via Western blot using a phospho-specific RSK antibody that recognizes phosphorylated RSK1, RSK2 and RSK3. RSK activation occurred in response to Ang II in WT/AT₁ cells and was maximal after 10 min of Ang II treatment and declined thereafter (Figure 4-1A and data not shown). However, Ang II-induced RSK activation was completely absent from SYF/AT₁ cells, indicating that c-Src/Yes/Fyn are necessary for the activation of RSK in response to Ang II. These results were recapitulated by pre-treating WT/AT₁ cells with PP2, a Src kinase inhibitor (Figure 4-1B). In addition, Ang II-induced RSK phosphorylation in WT/AT₁ cells was dependent upon ERK1/2 activity since pretreatment with the ERK activation inhibitor, PD98059, attenuated RSK activation in these cells (Figure 4-1C). RSK activation in response to Ang II is therefore mediated by Src kinases and ERK1/2.

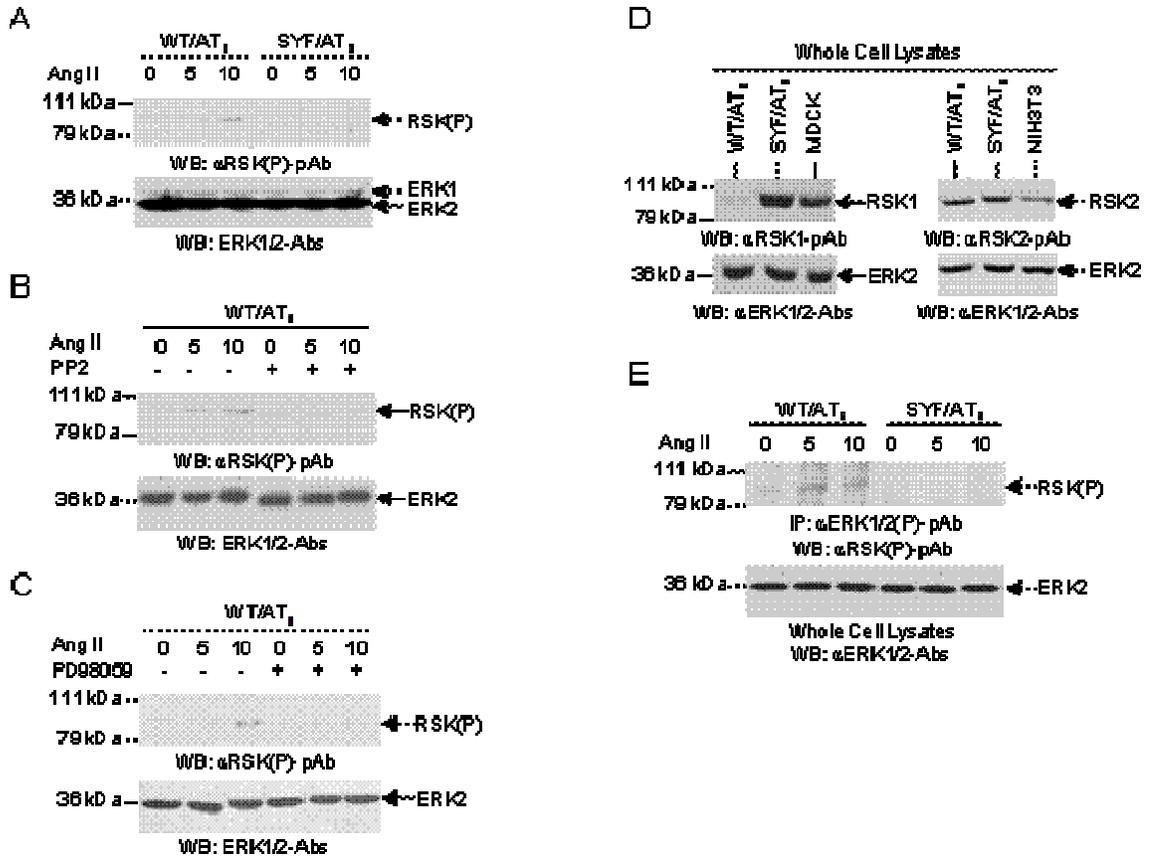


Figure 4-1. RSK2 phosphorylation and RSK2-ERK1/2 co-association are decreased in SYF/AT₁ cells. A: WT/AT₁ cells and SYF/AT₁ cells were stimulated with 100 nM Ang II for 0, 5, and 10 min and total RSK phosphorylation assessed via Western blot (WB) using the indicated antibody (top). Total protein loading was demonstrated by stripping the membrane and reprobing for ERK1/2 (bottom). WT/AT₁ cells were pretreated with either 30 μM PP2 (B) or 50 μM PD98059 (C) for 30 min, and stimulated with 100 nM Ang II for 0, 5, and 10 min. Total RSK phosphorylation was assessed by Western blot using the indicated antibody (top). Total protein loading was demonstrated by stripping the membrane and reprobing for ERK1/2 (bottom). D: The presence or absence of RSK1 and RSK2 in cells was examined by Western blotting WT/AT₁ and SYF/AT₁ whole cell lysates with the indicated antibodies (top). Control MDCK and NIH3T3 whole cell lysates were also run on the same gel. Total protein loading was demonstrated by stripping the membrane and reprobing for ERK1/2 (bottom). E: Active RSK2-ERK co-association was assessed in WT/AT₁ and SYF/AT₁ whole cell lysates by immunoprecipitating (IP) and Western blotting with the indicated antibodies (top). Total protein loading was demonstrated by blotting whole cell lysates with the indicated antibodies (bottom). All Westerns are representative of at least three independent blots. This figure is used with permission from (43).

The specific isoform(s) of RSK phosphorylated in response to Ang II within the WT/AT₁ and SYF/AT₁ cells was next determined. Of the three known RSK isoforms, only RSK1 and RSK2 are phosphorylated by ERK1/2 (37). Whole cell lysates from WT/AT₁ and SYF/AT₁ cells were prepared and Western blotted with RSK1 or RSK2 specific antibodies alongside positive control whole cell lysates. RSK1 was not expressed in WT/AT₁ cells, but was expressed in SYF/AT₁ cells (Figure 4-1D). These expression levels did not change with the addition of Ang II (data not shown). However, RSK2 was expressed equally in both cell types. Therefore, RSK2 is most likely the isoform phosphorylated in the presence of c-Src/Yes/Fyn. As an aside, c-Src/Yes/Fyn may regulate RSK1 protein degradation since RSK1 was present in c-Src/Yes/Fyn deficient cells, but absent from cells containing these proteins.

Previous reports have shown that active ERK1/2 bind RSK proteins via the ERK docking site and, once bound, modulate RSK activity (121, 128, 132). It was next determined whether RSK and ERK1/2 co-association is controlled by c-Src/Yes/Fyn, in order to determine if the ERK1/2 activated by c-Src/Yes/Fyn binds to RSK. WT/AT₁ and SYF/AT₁ whole cell lysates were immunoprecipitated with a phospho-specific ERK1/2 antibody, and then Western blotted with the phospho-specific RSK antibody. ERK1/2-RSK co-association was evident after 5 min of Ang II treatment in WT/AT₁ cells, but was completely absent from SYF/AT₁ cells (Figure 4-1E). As such, these data demonstrate that ERK1/2 and RSK co-associate in response to Ang II, and this is c-Src/Yes/Fyn-dependent.

RSK Nuclear Translocation Is Src Kinase Dependent, While ERK1/2 Nuclear Translocation Is PKC ζ Dependent in Response to Angiotensin II

Phosphorylated RSK has been shown to translocate into the nucleus and modulate the transcriptional activity of target genes in response to stimulation of various cytokine and growth factor receptors (16, 162). It was next determined whether RSK nuclear translocation occurs in response to Ang II, and whether this event is regulated by c-Src/Yes/Fyn. WT/AT₁ and SYF/AT₁ cells were pretreated with leptomycin B (LMB) to prevent the nuclear exportation of proteins, and then stimulated with Ang II. RSK nuclear accumulation was then assessed by immunofluorescence. Translocation of RSK2 into the nucleus occurred in response to Ang II treatment in WT/AT₁ cells, and the nuclear accumulation of RSK2 was confirmed by merging the RSK2 image with DAPI (Figures 4-2A & 4-2B, 4-2E & 4-2F). However, Ang II-induced RSK2 nuclear translocation did not occur in the absence of c-Src/Yes/Fyn (Figures 4-2C & 4-2D, 4-2G & 4-2H). Pretreatment of WT/AT₁ cells with PP2, a Src family kinase inhibitor, also prevented Ang II-induced RSK2 nuclear accumulation (Figures 4-2I & 4-2J, 4-2K & 4-2L). Quantification of nuclear and cytoplasmic fluorescence confirmed that RSK2 nuclear translocation occurred in WT/AT₁ cells stimulated with Ang II, but was blocked in SYF/AT₁ cells or in WT/AT₁ cells pretreated with PP2 (Figure 4-2M). Collectively, these data show that Ang II-induced RSK nuclear translocation is regulated by c-Src/Yes/Fyn.

Ang II-induced ERK1/2 nuclear translocation and RSK2 nuclear translocation patterns were next assessed in WT/AT₁ and SYF/AT₁ cells. All cells were pretreated

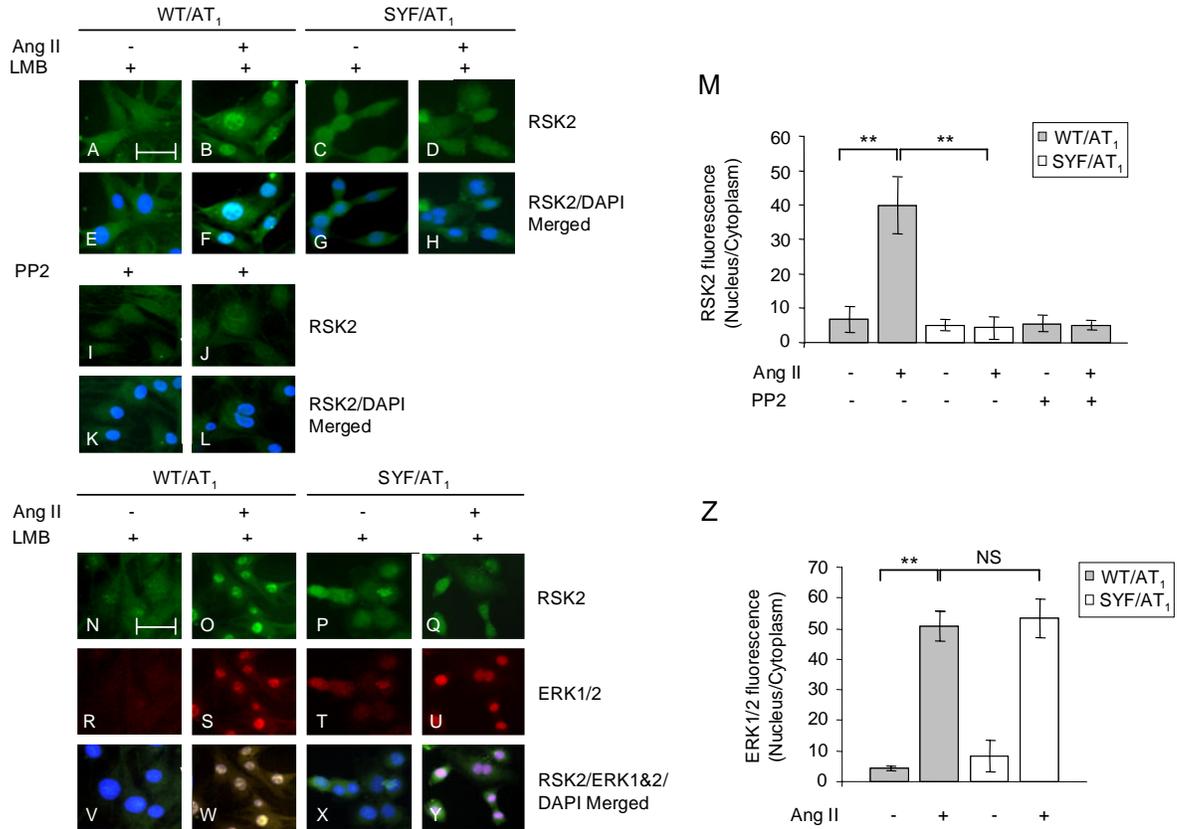


Figure 4-2. RSK2 and ERK1/2 nuclear phosphorylation in response to Ang II in WT/AT₁ and SYF/AT₁ cells. A-L, N-Y: WT/AT₁ and SYF/AT₁ cells were pretreated with 0.005 μ g Leptomycin B for 5 min prior to stimulation with 100 nM Ang II for 10 min. All cells were incubated with antibodies specific for the indicated proteins (right) and respective fluorochrome-conjugated secondary antibody. E-H, K, L, V-X: Images were DAPI stained and merged with the respective fluorescent protein images. I-L: WT/AT₁ cells were pretreated with 20 μ M PP2 for 1 hr prior to stimulation with 100 nM Ang II. M: Nuclear fluorescence from A - L was quantified and normalized to cytoplasmic fluorescence. Z: Nuclear fluorescence from R - U was quantified and normalized to cytoplasmic fluorescence. All images are representative of the entire field and were taken at 40X magnification. Bar represents 15 microns. This figure is used with permission from (43).

with LMB and then stimulated with Ang II. RSK2 and ERK1/2 nuclear translocation were then assessed via immunofluorescence. Both RSK2 and ERK1/2 translocated into the nucleus in response to Ang II in WT/AT₁ cells (Figures 4-2N & 4-2O, 4-2R & 4-2S). Merging of RSK2 and ERK1/2 images confirmed that RSK2 and ERK1/2 nuclear

translocation patterns were the same in these cells (Figures 4-2V and 4-2W). However, nuclear translocation of ERK1/2 persisted in SYF/AT₁ cells while RSK2 nuclear translocation was attenuated (Figures 4-2P and 4-2Q, 4-2T and 4-2U). Merging of the ERK1/2 and RSK2 images confirmed that these two proteins exhibit different patterns of nuclear translocation in SYF/AT₁ cells (Figures 4-2X and 4-2Y). Quantification of nuclear and cytoplasmic fluorescence revealed that GFP-ERK2 nuclear translocation was similar in WT/AT₁ and SYF/AT₁ cells (Figure 4-2Z). As such, these data demonstrate that ERK1/2 nuclear translocation is regulated by PKC ζ -dependent signaling whereas RSK2 nuclear translocation is controlled by c-Src/Yes/Fyn.

SRF and TCF Binding Within the *c-fos* Promoter Are Mediated in A RSK And A ERK1/2-dependent Manner, Respectively

c-fos transcription is regulated via the binding of specific transcription factors to the serum response element (SRE) within the *c-fos* promoter, namely the serum response factor (SRF) and ternary complex factor (TCF) (71, 78, 84, 162). RSK has been implicated in the phosphorylation of the SRF while ERK1/2 have shown to phosphorylate TCF proteins, thereby increasing the activity of these transcription factors (78, 84, 142, 162). However, the roles of the SRF and the TCF during Ang II-induced *c-fos* transcription are still unknown.

Whether SRF and TCF activity are regulated by either PKC ζ -dependent or c-Src/Yes/Fyn-dependent signaling in response to Ang II was next examined. WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II, and SRF or TCF nuclear phosphorylation assessed via immunofluorescence. Nuclear SRF phosphorylation occurred in response to Ang II treatment in WT/AT₁ cells (Figures 4-3A and 4-3B, 4-3F and 4-3G). Ang II-induced SRF phosphorylation was completely lost in SYF/AT₁ cells stimulated with Ang

II (Figures 4-3D and 4-3E, 4-3I and 4-3J). In addition, pretreatment of WT/AT₁ cells with PKC ζ MP did not affect Ang II-induced SRF phosphorylation (Figures 3C and 3H). Quantification of nuclear and cytoplasmic fluorescence revealed that Ang II-induced SRF phosphorylation in WT/AT₁ cells was not affected by PKC ζ MP pre-treatment, while Ang II-induced SRF phosphorylation did not occur in SYF/AT₁ cells (Figure 4-3Q). Thus, SRF phosphorylation is dependent upon c-Src/Yes/Fyn-dependent signaling and not PKC ζ .

The nuclear phosphorylation of elk, a TCF activated in response to ERK1/2, was assessed in the same manner. Elk1 phosphorylation occurred in response to Ang II in WT/AT₁ cells (Figures 4-3K and 4-3L, Figures 4-3N and 4-3O). In addition, Ang II-induced elk1 phosphorylation persisted in SYF/AT₁ cells (data not shown). Pretreatment with PKC ζ MP attenuated elk1 nuclear phosphorylation (Figures 4-3M and 4-3P). Quantification of nuclear and cytoplasmic fluorescence revealed that Ang II-induced elk1 phosphorylation in WT/AT₁ cells was blocked by PKC ζ MP pretreatment (Figure 4-3R). Thus, TCF phosphorylation appears to be mediated by PKC ζ -dependent signaling and not by c-Src/Yes/Fyn.

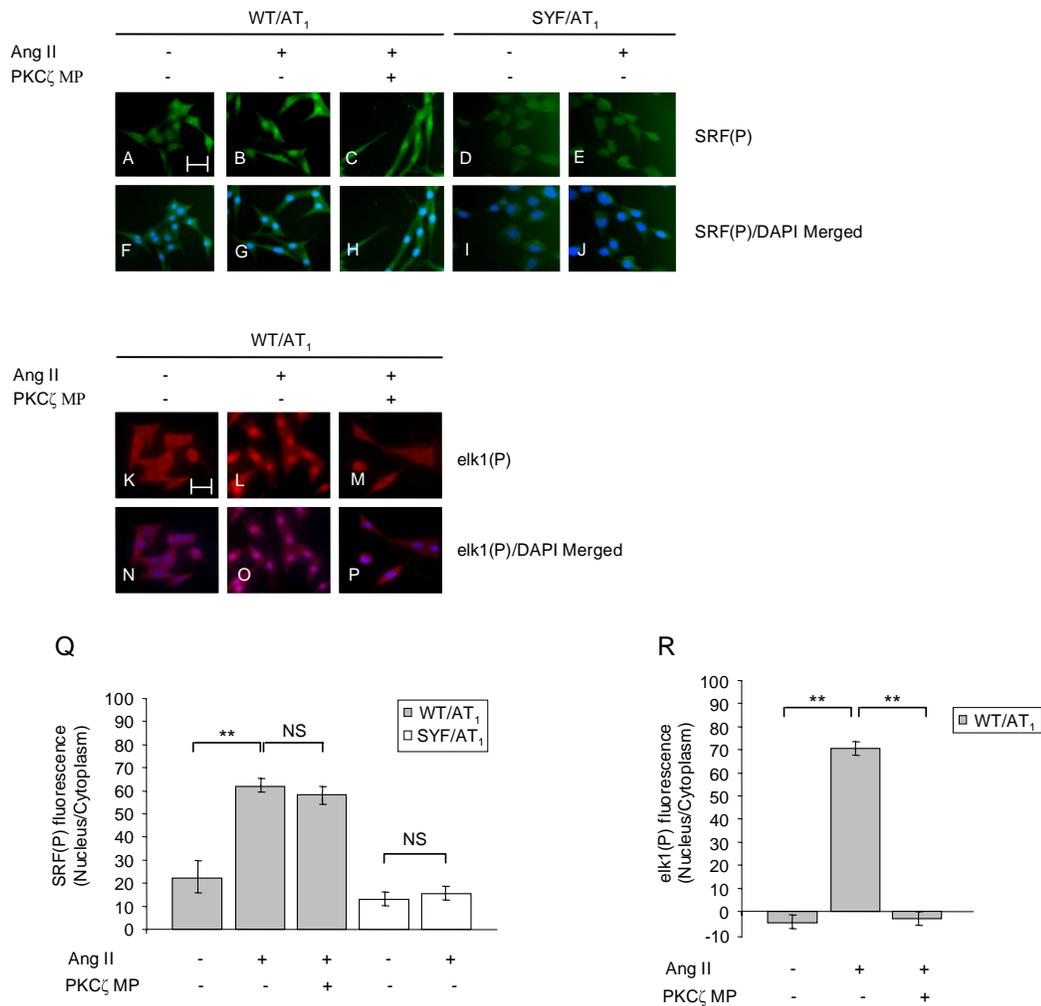


Figure 4-3. Ang II-induced SRF and elk1 nuclear phosphorylation in WT/AT₁ and SYF/AT₁ cells. A-P: WT/AT₁ or SYF/AT₁ cells were stimulated with 100 nM Ang II for 10 min. All cells were incubated with antibodies specific for the indicated proteins (right) and their respective fluorochrome-conjugated secondary antibody. F-J, N-P: Protein fluorescence images were merged with DAPI stained images. C & H, M & P: Cells were pretreated with 1 μ M PKC ζ MP for 1 hr prior to Ang II treatment. Q: Nuclear fluorescence from A - E was quantified and normalized to cytoplasmic fluorescence. R: Nuclear fluorescence from K - M was quantified and normalized to cytoplasmic fluorescence. These results are representative of three independent experiments. All images are representative of the entire field and were taken at 40X magnification. Bar represents 15 microns. This figure is used with permission from (43).

Both the SRF and TCF have been shown to modulate *c-fos* transcriptional activity in response to growth hormone treatment (71). Whether SRF and TCF binding of the *c-*

fos SRE occurred in response to Ang II, and, if these events were altered by the absence of c-Src/Yes/Fyn, were next tested. WT/AT₁ and SYF/AT₁ cells were transiently transfected with wild-type or mutated SRE-luciferase plasmids and then stimulated with Ang II. The wild-type SRE plasmid (SRE_w/Luc) alone mediated Ang II-induced luciferase expression in both cell types (Figure 4-4). Ang II-induced luciferase activity was reduced by about 50% in SRE_w/Luc transfected SYF/AT₁ cells relative to transfected WT/AT₁ cells, indicating that c-*fos* transcriptional activity is in part dependent upon c-Src/Yes/Fyn. Furthermore, mutation of either the SRF or TCF binding sites only partially blocked Ang II-induced luciferase expression in WT/AT₁ cells. In addition, Ang II-induced luciferase expression was completely blocked in SYF/AT₁ cells transfected with either the mSRF/Luc or mTCF/Luc plasmids. Note that the reporter plasmid alone (TK/Luc) consistently failed to respond to Ang II in both cell types. These data therefore indicate that the SRF and TCF transcription factors both partially modulate c-*fos* transcriptional activity in either a c-Src/Yes/Fyn or PKC ζ -dependent manner, respectively.

c-*fos* Protein Expression Is Dependent Upon Src Kinase Signaling And PKC ζ Signaling

It was next determined if the inhibition of PKC ζ -dependent and/or Src kinase-dependent signaling reduced c-*fos* protein levels. WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II and c-*fos* protein levels assessed via Western blot. c-*fos* protein exhibited an Ang II-dependent increase in both cell types, and was maximal after 60 min of Ang II treatment (Figure 4-5A). However, Ang II-induced c-*fos* protein production was reduced in SYF/AT₁ cells relative to WT/AT₁ cells. In fact, there was about a 50% reduction in c-*fos* in SYF/AT₁ cells compared to WT/AT₁ cells (Figure 4-5B).

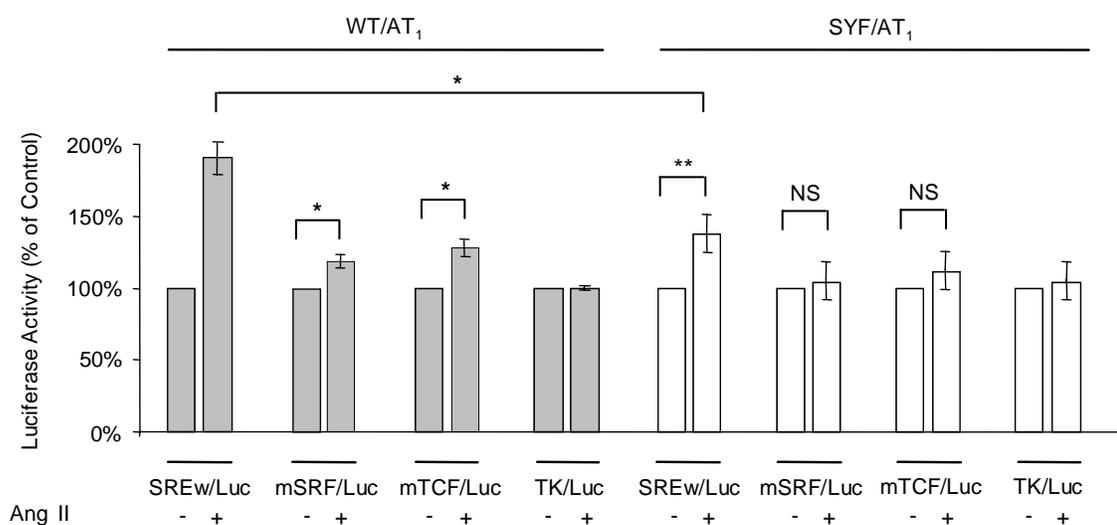


Figure 4-4. *c-fos* transcriptional activity in WT/AT₁ and SYF/AT₁ cells in response to Ang II. WT/AT₁ or SYF/AT₁ cells were transfected with the indicated plasmid and then stimulated with Ang II for 0 or 5 hr. Data are expressed as percentage of luciferase activity relative to unstimulated cells. These results are representative of three independent experiments. This figure is used with permission from (43).

Pretreatment of WT/AT₁ cells with PKC ζ MP also reduced *c-fos* protein amounts by 50%, while PKC ζ MP addition to Ang II-stimulated SYF/AT₁ cells resulted in a complete loss of Ang II-induced *c-fos* protein production. These data demonstrate that Ang II-induced *c-fos* protein synthesis is partially influenced by PKC ζ and partially by Src kinase-dependent signaling.

***c-fos* Phosphorylation Is Dependent Upon Src Kinase-RSK Signaling**

Previous reports have shown that *c-fos* activity is not only regulated at the transcriptional level, but also post-translationally via specific phosphorylation events (16). Specifically, phosphorylation of *c-fos* by RSK at Ser residues within the C-terminal tail increases the stability of *c-fos* and the subsequent growth-promoting effects associated with extended *c-fos* activity (16). Whether *c-fos* serine phosphorylation is

dependent upon c-Src/Yes/Fyn mediated ERK/RSK2 activation was next tested. WT/AT₁

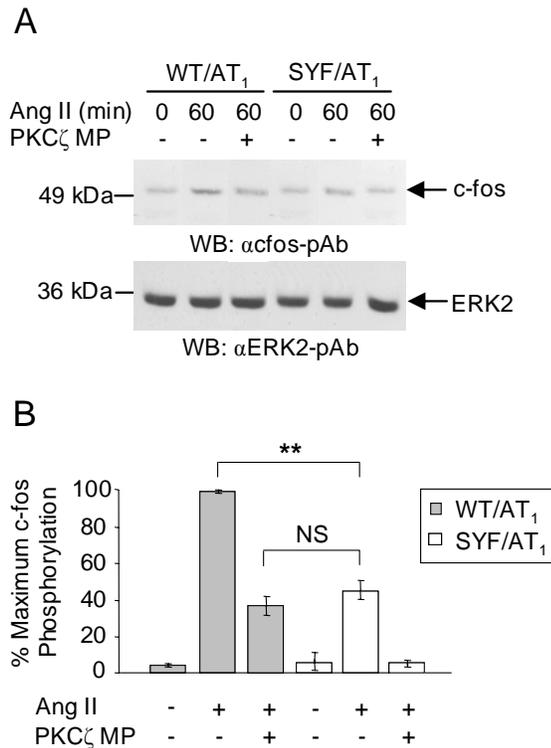


Figure 4-5. c-fos protein levels in response to Ang II in WT/AT₁ and SYF/AT₁ cells. A: WT/AT₁ or SYF/AT₁ cells were stimulated with 100 nM Ang II for 0 or 60 min, and c-fos protein production and total protein levels assessed by Western blot using the indicated antibodies. Some cells were pretreated with 1 μ M PKC ζ MP for 1 hr as indicated. B: These data are expressed as the percentage of maximum c-fos protein production in response to Ang II. Protein amounts were densitized and values were normalized to the amount of c-fos protein in WT/AT₁ cells treated with Ang II. These results are representative of three independent experiments. This figure is used with permission from (43).

and SYF/AT₁ cells were stimulated with Ang II and cell lysates were immunoprecipitated with anti-phosphoserine antibody and Western blotted with a c-fos specific antibody to assess for changes in c-fos phosphorylation at Ser residues. A marked four-fold increase in c-fos phosphorylation was observed in WT/AT₁ cells after 60 min of Ang II treatment (Figure 4-6A). Phosphorylated c-fos levels remained elevated after 120 min of Ang II treatment, and began to decline by 240 minutes. In contrast, a comparatively small

increase in c-fos phosphorylation was observed in Ang II-treated SYF/AT₁ cells after 60 min, and phosphorylated c-fos levels declined to baseline amounts by 120 min. These results are displayed graphically, and were normalized to total c-fos protein amounts to account for differences in total c-fos protein (Figure 4-6B). Collectively, these data suggest that c-fos phosphorylation is reduced in the absence of c-Src/Yes/Fyn and subsequent ERK/RSK2 activation in response to Ang II.

Angiotensin II-induced Cell Proliferation Requires RSK And PKC ζ Activity

The consequence of selective RSK inhibition on Ang II-induced cell proliferation was next assessed since RSK activation (under the influence of Src kinase activated ERK1/2) appears to modulate c-fos levels and activity. Recently, a highly selective and potent RSK inhibitor, SL0101, was isolated by Lannigan and colleagues (129).

Interestingly, this compound is a natural product derived from the *foresta refracta* plant found in the amazon rain forest. SL0101 has already been shown to selectively inhibit RSK without interfering with upstream activators of RSK like ERK, MEK, EGFR and PKC (77). Furthermore, this compound has been shown to prevent cell proliferation in cancer cells and thus has established RSK as a target for therapeutic intervention and SL0101 as an anti-cancer agent (18). However, a role for RSK in Ang II-induced cell proliferation remains to be established.

WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II, and cell proliferation assessed via a direct cell count. A marked 3-fold increase in cell number was observed in WT/AT₁ cells treated with Ang II (Figure 4-7A). Ang II-induced increases in cell number were reduced by 1.5 fold in SYF/AT₁ cells relative to WT/AT₁ cells.

Pretreatment with SL0101 reduced WT/AT₁ cell number to levels found in SYF/AT₁ cells treated with Ang II. However, SL0101 pretreatment did not affect Ang II-induced

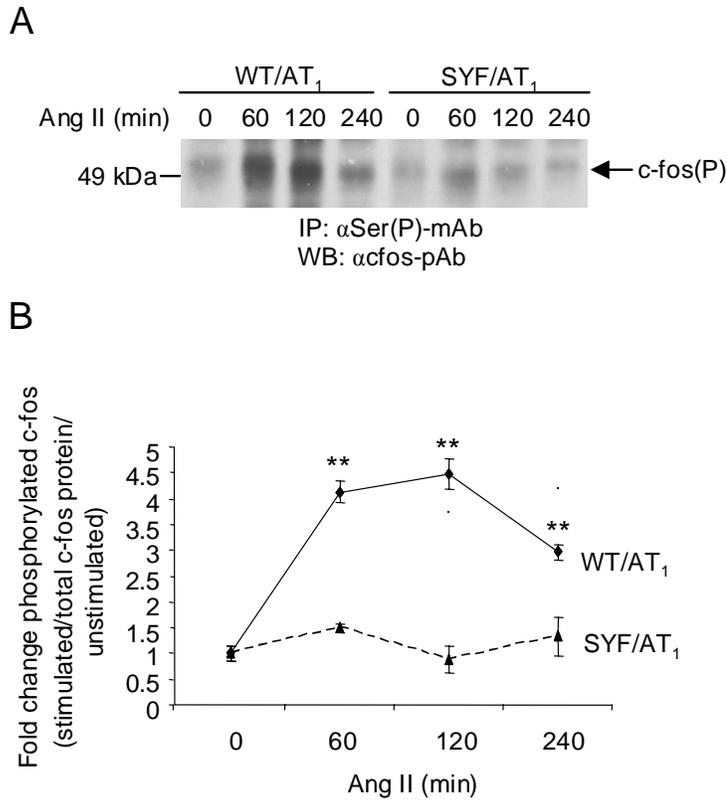


Figure 4-6. Ang II-induced c-fos phosphorylation in WT/AT₁ and SYF/AT₁ cells. WT/AT₁ or SYF/AT₁ cells were stimulated with 100 nM Ang II for 0, 60, 120, or 240 min. A: c-fos phosphorylation was then assessed by immunoprecipitating and Western blotting with the indicated antibodies. B: Protein bands were densitized and values were expressed as a fold change in c-fos phosphorylation relative to unstimulated cells as a function of total c-fos protein amounts. These results are representative of three independent experiments. This figure is used with permission from (43).

increases in SYF/AT₁ cell number, suggesting that Ang II-induced cell proliferation occurring independent of c-Src/Yes/Fyn is not dependent upon RSK2 and also that SL0101 exhibits low toxicity. Finally, PKC ζ MP pretreatment completely attenuated Ang II-induced increases in SYF/AT₁ cell number but only partially attenuated Ang II-induced increases in WT/AT₁ cell number. Furthermore, the addition of PKC ζ MP and SL0101 to WT/AT₁ cells blocked all Ang II-induced increases in cell number. These data therefore demonstrate that PKC ζ partially regulates Ang II-induced cell proliferation

independent of RSK2. Furthermore, RSK2 mediates Ang II-induced cell proliferation downstream of c-Src/Yes/Fyn since the addition of SL0101 to SYF/AT₁ cells did not further lower the already reduced amount of Ang II-induced cell proliferation exhibited by these cells.

To recapitulate these findings in another cell type, the effect of SL0101 addition on cells devoid of heterotrimeric G protein activation (CHO/AT₁-M5 cells) was examined. A marked 3-fold increase in cell number was observed in CHO/AT₁ cells treated with Ang II (Figure 4-7B). Ang II-induced increases in cell number were reduced by about 0.5 fold in CHO/AT₁-M5 cells relative to CHO/AT₁ cells. Pretreatment of CHO/AT₁-M5 cells with SL0101 completely blocked Ang II-induced increases in cell number, whereas SL0101 partially attenuated cell number in CHO/AT₁ cells stimulated with Ang II to levels found in Ang II-treated CHO/AT₁-M5 cells. Thus, RSK2 does not regulate Ang II-induced cell proliferation through heterotrimeric G protein-dependent mechanisms. The pretreatment of CHO/AT₁ cells with PKC ζ MP partially reduced Ang II-induced cell proliferation; however, CHO/AT₁-M5 cell number was not affected by the addition of PKC ζ MP. Therefore, PKC ζ appears to partially mediate Ang II-induced cell proliferation, and it does so downstream of heterotrimeric G proteins. Finally, all Ang II-induced cell proliferation in CHO/AT₁ cells was blocked by pretreatment with PKC ζ MP and SL0101. Collectively, these data further demonstrate that Ang II-induced cell proliferation is regulated by heterotrimeric G protein/PKC ζ and c-Src/Yes/Fyn/RSK-dependent signaling

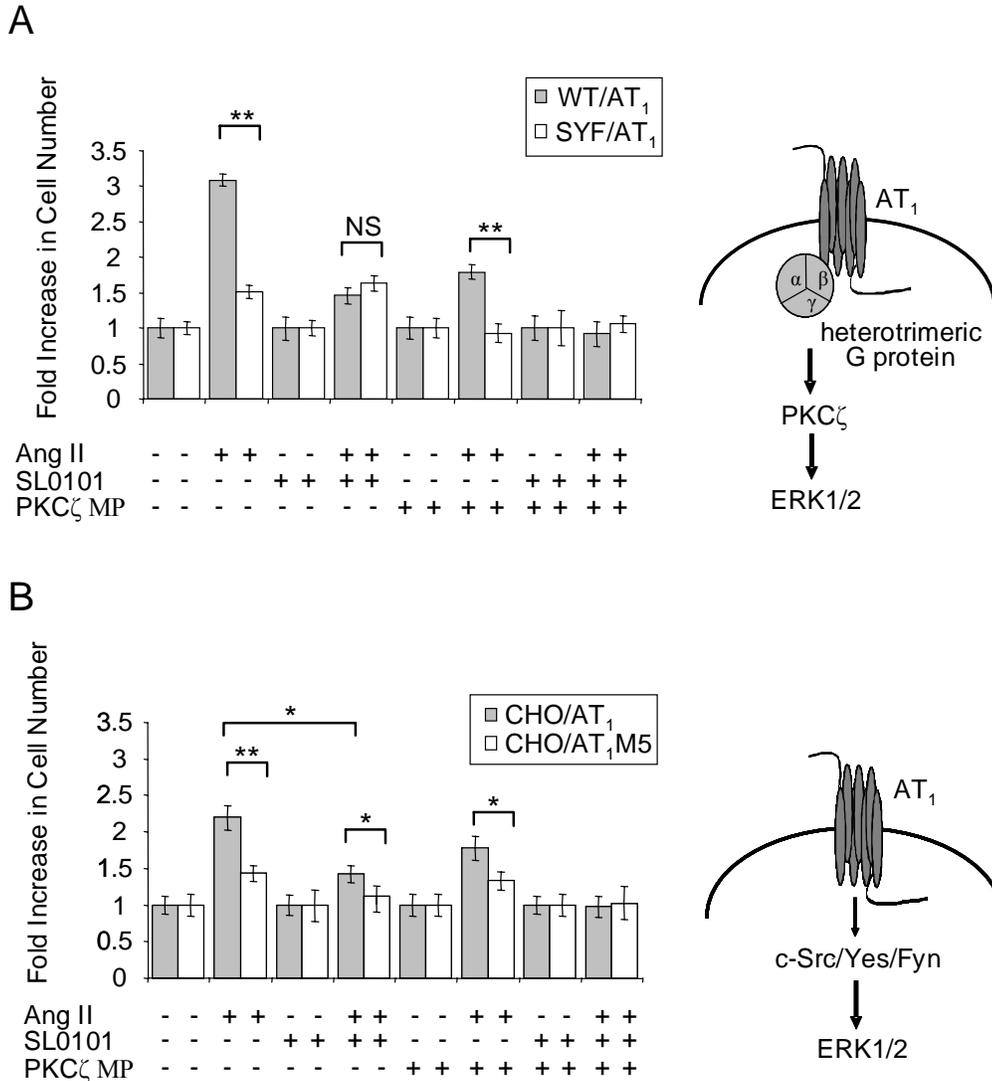


Figure 4-7. Ang II-induced cell proliferation in response to RSK and PKC ζ inhibition. A: WT/AT₁ and SYF/AT₁ cells were stimulated with Ang II for 0 (-) or 24 (+) hr. Some cells were pretreated with either 30 μ M SL0101, 1 μ M PKC ζ MP, or both inhibitors in combination for 1 hr as indicated. Cells were counted and fold changes in cell number plotted relative to unstimulated cells (left). The drawing on the right illustrates the intact endogenous ERK1/2 activation pathway in the SYF/AT₁ cells. B: CHO/AT₁ and CHO/AT₁-M5 cells were stimulated with Ang II for 0 (-) or 24 (+) hr. Some cells were pretreated with either 30 μ M SL0101, 1 μ M PKC ζ MP, or both inhibitors in combination for 1 hr as indicated. Cells were counted and fold changes in cell number plotted relative to unstimulated cells (left). The drawing on the right illustrates the intact endogenous ERK1/2 activation pathway in CHO/AT₁-M5 cells. These results are representative of three independent experiments. This figure is used with permission from (43).

Angiotensin II-induced ERK1/2 Activation Is Mediated By Both Src Kinases and PKC ζ in Vascular Smooth Muscle Cells

Thus far, it has been demonstrated that Ang II-induced ERK1/2 activation is mediated by Src kinase and heterotrimeric G protein/PKC ζ signaling in both MEF and CHO cells. It was next determined if Ang II-induced ERK1/2 activation is also mediated by both c-Src/Yes/Fyn and heterotrimeric G protein/PKC ζ signaling in cells which endogenously express the AT₁ receptor. Here, primary cultures of VSMCs isolated from rat aortas were utilized. c-Src/Yes/Fyn or PKC ζ activity was then blocked using pharmacological inhibitors. VSMC were pretreated with either PP2, PKC ζ MP or PKC ζ MP and PP2 in combination. Cells were then stimulated with 100 nM Ang II for 0, 5 and 10 min. Whole cell lysates were prepared and Western blotted with phospho-specific ERK1/2 antibodies to identify changes in ERK1/2 activation. Ang II-induced ERK1/2 activation occurred after 5 min of Ang II treatment in VSMC (Figure 4-8A). ERK1/2 activation was significantly reduced in VSMC treated with either PP2 or the PKC ζ pseudosubstrate alone, and treatment with either of these inhibitors alone resulted in about a 50% reduction in ERK2 phosphorylation (Figure 4-8B). Furthermore, ERK1/2 activation was completely reduced in cells treated with both PP2 and the PKC ζ pseudosubstrate in combination. Collectively, these data confirm that the mechanisms of intracellular ERK1/2 activation are the same in the AT₁ receptor-transfected MEF and CHO cells as in VSMCs which endogenously express the AT₁ receptor. VSMC ERK1/2 activation occurs via c-Src/Yes/Fyn or heterotrimeric G protein/PKC ζ -dependent signaling in response to angiotensin II.

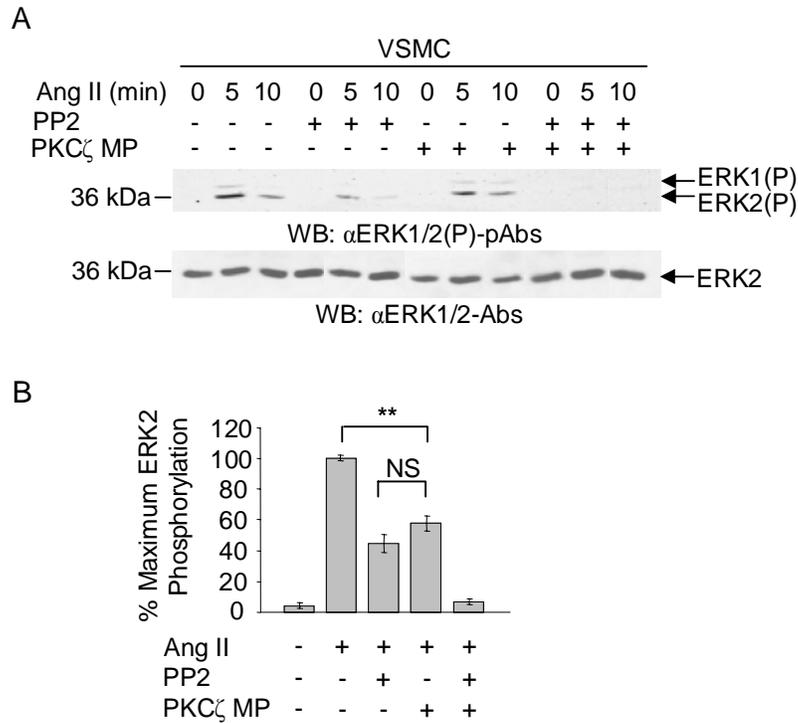


Figure 4-8. Ang II-induced ERK1/2 activation is mediated by c-Src/Yes/Fyn and PKC ζ -dependent signaling in VSMC. **A:** VSMCs were pretreated with either DMSO, 30 μ M PP2, 1 μ M PKC ζ myristoylated pseudosubstrate, or both PP2 and PKC ζ MP in combination. All inhibitor treatment times were 1 hr. ERK1/2 activation was then measured via Western blot analysis using the indicated antibodies (top panel). Total ERK1/2 protein loading was demonstrated by stripping the membrane and reprobing with the indicated antibodies (bottom panel). **B:** Three representative Western blots of **A** were scanned and densitized and the percent maximum ERK2 phosphorylation was calculated by dividing by active ERK2 amounts in non-inhibitor treated cells after 5 min of Ang II stimulation and multiplying by 100. This figure is used with permission from (43).

Angiotensin II-induced Cell Migration Is Attenuated in VSMCs Treated With SL0101

Previous work has demonstrated that aberrant migration and proliferation of VSMCs is triggered by angiotensin II during cardiovascular diseases such as atherosclerosis (72). VSMC migration precedes proliferation, and results in the

formation of a fibrous plaque. It was next tested whether Ang II-induced VSMC migration could be attenuated by pretreating with SL0101.

VSMCs were pretreated for 1 hour with the indicated concentration of SL0101 or vehicle control, and then stimulated with 100 nM angiotensin II for 24 hours. Cells were allowed to migrate across a nylon membrane where they were then stained, photographed and counted via quantification of the stain using a spectrophotometer. Little to no cell migration occurred in the absence of Ang II; however, cell migration did occur in response to Ang II (Figure 4-9). Furthermore, Ang II-induced cell migration was attenuated in a dose-dependent manner through the addition of SL0101. Maximum reductions in Ang II-induced cell migration were observed with the addition of 100 μ M SL0101. As such, some Ang II-induced cell migration is RSK-dependent.

Discussion

In Chapters 2 and 3, intracellular ERK1/2 activation is shown to be mediated by both c-Src/Yes/Fyn-dependent and heterotrimeric G protein/PKC ζ -dependent signaling, and both of these signaling pathways contribute equally to cell proliferation in response to Ang II (42). Here, these findings are extended by defining the mechanism as to how this occurs (summarized in Figure 4-10). The key to these findings is that heterotrimeric G protein/PKC ζ -dependent signaling dictates whether ERK1/2 translocates into the nucleus and phosphorylates specific transcription factors like elk1, leading to increased c-fos transcriptional activity. c-Src/Yes/Fyn-signaling, on the other hand, phosphorylates ERK1/2 in the cytoplasm, where ERK1/2 remains and complexes with RSK2. RSK2 becomes activated, and then translocates into the nucleus to modulate *c-fos* transcription and c-fos protein activity. As such, these signaling events coordinately regulate proliferation in response to Ang II.

Effect of RSK inhibition on angiotensin II-induced cell migration

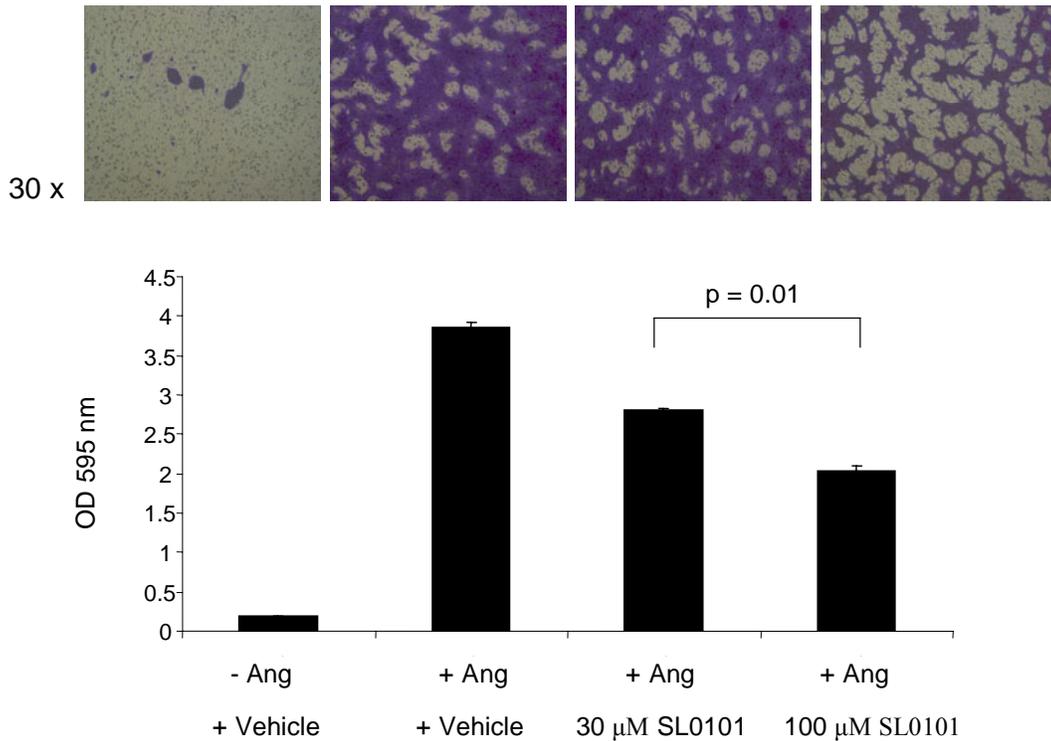


Figure 4-9. Angiotensin II-induced cell migration is attenuated through selective RSK inhibition. Cells were pretreated with the indicated concentration of SL0101 or vehicle control for 1 hour. Cells were then stimulated with 100 nM Ang II for 24 hr, and allowed to migrate. Migratory cells (purple) were photographed after Ang II treatment, and cell number was assessed through spectrophotometry. These data are representative of three independent experiments. This figure is used with permission from (43).

These findings support the idea that two separate pools of ERK1/2 exist within the cell: a pool of ERK1/2 which complexes with and activates RSK2 and a pool of ERK1/2 which translocates directly into the nucleus. However, ERK2 activation resulted in the dissociation of the ERK2-RSK complex within these cells. Other studies in COS7 cells ectopically expressing the three RSK isoforms demonstrated that ERK-RSK complexes

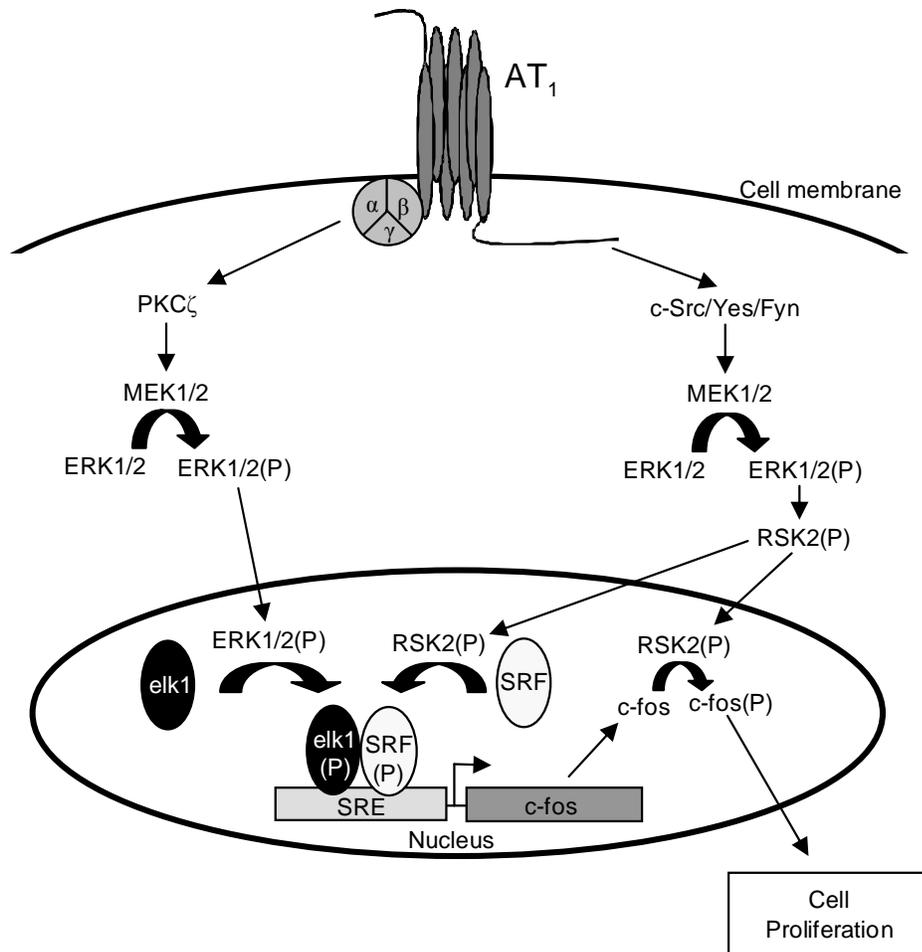


Figure 4-10. Mechanistic diagram illustrating how Src kinase and PKC ζ -dependent ERK1/2 activation pathways dually regulate Ang II-induced cell proliferation. ERK1/2 activation is separately mediated by Src family tyrosine kinase and heterotrimeric G protein/PKC ζ signaling in response to Ang II. With regards to PKC ζ regulation, ERK1/2 translocates into the nucleus upon stimulation of the AT $_1$ receptor. Here, ERK1/2 phosphorylates elk1, which binds to the c-fos SRE and partially regulates c-fos transcriptional activity. c-fos transcriptional activity is also regulated by binding of the SRF. The SRF is phosphorylated in response to nuclear RSK2, which translocates into the nucleus after being phosphorylated by ERK1/2 in the cytoplasm. Cytoplasmic ERK1/2 phosphorylation is regulated by c-Src/Yes/Fyn-dependent signaling, independent of heterotrimeric G protein/PKC ζ activity. Additionally, nuclear RSK2 directly phosphorylates c-fos and increases the activity and stability of this protein. Thus, two independent pathways of ERK1/2 activation coordinately regulate Ang II-induced cell proliferation by inducing c-fos transcription and increasing c-fos activity through the post-translational modification of this protein. This figure is used with permission from (43).

did not dissociate when ERK1/2 was activated by EGF (161). Here, ERK1/2 and RSK2 rapidly co-associate in response to Ang II, with maximum co-association occurring after 5 min of Ang II treatment. This co-association is completely dependent upon c-Src/Yes/Fyn signaling. Other pools of ERK1/2 do not co-associate with RSK since nuclear accumulation of ERK1/2 occurs well before RSK2 nuclear translocation, indicating that some ERK1/2 does not complex with RSK2. Additionally, this nuclear translocation of ERK1/2 occurs at the same time as a portion of ERK1/2 complexes with RSK2. Note that RSK2-ERK1/2 complexes do not translocate into the nucleus together since pretreatment of cells with PKC ζ MP blocked all ERK1/2 nuclear translocation but did not affect RSK2 nuclear translocation (data not shown). Therefore, ERK1/2 and RSK2 nuclear translocation occur separately in response to Ang II; some ERK1/2 directly enters the nucleus whereas another portion complexes with RSK2. Previous studies have also shown that a pool of ERK complexes with RSK, though RSK-ERK co-association patterns may differ depending upon the receptor activated (12).

The duration as well as the magnitude of ERK1/2 activation has also been proposed to regulate gene expression and other specific intracellular responses. Catt and colleagues have shown that the magnitude and duration of ERK1/2 activation in response to gonadotropin-releasing hormone (GnRH) depends upon the mechanism whereby ERK1/2 is activated, with a more sustained pattern of ERK activation occurring through G α q/PKC-dependent signaling, and a more transient pattern of ERK activation caused by transactivation of the EGFR by the GnRH receptor (123). Sustained GnRH-induced ERK1/2 activation lead to ERK1/2 nuclear accumulation, whereas ERK1/2 activated transiently failed to accumulate in the nucleus. Other reports have shown that sustained

vs. transient patterns of ERK1/2 lead to different cellular responses including cell death or cell growth/proliferation (98). In Chapters 2 and 3, it was demonstrated that sustained patterns of Ang II-induced ERK1/2 activation occurred in cells in which both c-Src/Yes/Fyn and heterotrimeric G protein/PKC ζ signaling were intact (44). More transient patterns of ERK1/2 activation are evident when either of these pathways was disrupted. In addition, longer and more robust ERK1/2 activation pattern achieved through the simultaneous activation of both signaling cascades resulted in greater amounts of Ang II-induced cell proliferation. Less robust and transient patterns of ERK1/2 activation achieved by the disruption of one pathway resulted in a diminished amount of cell proliferation in response to Ang II. Cell proliferation was found to be dependent upon the amount of *c-fos* transcribed and phosphorylated, with a greater amount of *c-fos* transcription occurring when both heterotrimeric G protein/PKC ζ and c-Src/Yes/Fyn signaling pathways were dually activated. Furthermore, c-fos protein stability was extended when c-Src/Yes/Fyn-dependent signaling was intact. Thus, the magnitude and duration of ERK1/2 activation affect Ang II-induced cell proliferation as well.

Ang II-induced cell proliferation is associated with aberrant Ang II release and subsequent cell growth and proliferation during the progression of various cardiovascular diseases and cancers (24, 140, 145, 157, 159). Here, we show that both heterotrimeric G protein and Src family tyrosine kinase signaling must be blocked in order to completely attenuate Ang II induced ERK1/2 activation. RSK2 can also be inhibited, as downstream target for ERK1/2 activated by c-Src/Yes/Fyn-dependent signaling. Thus SL0101, a compound previously shown to be an anti-cancer agent, may

also provide possible therapeutic benefits to patients suffering from certain cardiovascular diseases.

In summary, Ang II-induced cell proliferation is mediated by two mechanistically different signaling pathways, both dependent on ERK1/2. Whether ERK1/2 activates RSK2 or translocates into the nucleus in order to mediate cell proliferation is determined by c-Src/Yes/Fyn or heterotrimeric G protein/PKC ζ signaling, respectively. Interestingly, both of these pathways positively regulate c-fos transcription and c-fos protein activity via the phosphorylation of different transcription factors which initiate cell proliferation. Thus, this work demonstrates that the AT₁ receptor, a prototypical GPCR, coordinately utilizes both heterotrimeric G protein and Src family tyrosine kinase-dependent signaling pathways in order to achieve angiotensin II-induced proliferation.

CHAPTER 5
THE N-TERMINAL SH2 DOMAIN OF THE TYROSINE PHOSPHATASE, SHP-2, IS
ESSENTIAL FOR JAK2-DEPENDENT SIGNALING VIA THE ANGIOTENSIN II
TYPE 1 RECEPTOR

Introduction

Chapters 2-4 have focused on the role of Src family tyrosine kinases in angiotensin II-dependent signaling. In addition to Src kinases, members of the *Janus* family of tyrosine kinases are also critical mediators of angiotensin II-dependent signaling. Specifically, Jak2 has been shown to play a role in a number of signaling processes occurring downstream of the AT₁ receptor (112). However, the cellular events underlying Jak2-dependent signaling downstream of this prototypical G protein-coupled receptor are not completely understood.

It is clear that angiotensin II is a potent activator of Jak2, *in vitro* and *in vivo* (3, 79, 83). Once activated, Jak2 is recruited to the AT₁ receptor signaling complex, where it mediates STAT activation and subsequent transcriptional regulation (2, 3, 116). Under certain conditions however, the signaling properties of angiotensin II can be maladaptive and lead to cardiovascular pathologies (82, 95, 96, 100). Interestingly, Jak2 has also been linked to many of the same disease states that angiotensin II has been linked (91, 95). Furthermore, blockage of either angiotensin II signaling with AT₁ receptor antagonists or inhibition of Jak2 function using the Jak2 pharmacological inhibitor, AG490, has the same beneficial effect of ameliorating these cardiovascular diseases. Thus, some have suggested that the deleterious actions of angiotensin II may be mediated, in part, by the intracellular actions of Jak2.

How Jak2 is activated by and recruited to the AT₁ receptor is poorly understood. Previous work suggests that Jak2 is first activated in the cytoplasm and then recruited to the AT₁ receptor (3). The amino acids on the AT₁ receptor that facilitate this interaction are encoded by the ³¹⁹YIPP motif (3). Once bound to the receptor, Jak2 then acts as a molecular bridge in recruiting STAT1 to the receptor complex (2). STAT1 is then phosphorylated by Jak2 and translocates into the nucleus where it modulates gene transcription. Work by Marrero and colleagues suggest that the protein tyrosine phosphatase SHP-2 may act to facilitate Jak2-dependent signaling via the AT₁ receptor (81). However, the mechanism by which this occurs is not known.

Here, it was hypothesized that the N-terminal SH2 domain of SHP-2 is required for angiotensin II-mediated, Jak2-dependent signaling. To test this, angiotensin II-dependent signaling was measured in fibroblasts derived from mice in which the N-terminal SH2 domain of SHP-2 was lacking (SHP-2^{Δ46-110}). Fibroblasts from wild type litter mates served as controls (SHP-WT). SHP-2 was found to be constitutively bound to the AT₁ receptor, and this occurs independent of the N-terminal SH2 domain of SHP-2.

While the SHP-2^{Δ46-110} cells were capable of activating Jak2 tyrosine kinase, they were unable to facilitate AT₁ receptor/Jak2 co-association, STAT activation and subsequent Ang II-mediated gene transcription when compared to wild type controls. These data therefore suggested that the N-terminal SH2 domain of SHP-2 was acting to recruit Jak2 to the AT₁ receptor signaling complex. Furthermore, the N-terminal SH2 domain of SHP-2 bound Jak2 predominantly, but not exclusively at Jak2 tyrosine residue 201. When this tyrosine was converted to phenylalanine, the ability of Jak2 to activate subsequent downstream signaling events was reduced. Finally, wild type SHP-2

transfected back into the SHP-2^{Δ46-110} cells restored Ang II-mediated, Jak2-dependent signaling. Collectively, these data suggest that the N-terminal SH2 domain of SHP-2 is essential for Ang II-mediated, Jak2-dependent signaling. The functional consequence of this interaction is to recruit Jak2 to the AT₁ receptor signaling complex and in turn promote downstream Jak2-dependent signaling.

Materials and Methods

Cell Culture

SHP-2^{Δ46-110} and wild type litter mate control fibroblasts (SHP-2 WT) were generated as described (114). The cells were passaged weekly with DMEM media containing 4.5 g/L of glucose and supplemented with 10% fetal bovine serum. The cells were transfected with 10 μg of an HA-tagged AT₁ receptor cDNA expression plasmid (111) using 10 μl Lipofectin, following the manufacturer's instructions (Invitrogen). Cells were subsequently growth arrested in serum-free DMEM for 48 hrs prior to experimental use. All cell culture reagents were obtained from Invitrogen. All other reagents were purchased from Sigma Chemical or Fisher Scientific.

Immunoprecipitation

Normalized cellular lysates (~0.5 mg/ml) were immunoprecipitated exactly as described in Chapter 2 (117). The immunoprecipitating anti-phosphotyrosine antibody (PY20) was from BD Transduction Laboratories. The immunoprecipitating anti-HA (F7) and anti-Jak2 (HR758) antibodies were from Santa Cruz Biotechnology.

Western Blotting

Proteins were detected using enhanced chemiluminescence exactly as described in Chapter 2 (117). The anti-Stat1, anti-Stat3, anti-SHP-2, anti-phosphotyrosine (PY99) and

anti-GST blotting antibodies were from Santa Cruz Biotechnology. The anti-Jak2 (HR758) blotting antibody was from Upstate Biotechnology, Inc.

GST Pull Down Assays

The creation, expression and purification of the four GST/Jak2 fusion proteins has been described (163). Protein lysates were prepared from SHP-2^{Δ46-110} and wild type litter mate control fibroblasts as described. Cell lysates were first precleared with 7.5 μg of Sepharose-bound-GST for 1 h at 4°C. To each sample, 1.0 nmol of the indicated GST/Jak2 fusion protein was then added and incubated for 90-120 min at 4°C on a rocking platform. The beads were washed 4-5 times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) and resuspended in sample buffer. Sample buffer containing proteins were boiled, separated by SDS-PAGE, transferred onto nitrocellulose membranes, and Western blotted as described above.

Luciferase Assay

Cells were transfected with 10 μg HA-tagged AT₁ receptor cDNA expression plasmid and 5 μg of a luciferase reporter construct containing four tandem repeats of the IFN-γ Activating Sequence (GAS) element, upstream of a minimal TK promoter, in 10 μl Lipofectin. The cells were subsequently seeded into 6-well plates at 5x10⁵ cells per well and allowed to attach overnight. The cells were serum starved for 48 hrs and treated as indicated. Luciferase activity was measured from detergent extracts in the presence of ATP and luciferin using the Reporter Lysis Buffer System (Promega) and a luminometer (Monolight Model 3010).

Molecular Model of Jak2

A molecular model encoding full length, murine Jak2 has been described (41). The coordinates of the model were kindly provided to us in PDB format and regenerated

using the PDB software program (www.rcsb.org/pdb). Side chain surface accessibility of tyrosine 201 was calculated using the volume area dihedral angle reporter program, VADAR (154).

Statistical Analysis

Statistical comparisons were made by Student's *t* Test. Data are represented as the mean +/- SEM.

Results

SHP-2^{Δ46-110} and SHP-2 WT Cells

Figure 6-1A provides a brief overview of the cell lines used in these studies, which have previously been described (114). The SHP-2^{Δ46-110} cells are mouse embryonic fibroblasts which lack the N-terminal SH2 domain (amino acids ⁴⁶⁻¹¹⁰) on both alleles. The control cells are fibroblasts derived from wild type litter mates, and express the full length 64 kDa isoform of SHP-2. The SHP-2^{Δ46-110} cells express a 57 kDa truncated form of the protein at ~25% of wild type levels, presumably due to inefficient splicing of the mutant allele (114). We examined the cells and found that while they express detectable levels of Jak2, they express little to no AT₁ receptor protein (data not shown).

Thus, for the studies described below, the AT₁ receptor was transiently transfected into both cell types in order to establish a viable signaling system. Furthermore, both cell types were transfected with similar efficiency.

Jak2 Phosphorylation Is Not Influenced by the N-terminal SH2 Domain of SHP-2

It was first tested whether there was any difference in how the two cell lines activated Jak2. For this, both sets of cells were transiently transfected with the AT₁ receptor and then treated with 100 nM angiotensin II for the indicated times. Protein lysates were then prepared and subsequently immunoprecipitated with anti-Jak2

antibody. The precipitates were Western blotted with anti-Tyr(P) antibodies in order to measure Jak2 tyrosine phosphorylation levels (Figure 5-1B, top). These results show that angiotensin II was able to increase tyrosine phosphorylation levels of Jak2 in both cell types. To show that all lanes were precipitated equally, the membrane was Western blotted with anti-Jak2 antibody (Figure 5-1B, bottom).

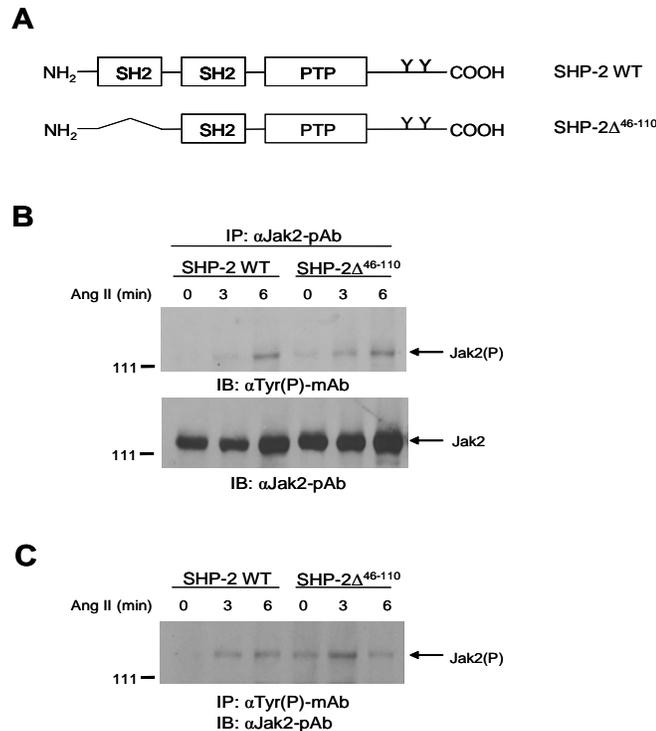


Figure 5-1. Jak2 tyrosine phosphorylation in SHP-2 WT or SHP-2Δ⁴⁶⁻¹¹⁰ fibroblast cells. A: Cartoon representing the structure of the full-length 64 kDa SHP-2 (SHP-2 WT) and a truncated 57 kDa isoform (SHP-2Δ⁴⁶⁻¹¹⁰). SH2= phosphotyrosine binding SH2 domain, PTP= protein tyrosine phosphatase domain, YY= the YY regulatory domain. B: SHP-2 WT or SHP-2Δ⁴⁶⁻¹¹⁰ cells were stimulated with 100 nM angiotensin II for 0, 3 and 6 min. Cellular lysates were immunoprecipitated (IP) with anti-Jak2 pAb and immunoblotted (IB) with anti-Tyr(P) mAb in order to determine total Jak2 phosphorylation (top). The membrane was then stripped and reprobed with anti-Jak2 pAb to demonstrate equal protein loading (bottom). C: Cellular lysates were immunoprecipitated with anti-phosphotyrosine mAb and immunoblotted with anti-Jak2 pAb to determine Jak2 tyrosine phosphorylation levels. All Westerns are representative of three independent experiments. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

To measure this in an alternate manner, a reciprocal precipitation was performed whereby lysates were immunoprecipitated with anti-Tyr(P) antibody and then Western blotted with anti-Jak2 antibody (Figure 5-1C). These results were similar to those in Figure 6-1B as both cell types were able to increase the tyrosine phosphorylation levels of Jak2, in response to angiotensin II. Collectively, the data in Figure 5-1 suggest that the N-terminal SH2 domain of SHP-2 is not required for angiotensin II-mediated Jak2 activation.

STAT1 and STAT3 Phosphorylation and STAT-mediated Gene Transcription Require the N-terminal SH2 Domain of SHP-2

Distal to Jak2 activation is STAT activation and gene transcription. To determine whether the N-terminal SH2 domain of SHP-2 had any effect on these cellular events, the ability of angiotensin II to promote STAT phosphorylation and STAT-mediated gene transcription was measured. Both cell types were again transfected with the AT₁ receptor and then treated with 100 nM angiotensin II for the indicated times. The lysates were subsequently immunoprecipitated with anti-Tyr(P) antibody and then Western blotted with anti-STAT1 antibody (Figure 5-2A). These data showed that while the Control cells were able to markedly increase the tyrosine phosphorylation levels of STAT1, the SHP-2^{Δ46-110} cells were not. When STAT3 levels were measured, a similar pattern was observed; the Control cells had markedly increased STAT3 tyrosine phosphorylation levels, while the SHP-2^{Δ46-110} cells did not.

To determine whether the N-terminal SH2 domain had any effect on STAT mediated gene transcription, both sets of cells were transfected with the AT₁ receptor plasmid and a luciferase reporter plasmid encoding a STAT responsive element. It has

previously been shown that this plasmid is a good indicator of Jak/STAT-mediated transcription (149). The cells were treated with 100 nM angiotensin II for the indicated

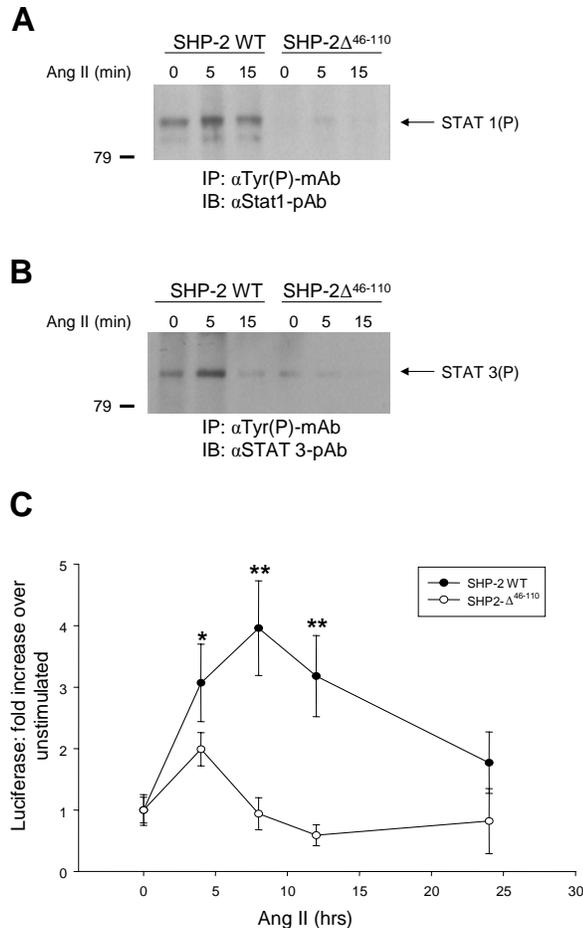


Figure 5-2. STAT1/3 phosphorylation and STAT-induced luciferase activity in SHP-2 WT or SHP-2 Δ^{46-110} transfected fibroblast cells. A: SHP-2 WT or SHP-2 Δ^{46-110} transfected cells were stimulated with 100 nM angiotensin II for 0, 5 and 15 min. Cellular lysates were immunoprecipitated with anti-Tyr(P) mAb and immunoblotted with anti-STAT1 pAb in order to determine total STAT1 phosphorylation. B: SHP-2 WT or SHP-2 Δ^{46-110} transfected cells were stimulated with 100 nM angiotensin II for 0, 5 and 15 min. Cellular lysates were immunoprecipitated with anti-Tyr(P) mAb and immunoblotted with anti-STAT3 pAb in order to determine STAT3 tyrosine phosphorylation. C: SHP-2 WT or SHP-2 Δ^{46-110} transfected cells were co-transfected with the luciferase reporter plasmid encoding a STAT responsive element. Cells were then stimulated with 100 nM angiotensin II for the indicated times and luciferase activity assessed. Data represent mean fold increase in luciferase activity relative to unstimulated cells. * = $p < 0.05$, ** = $p < 0.01$. All data are representative of three independent experiments. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

times and luciferase activity was measured (Figure 5-2C). SHP-2^{Δ46-110} cells had a marked reduction in their ability to generate luciferase activity in response to angiotensin II when compared to the wild type control cells. Collectively, the data in Figure 5-2 suggest that the N-terminal SH2 domain of SHP-2 is required for angiotensin II-mediated STAT1 and STAT3 tyrosine phosphorylation and angiotensin II-mediated Jak/STAT-dependent gene transcription.

Jak2-AT₁ Receptor Co-association Is Mediated by SHP-2

Previous work has suggested that SHP-2 can act as a positive mediator of angiotensin II-mediated, Jak2-dependent signaling (81). Therefore, the relationship between the AT₁ receptor, Jak2 and SHP-2 was next determined, within these two cell types. Here, both sets of cells were transfected with an HA-tagged AT₁ receptor plasmid and subsequently treated with 100 nM angiotensin II for the indicated times. The AT₁ receptor was immunoprecipitated from the lysates via the addition of anti-HA antibody. First, the precipitates were Western blotted with anti-SHP-2 antibody in order to access AT₁/SHP-2 interactions (Figure 5-3A). In both cell types, SHP-2 was constitutively bound to the AT₁ receptor and this did not change with ligand addition. It is worth noting however, that overall, there is less SHP-2^{Δ46-110} protein when compared to wild type SHP-2, but presumably this is due to the fact that the SHP-2^{Δ46-110} protein is expressed at ~25% the levels that of wild type. Thus, it appears that the N-terminal SH2 domain has no effect on AT₁/SHP-2 interactions.

HA immunoprecipitates were next Western blotted with anti-Jak2 antibody to access AT₁/Jak2 co-association. While angiotensin II was able to promote AT₁/Jak2 co-association in the wild type cells, it was unable to do this in the SHP-2^{Δ46-110} cells (Figure 5-3B). Furthermore, overnight ECL exposure failed to yield any Jak2 bands in the SHP-

$2^{\Delta 46-110}$ cells (data not shown). Thus, it appears that the N-terminal SH2 domain of SHP-2 is required for angiotensin II-dependent, AT₁/Jak2 co-association. Cells were again transfected with the AT₁ receptor and this time the lysates were immunoprecipitated with anti-Jak2 and then Western blotted with anti-SHP-2 antibody in order to access Jak2/SHP-2 co-association (Figure 5-3C, top). While angiotensin II induced Jak2/SHP-2 co-association in the wild type cells, this was completely lacking in the SHP-2 $^{\Delta 46-110}$ cells. Once again, overexposure of the film demonstrated that no Jak2/SHP-2 co-association was present in SHP-2 $^{\Delta 46-110}$ cells (data not shown). To demonstrate that all lanes were loaded similarly, the same membrane was Western blotted with anti-Jak2 antibody (Figure 5-3C, bottom). Thus, these data suggest that the N-terminal SH2 domain is required for angiotensin II-dependent Jak2/SHP-2 co-association.

Lastly, it has been previously demonstrated that the angiotensin II-dependent physical interaction of Jak2 with the AT₁ receptor requires AT₁ receptor amino acids 319-322, encoding the ³¹⁹YIPP motif. When ³¹⁹YIPP was mutated to ³¹⁹FAAA, the AT₁ receptor was unable to bind Jak2 in response to angiotensin II (3). Here, the role of the ³¹⁹YIPP motif on AT₁/SHP-2 interaction was determined. For this, the wild type cells were transfected with a plasmid encoding either an HA-tagged, wild type AT₁ receptor cDNA plasmid or an HA-tagged AT₁ receptor cDNA in which the ³¹⁹YIPP motif was converted to ³¹⁹FAAA. The cells were treated with 100 nM angiotensin II for the indicated times and AT₁ receptor protein was immunoprecipitated from the lysates via the addition of anti-HA antibody. The precipitates were then Western blotted with anti-SHP-2 antibody to access AT₁/SHP-2 interactions (Figure 5-3D). SHP-2 was found to be constitutively bound to the AT₁ receptor, and this did not change with ligand addition

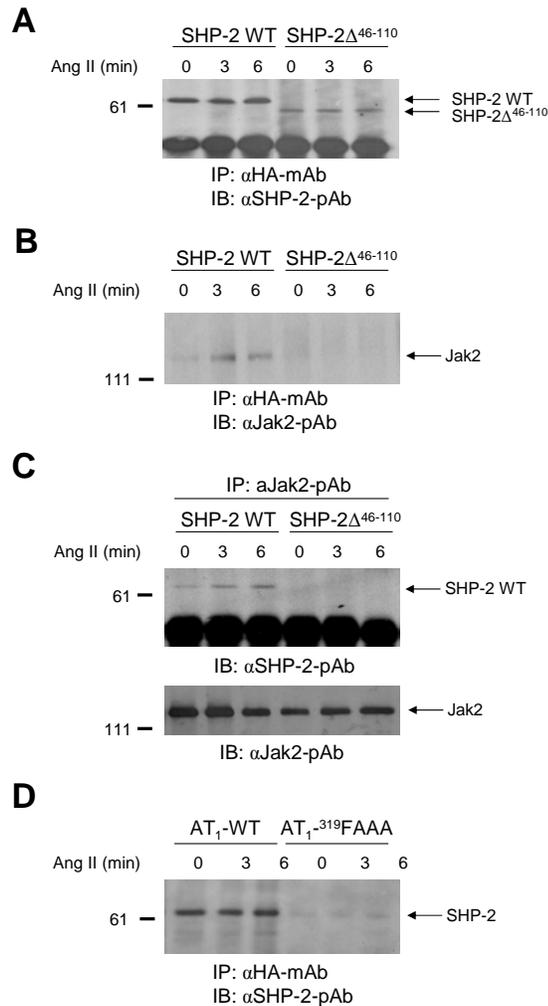


Figure 5-3. The recruitment of Jak2 to the AT₁ receptor is dependent upon SHP-2. A: AT₁/SHP-2 co-association was examined in SHP-2 WT or SHP-2 Δ ⁴⁶⁻¹¹⁰ transfected fibroblasts. Cells were stimulated with 100 nM angiotensin II for 0, 3 and 6 min. Cellular lysates were immunoprecipitated with anti-HA mAb and immunoblotted with anti-SHP-2 pAb. B: Cellular lysates were immunoprecipitated with anti-HA mAb and immunoblotted with anti-Jak2 pAb. C: Cells were stimulated with 100 nM angiotensin II for 0, 3 and 6 min. Cellular lysates were immunoprecipitated with anti-Jak2 pAb and immunoblotted with anti-SHP-2 pAb (top). The membrane was stripped and reprobed with anti-Jak2 pAb to demonstrate equal protein loading (bottom). D: AT₁/SHP-2 co-association was examined in AT₁-WT and AT₁-319FAAA transfected cells. Cellular lysates were immunoprecipitated with anti-HA mAb and immunoblotted with anti-SHP-2 pAb. All Westerns are representative of three independent experiments. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

(lanes 1-3). However, when cells were transfected with the $^{319}\text{FAAA}$ mutant receptor, no $\text{AT}_1/\text{SHP-2}$ co-association was observed, thus demonstrating that the $^{319}\text{YIPP}$ motif is critical for angiotensin II-mediated, $\text{AT}_1/\text{SHP-2}$ co-association.

Transfecting Wild Type SHP-2 back into SHP-2 $^{\Delta 46-110}$ Cells Restores STAT1 and STAT3 Phosphorylation and STAT-mediated Gene Transcription

These data in Figures 6-2 and 6-3 demonstrate that in the SHP-2 $^{\Delta 46-110}$ cells, signaling distal to Jak2 is virtually lost. Previous work has shown that the Δ^{46-110} mutation is recessive in nature and therefore does not act in a dominant negative fashion (114). Therefore, it was hypothesized that transfecting wild type SHP-2 back into these cells would restore these angiotensin II-mediated, Jak2-dependent signaling events, via the introduction of the N-terminal SH2 domain of SHP-2. To test this hypothesis, SHP-WT cells transfected with AT_1 receptor, and SHP-2 $^{\Delta 46-110}$ cells co-transfected with AT_1 receptor and wild type SHP-2, were utilized. The samples were immunoprecipitated with anti-HA antibody and then Western blotted with anti-Jak2 antibody to measure $\text{AT}_1/\text{Jak2}$ co-association (Figure 5-4A). These results indicate that addition of wild type SHP-2 into the SHP-2 $^{\Delta 46-110}$ cells restored the ability of Jak2 to bind the AT_1 receptor in response to angiotensin II. Next, angiotensin II-dependent STAT1 phosphorylation was examined (Figure 5-4B). These results similarly show that placement of wild type SHP-2 into the SHP-2 $^{\Delta 46-110}$ cells restores angiotensin II-dependent STAT1 tyrosine phosphorylation. Finally, we again measured Jak2-dependent gene transcription via the GAS-luciferase construct (Figure 5-4C). These data show that transfection of wild type SHP-2 cDNA into the SHP-2 $^{\Delta 46-110}$ cells restores angiotensin II-mediated, Jak2-dependent gene transcription when compared to similarly treated wild type SHP-2 cells.

In summary, these data in Figure 5-4 demonstrate that when wild type SHP-2 is added back to the SHP-2^{Δ46-110} cells, the ability of Jak2 to signal distal to its autophosphorylation is fully restored. Collectively, these data demonstrate that the N-terminal SH2 domain of SHP-2 is essential for angiotensin II-mediated AT₁/Jak2 co-association, STAT1 tyrosine phosphorylation and Jak2-dependent gene transcription.

Jak2 Tyrosine 201 Mediates Jak2-SHP2 Interactions

In Figure 5-3, it was demonstrated that Jak2 and SHP-2 form a physical co-association in response to angiotensin II and that this interaction requires the N-terminal SH2 domain of SHP-2. It was next determined which region(s) of Jak2 were mediating this interaction. For these experiments, a series of GST/Jak2 fusion proteins were utilized, and GST pull down assays were performed *in vitro*. Figure 5-5A is a cartoon showing each specific GST/Jak2 fusion protein used in this assay. First, wild type cells were treated with angiotensin II for the indicated times and protein lysates were prepared. Equal molar amounts of each GST/Jak2 fusion protein were added as indicated. The pull downs were eventually separated by SDS-PAGE and Western blotted with anti-SHP-2 antibody to detect SHP-2 binding (Figure 5-5B). It was found that the GST/Jak2 construct encoding amino acids 1-294 strongly bound SHP-2 in a ligand-dependent manner. Additionally, the second construct encoding amino acids 295-522 also showed some, albeit reduced, SHP-2 binding capability. However, when the same pull down assay was performed on the SHP-2^{Δ46-110} cells, no SHP-2 binding was observed (Figure 5-5C). These data therefore suggest that the physical co-association of SHP-2 with the GST/Jak2 fusion proteins is absolutely dependent on the N-terminal SH2 domain of SHP-2 as the GST/Jak2 fusion proteins bound wild type SHP-2, but failed to bind SHP-2^{Δ46-110}.

Tyrosine residue(s) on Jak2 that were binding SHP-2 were next examined.

Phosphotyrosine motifs that bind SH2 domains have a general consensus sequence of

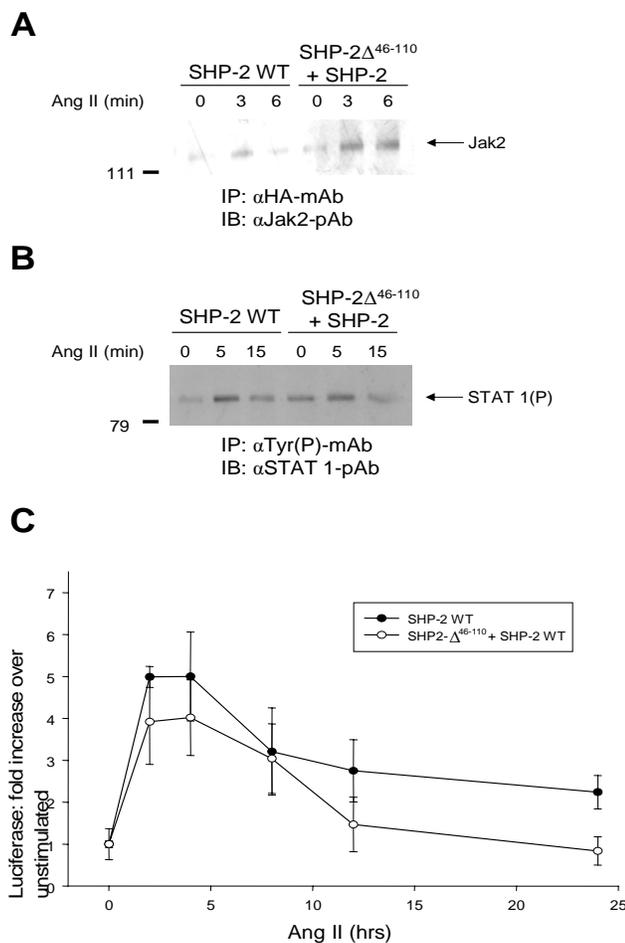


Figure 5-4. AT₁/Jak2 co-association, STAT1 phosphorylation and STAT-induced luciferase activity are restored in SHP-2 Δ^{46-110} fibroblasts transfected with wild type SHP-2. A: AT₁/Jak2 co-association was examined in SHP-2 WT or SHP-2 Δ^{46-110} + SHP-2 transfected fibroblasts. Cells were stimulated with 100 nM angiotensin II for 0, 3 and 6 min. Cellular lysates were immunoprecipitated with anti-HA mAb and immunoblotted with anti-Jak2 pAb. B: STAT1 phosphorylation was examined in SHP-2 WT or SHP- Δ^{46-110} + SHP-2 transfected fibroblasts. Cellular lysates were immunoprecipitated with anti-Tyr(P) mAb and immunoblotted with anti-STAT1 pAb. C: SHP-2 WT or SHP-2 Δ^{46-110} + SHP-2 transfected cells were co-transfected with the luciferase reporter plasmid encoding a STAT responsive element. Cells were then stimulated with 100 nM angiotensin II for the indicated times and luciferase activity assessed. Data represent mean fold increase in luciferase activity relative to unstimulated cells. All data are representative of three independent experiments. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

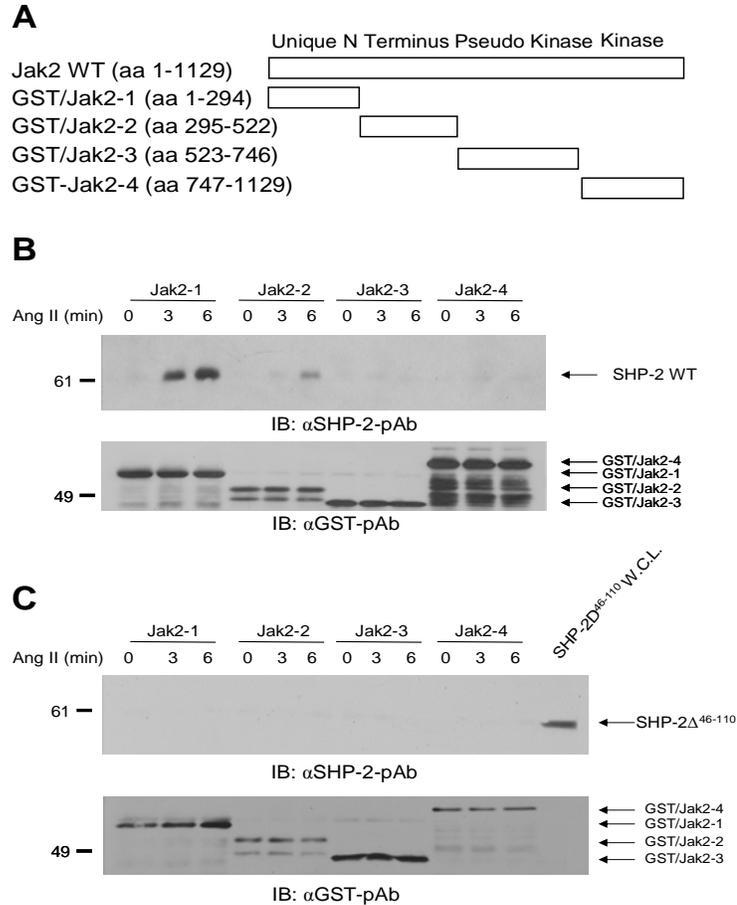


Figure 5-5. SHP-2/Jak2 co-association occurs mainly through interaction of Jak2 amino acids 1-294 and the N terminal SH2 domain of SHP-2. **A**: Cartoon illustrating the various GST/Jak2 fusion proteins used in **B** and **C**. The white box indicates the portion of wild type Jak2 contained within the fusion protein and the corresponding domains. Encompassing Jak2 amino acid sequences are listed for each GST fusion protein. **B**: GST pull down assays measuring the ability of each GST/Jak2 fusion protein to co-associate with SHP-2 WT. SHP-2 WT transfected cells were stimulated with 100 nM angiotensin II for 0, 3 and 6 min. Cellular lysates were separately incubated with each GST/Jak2 fusion protein as indicated and immunoblotted with anti-SHP-2 pAb (top). The membrane was then stripped and reprobed with anti-GST pAb to demonstrate equal protein loading (bottom). **C**: GST pull down assays measuring the ability of each GST/Jak2 fusion protein to co-associate with SHP-2Δ⁴⁶⁻¹¹⁰. SHP-2Δ⁴⁶⁻¹¹⁰ transfected cells were stimulated with 100 nM angiotensin II for 0, 3 and 6 min. Cellular lysates were separately incubated with each GST/Jak2 fusion protein as indicated and immunoblotted with anti-SHP-2 pAb (top). The membrane was then stripped and reprobed with anti-GST pAb to demonstrate equal protein loading (bottom). All Westerns are representative of three independent experiments. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

YXX(L/V/M). Two tyrosines in the first 294 amino acids of Jak2 bear some similarity to this sequence. They are ¹⁵²YLFV and ²⁰¹YNSV. For these experiments, these two tyrosines were individually converted to phenylalanine in the context of the GST fusion protein encoding the first 294 amino acids of Jak2. GST pull down assays were then performed similar to those described above using wild type cells. It was found that while the Y152F mutant was able to bind SHP-2, the Y201F mutant was not (Figure 5-6A). As such, the data suggest that the binding occurring between the GST fusion protein encoding the initial 294 amino acids of Jak2 and SHP-2, is mediated by Jak2 tyrosine residue 201.

While the data in Figure 5-6A indicate that SHP-2 is able to bind Jak2 residue 201 in the context of a GST fusion protein encoding the initial 294 amino acids of Jak2, whether tyrosine 201 was solvent accessible in the context of the full length protein was still in question. Kroemer and colleagues previously generated a molecular model of full length Jak2 (41, 73). A recent work describing the crystal structure encoding a portion of the Jak2 kinase domain suggests that Kroemer's model is highly accurate (75). Hence, the PDB coordinates of the full length Jak2 model were obtained, and it was found that tyrosine 201 was very much on the surface of the structure and presumably capable of binding SH2 domains (Figure 5-6B). The side chain surface accessibility of tyrosine 201 was calculated to be 148.9 angstroms squared, when the criteria for designating a residue as being solvent accessible is 15-20 angstroms squared.

Collectively, these data in Figures 5-5 and 5-6 suggest that Jak2 binds SHP-2. This interaction is critically dependent on the N-terminal SH2 domain of SHP-2 as the GST/Jak2 fusion proteins can bind wild type SHP-2, but cannot bind SHP-2^{Δ46-110}.

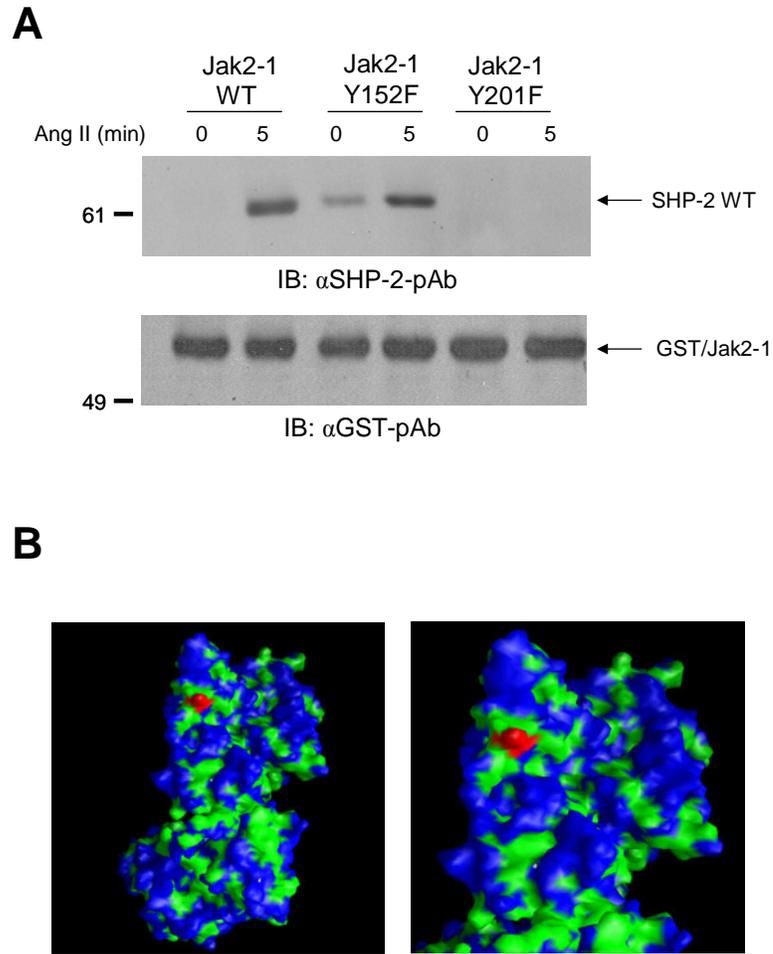


Figure. 5-6. Jak2 tyrosine 201 is critical for Jak2/SHP-2 interaction. A: GST pull down assays measuring the ability Jak2 to co-associate with SHP-2 WT when Jak2 tyrosine residues are mutated at either position 152 or 201. Cells were stimulated with 100 nM angiotensin II for 0 or 5 min. Cellular lysates were separately incubated with either Jak2-1 WT, Jak2-1 Y152F or Jak2-1 Y201F GST fusion proteins, and immunoblotted with anti-SHP-2 pAb (top). The membrane was then stripped and reprobed with anti-GST pAb to demonstrate equal protein loading (bottom). These Westerns are representative of three independent experiments. B: The position of tyrosine residue 201 (shown in red) in the context of full length Jak2. Shown is a lower magnification (left) and higher magnification (right) of the structure. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

Furthermore, Jak2 tyrosine residue 201 appears to be the major mediator of this phosphotyrosine/SH2 interaction.

Jak2 Tyrosine 201 Mediates AT₁ Receptor-Jak2 Co-association, STAT1 and STAT3 Activation and STAT-mediated Gene Transcription

The effect of tyrosine residue 201 on angiotensin II-mediated, Jak2-dependent signaling was next examined. Here, tyrosine 201 was converted to phenylalanine in the context of a full length, Jak2 cDNA expression plasmid. For these experiments, COS7 cells were transiently co-transfected with an HA-tagged AT₁ receptor plasmid and a plasmid encoding either a full length, wild type Jak2 cDNA (Jak2-WT), or a full length Jak2 cDNA in which tyrosine 201 was converted to phenylalanine (Jak2-Y201F). AT₁/Jak2 co-association, STAT1/3 phosphorylation and STAT-mediated gene transcription were then assessed. With regards to AT₁/Jak2 co-association, cell lysates were immunoprecipitated with anti-HA antibody and then Western blotted with anti-Jak2 antibody (Figure 5-7A). AT₁/Jak2 co-association occurred in response to angiotensin II treatment of Jak2-WT-transfected cells, but was reduced in cells transfected with the Jak2-Y201F plasmid. These data suggest that the tyrosine 201 residue contained within the JH6-JH7 domains of Jak2 contributes to AT₁/Jak2 co-association.

It was next assessed whether the Jak2-Y201F mutation also influenced STAT1/3 tyrosine phosphorylation. Lysates from cells transfected with either the Jak2-WT or Jak2-Y201F plasmids were immunoprecipitated with anti-Tyr(P) antibody and then Western blotted with either anti-STAT1 or anti-STAT3 antibodies. STAT1 phosphorylation occurred in response to angiotensin II treatment of JAK2-WT-transfected cells, but was attenuated in cells transfected with the Jak2-Y201F plasmid (Figure 5-7B). Likewise, STAT3 phosphorylation was also attenuated in cells transfected with Jak2-Y201F plasmid when compared to Jak2-WT-transfected cells (Figure 5-7C).

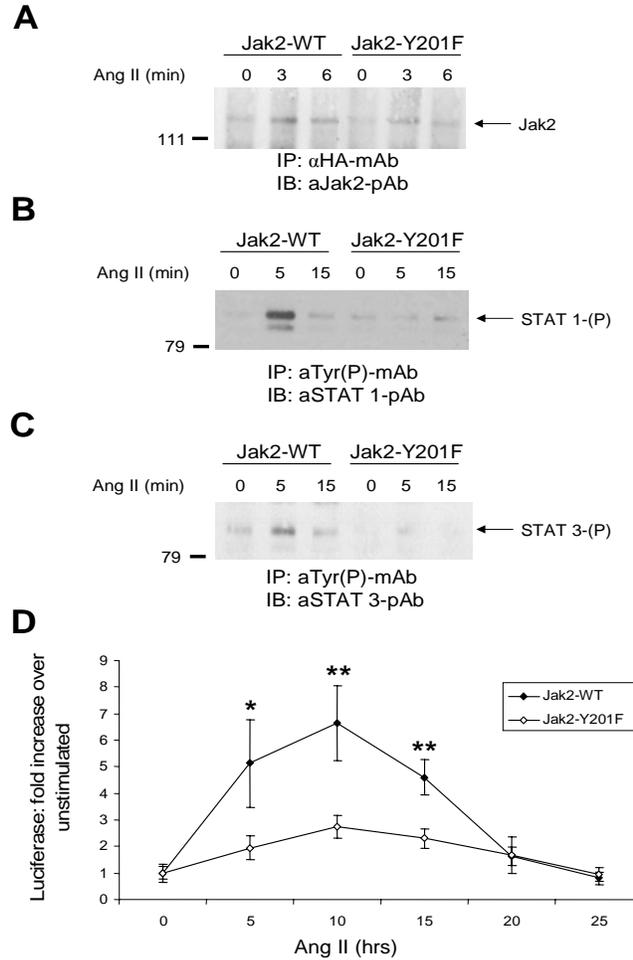


Figure 5-7. Mutation of Jak2 tyrosine 201 reduces Jak2-dependent signaling in response to angiotensin II. A: AT₁/Jak2 co-association was measured in Jak2-WT or Jak2-Y201 transiently-transfected COS7 cells. Cells were stimulated with 100 nM angiotensin II for 0, 3 or 6 min. Cell lysates were immunoprecipitated with anti-HA mAb, and then immunoblotted anti-Jak2 pAb. B: STAT1 phosphorylation was measured in Jak2-WT or Jak2-Y201 transiently-transfected COS7 cells. Cells were stimulated with 100 nM angiotensin II for 0, 5 or 15 min. Cell lysates were immunoprecipitated with anti-Tyr(P) mAb and then immunoblotted anti-STAT1 pAb. C: STAT 3 phosphorylation was measured in Jak2-WT or Jak2-Y201 transiently-transfected COS7 cells as in B with the exception that cellular lysates were immunoblotted with anti-STAT3 pAb instead. D: Jak2-WT or Jak2-Y201F transfected cells were co-transfected with the luciferase reporter plasmid encoding a STAT responsive element. Cells were then stimulated with 100 nM angiotensin II for the indicated times and luciferase activity assessed. Data represent mean fold increase in luciferase activity relative to unstimulated cells. All data are representative of three independent experiments. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

As such, Jak2 tyrosine 201 contributes to angiotensin II-mediated, Jak2-dependent STAT1/3 activation in addition to AT₁/Jak2 co-association.

Lastly, the effect of the Jak2 Y201F mutation on STAT-mediated gene transcription was assessed. COS7 cells were transfected with the AT₁ receptor plasmid and a luciferase reporter plasmid encoding a STAT responsive element. In addition, these same cells were co-transfected with either the Jak2-WT plasmid or the Jak2-Y201F plasmid. All cells were then treated with 100 nM angiotensin II for the indicated times and luciferase activity was measured. The Jak2-Y201F cells exhibited a significant reduction in their ability to generate luciferase activity in response to angiotensin II, when compared to the wild type control cells (Figure 5-7D). These data therefore suggest that Jak2 tyrosine 201 contributes to Jak2/STAT-dependent gene transcription in addition to Jak2/AT₁ co-association and STAT1/3 phosphorylation.

Discussion

Here, the functional role of the N-terminal SH2 domain of SHP-2 in angiotensin II-mediated, Jak2-dependent signal transduction was analyzed. It was found that SHP-2 appears to constitutively bind the AT₁ receptor and this is independent of the N-terminal SH2 domain. However, this SH2 domain appears essential for the recruitment of Jak2 to the AT₁ receptor and the signaling events that are distal to AT₁/Jak2 co-association. Specifically, in cells that lack the N-terminal SH2 domain of SHP-2, angiotensin II was unable to promote AT₁/Jak2 co-association, STAT tyrosine phosphorylation and Jak2-dependent gene transcription. Thus, the N-terminal SH2 domain of SHP-2 appears to be a rate limiting factor in angiotensin II-mediated, Jak2-dependent signal transduction.

Region(s) of Jak2 that specifically bind SHP-2 were also identified. GST pull down assays indicated that the initial 294 amino acids strongly bind wild type SHP-2, but

not a mutant form of SHP-2 lacking the N-terminal SH2 domain. Additionally, a GST fusion protein containing Jak2 amino acids 295-522 also bound wild type SHP-2, but not the mutant. However, this second fusion protein bound SHP-2 with about 10% the efficiency of the construct containing amino acids 1-294. Site directed mutagenesis of the GST fusion protein containing Jak2 amino acids 1-294 indicated that Jak2 tyrosine residue 201 was the principal mediator of SHP-2 binding as conversion of this tyrosine residue to phenylalanine abolished this interaction. Furthermore, analysis of a molecular model encoding full length Jak2 suggests that Y201 is on the surface of the full length protein.

The data in Figure 6-7 demonstrate that mutation of Jak2 tyrosine residue 201 markedly reduces, but does not fully abolish signaling events distal to Jak2. There are several possible explanations for this. First, the GST fusion protein containing amino acids 295-522 was found to specifically bind the N-terminal SH2 domain of SHP-2, although not as efficiently as the construct containing amino acids 1-294 (Figure 5-5). Thus, it is possible that, in addition to tyrosine 201, a tyrosine residue located between amino acids 295 and 522 may be binding the SH2 domain. Examination of this region of Jak2 identified nine tyrosine residues. However, none bear a reasonable similarity to the consensus SH2-binding motif, YXX(L/V/M). Thus, if one or more of these tyrosines is binding the N-terminal SH2 domain of SHP-2, it is doing so through a less conserved binding motif.

Second, it has been previously demonstrated that the Jak2 tyrosine motif, ²³¹YRFRR, is required for binding the AT₁ receptor in transfected COS7 cells (116). When this sequence was converted to ²³¹FAAAA, Jak2 was unable to bind the AT₁

receptor. It appears that the ²³¹YRFRR motif provides a critical structural function rather than a phosphotyrosine interaction as a single Y231F mutation does not prevent AT₁/Jak2 co-association. The amino terminal half of Jak2 has nineteen “band 4.1” domains. These domains are characterized as having hydrophobic clusters among charged amino acids. Homology between domains is not determined by primary amino acid sequence, but rather by secondary structure prediction which allows for the detection of hydrophobic clusters within domains of very low sequence identity. The domains are separated by intervening unrelated sequences of variable lengths. Analysis of the region around amino acids 200-235 indicates that tyrosine 201 resides between domains 8 and 9 while the ²³¹YRFRR motif is in the middle of domain 10. Thus, one possible explanation for the inability of the Jak2-²³¹FAAAA mutant to bind the AT₁ receptor, even with an intact tyrosine at position 201, is that the Jak2-²³¹FAAAA substitution mutation alters the tertiary structure of domain 10, which in turn reduces the otherwise exposed nature of tyrosine 201.

What is known, however, is that the N-terminal SH2 domain of SHP-2 is singularly essential for binding Jak2 and bringing Jak2 to the AT₁ receptor signaling complex; when this single domain is deleted, the ability of Jak2 to bind the AT₁ receptor is completely lost. In contrast however, there appears to be some redundancy in how Jak2 binds SHP-2 and in turn the AT₁ receptor; areas showing some binding ability, either directly or via a tertiary structure, include tyrosine 201, the ²³¹YRFRR motif and possibly a tyrosine located between amino acids 295-522. A proposed model for the role of SHP-2 in promoting AT₁ receptor-induced JAK/STAT signaling is summarized in Figure 5-8.

Whether the AT₁-Jak2-SHP2 complex recruits other proteins still remains to be elucidated.

In summary, this work provides novel insight into the proximal signaling elements that facilitate angiotensin II-mediated, Jak/STAT-dependent signaling. As such, these studies may provide insight into the signaling associated with other GPCRs that activate Jak2.

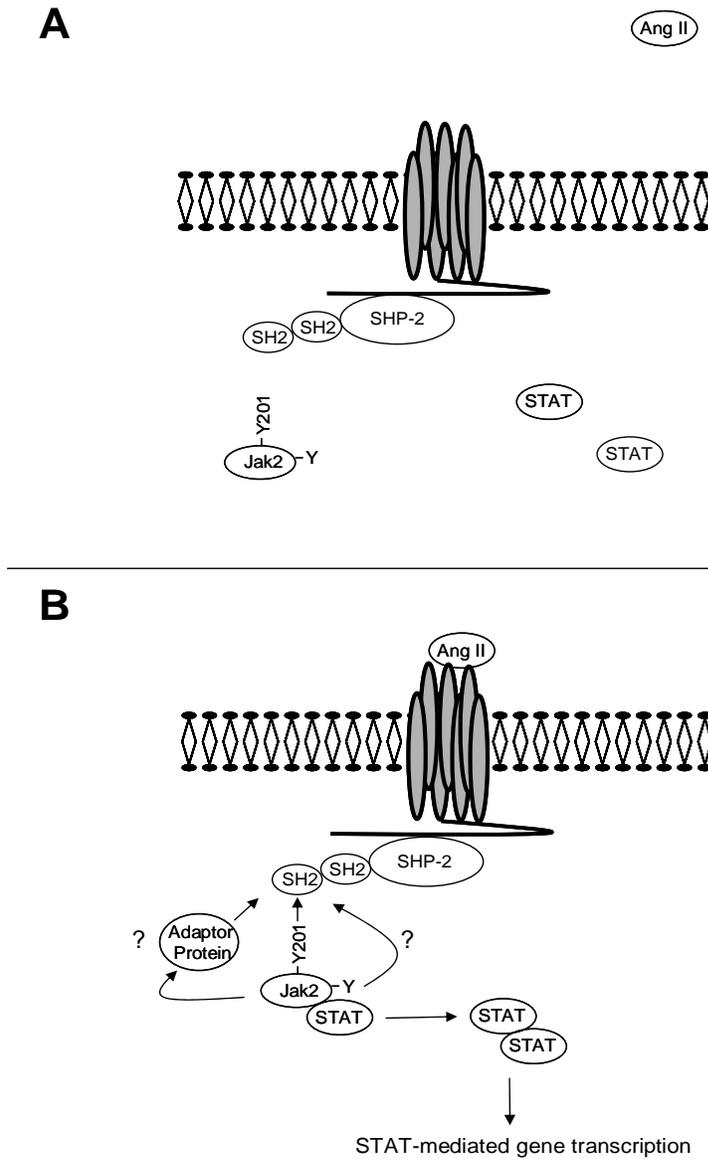


Figure 5-8. Proposed mechanism for Jak2/SHP-2 interactions upon stimulation of the AT₁ receptor. A: In the absence of ligand, SHP-2 is bound to the AT₁ receptor while Jak2 and STATs remain within the cytoplasm. B: Upon binding of angiotensin II to the AT₁ receptor, phosphorylated Jak2 binds SHP-2 at the N-terminal SH2 domain. With respect to Jak2, this interaction predominately occurs at an exposed tyrosine 201 residue on the surface of Jak2. Other Jak2 tyrosine residues or other adaptor proteins may also mediate Jak2/SHP-2 interactions. Once the AT₁/Jak2/SHP-2 complex is formed, Jak2 tyrosine phosphorylates the STATs, resulting in STAT-dependent gene transcription. Reproduced from Cellular Signaling, in press article, Copyright 2006 with permission from Elsevier.

CHAPTER 6 CONCLUSIONS AND IMPLICATIONS

Summary of Results

Since the discovery in the 1990's that the AT₁ receptor—a prototypical G protein-coupled receptor—can activate tyrosine kinases, a wave of research has been aimed at elucidating the roles of these kinases in angiotensin II signaling as well as the mechanisms leading to their activation. This work expands upon the current knowledge of angiotensin II, tyrosine kinase-mediated signaling. First, the role of Src family tyrosine kinases in intracellular ERK1/2 activation and a mechanism for how these proteins mediate angiotensin II-induced cell proliferation is established. It appears that Src kinases activate as much as 50% of ERK1/2 within the cell, and Src kinase-activated ERK1/2 mediates cell proliferation through the cytoplasmic activation of RSK. In addition, it is demonstrated that the remaining 50% of ERK1/2 activation and Ang II-induced cell proliferation is mediated by heterotrimeric G protein/PKC ζ signaling, and occurs in a Src kinase-independent manner. ERK1/2 activated by these signaling proteins directly translocates into the nucleus and modulates a portion of early response gene transcription and cell proliferation. Next, RSK is for the first time implicated in angiotensin II-induced cell proliferation. Given the recent discovery of a potent and highly-selective naturally occurring inhibitor of RSK (77), RSK inhibition represents a promising new area of therapeutic intervention for the treatment of Ang II-associated diseases. Finally, this work also identifies an SHP-2 dependent mechanism for the activation of another important tyrosine kinase, Jak2. As such, more insight is gained

into how the AT₁ receptor activates this kinase and initiates Jak2/STAT signaling inside the cell. Collectively, the work in this dissertation defines important aspects of tyrosine kinase-mediated signaling downstream of the AT₁ receptor, and helps to redefine the angiotensin II signaling paradigm.

Src Kinases and Angiotensin II-induced Cell Proliferation

Previously published findings have had discrepancies with regards to the role of Src kinases in Ang II-induced ERK1/2 activation. Some reports claim that these events are entirely mediated by Src kinase-dependent signaling (53, 115, 140), whereas other work has shown that ERK1/2 activation can occur via other signaling pathways not traditionally thought to be Src-dependent (33, 44-46, 70, 86, 124, 153). Some caveats to the findings dealing specifically with Src kinases were that these studies relied on either non-specific pharmacological inhibitors, or only inhibited c-Src activity and did not take into account other functionally redundant Src family members, such as Yes and Fyn. Therefore, a role for Src kinases in angiotensin II-induced ERK1/2 activation had not yet firmly been established. Here, MEF cells isolated from c-Src, Yes, Fyn compound knockout mice were used to discern the role of Src kinases in Ang II-induced ERK1/2 activation and cell proliferation, providing the advantage of studying these processes in a Src kinase-deficient background.

These results from Chapters 2-3 indicate that Ang II-induced ERK1/2 activation does not rely solely on Src kinase signaling. Roughly about 50% of intracellular ERK1/2 activation is mediated via Src kinase-dependent signaling. In response to Ang II, Src kinases are most likely activated through the Shc/Grb2/Sos signaling cascade as previously described (115). Sos then serves as a guanine exchange factor (GEF), and activates RAS by exchanging GDP for GTP. Once Ras becomes activated, it activates

ERK1/2 via the traditional MAP kinase signaling cascade, consisting of Ras induced Raf1 phosphorylation, followed by Raf1-dependent MEK1/2 activation and finally MEK1/2-induced ERK1/2 activation. These events have all been previously described, and provide a mechanism whereby Src kinases activate ERK1/2 in response to AT₁ receptor activation.

Src kinases are a rate-limiting step in the activation of as much as 50% of total phosphorylated ERK1/2. Treatment of SYF/AT₁ cells with Ang II resulted in a reduced amount of ERK1/2 activation when compared to WT/AT₁ control cells. These results clearly show that a portion of Ang II-induced ERK1/2 activation is Src-dependent. In addition, inhibition of either heterotrimeric G protein or PKC ζ activity completely attenuated the remaining portion of ERK1/2 activation. Thus, PKC ζ and heterotrimeric G protein activation are rate-limiting steps in the activation of ERK1/2 independent of Src kinases. As such, ERK1/2 activation is controlled by two-independent signaling pathways.

There is also a difference in what happens to ERK1/2 when activated by either Src kinase or heterotrimeric G protein/PKC ζ -dependent signaling as described in Chapter 4. In response to c-Src/Yes/Fyn signaling, ERK1/2 activates RSK2. This presumably occurs in the cytoplasm since GFP-ERK2 studies found that ERK2 does not translocate into the nucleus when activated by Src kinases (Chapter 2). In response to heterotrimeric G protein/PKC ζ signaling, ERK1/2 translocates into the nucleus. Therefore, the ability of ERK1/2 to enter into the nucleus or remain within the cytoplasm and phosphorylate substrates like RSK is determined by the upstream signaling pathway activating ERK1/2. A looming question is how exactly does intracellular ERK1/2 distinguish that it has been

activated by either c-Src/Yes/Fyn or heterotrimeric G protein/PKC ζ signaling, and know to either enter the nucleus or phosphorylate RSK within the cytoplasm.

The exact answer to this question is still unresolved; however, a number of possible mechanisms can explain how the cell distinguishes between Src kinase or heterotrimeric G protein/PKC ζ activated ERK1/2 (Figure 6-1). For example, the subcellular distribution of ERKs may be altered depending upon the upstream signaling initiating ERK1/2 activation. Previous work has suggested that ERK1/2 nuclear localization is in part mediated by the nuclear exportin protein, Crm-1 (122). Overexpression of Crm-1 inhibits nuclear translocation of phospho-ERK1/2 as well as elk1 phosphorylation in response to Ang II, presumably by causing ERK1/2 to rapidly exit the nucleus, thereby preventing nuclear accumulation (122). In addition, inhibition of Crm-1 restores the ability of ERK1/2 to translocate into the nucleus in cells lacking heterotrimeric G protein signaling or treated with PKC inhibitor. Furthermore, it has been suggested that Crm-1 activity is negatively regulated by heterotrimeric G protein and PKC signaling (122). Therefore, Crm-1 protein appears to govern whether or not ERK1/2 enters the nucleus, and is regulated by heterotrimeric G protein/PKC signaling. Perhaps heterotrimeric G protein/PKC ζ activation shuts off a portion of Crm-1 nuclear export activity, causing some ERK1/2 to enter into the nucleus. In addition, a portion of ERK1/2 would be activated by Src kinase dependent signaling, but would be sequestered in the cytoplasm by the remaining active Crm-1. As such, ERK1/2 subcellular localization would be regulated at the point of Crm-1 by the presence or absence of heterotrimeric G protein/PKC ζ signaling. A more detailed study of Crm-1 function with respect to

ERK1/2 distribution will need to be performed in order to examine the validity of this hypothesis. This proposed mechanism is illustrated in Figure 6-1.

ERK1/2 nuclear localization could also be influenced via specific phosphorylation/dephosphorylation events dependent upon the upstream activation pathway. It has been well established that ERK1/2 activation occurs via phosphorylation at a conserved T-E-Y motif within the activation loop by MEK. Perhaps other phosphorylation or dephosphorylation events mediate whether ERK1 and ERK2 translocate into the nucleus or remain within the cytoplasm. For example, previous studies have demonstrated that conversion of amino acid residues 312-320 to alanine within GFP-ERK2 prevented ERK2 from being retained in the cytoplasm (106). Further work by Takishima and colleagues demonstrated that a specific Y314A mutation within the GFP-ERK2 fusion protein was sufficient to block the cytoplasmic accumulation of ERK2 (125). This mutation blocked the ability of ERK2 to be anchored in the cytoplasm by PTP-SL, a protein tyrosine phosphatase which has been shown to retain phosphorylated ERK2 within the cytoplasm when overexpressed (165). Thus, perhaps the dephosphorylation of ERK2 at tyrosine 314 by PTP-SL is triggered by Src kinase-dependent signaling, effectively anchoring this protein within the cytoplasm so that it can dock to and activate RSK. Alternatively, dephosphorylation of ERK1/2 by PTP-SL could prevent ERK1/2 dimerization, an event which is thought to be necessary for ERK1/2 nuclear translocation to occur. As such, ERK1/2 would effectively be retained within the cytoplasm. This mechanism is illustrated in Figure 6-2.

Another possibility is that the ability of phosphorylated ERK1/2 to either be targeted to the nucleus or cytoplasm is dependent upon substrate-recognition. It has been

shown that active ERK1/2 localize to effectors containing DEF (docking site for ERK, (F)/(Y) -X-(F)/(Y) -P) or D-domain (docking domain) motifs (97). Furthermore,

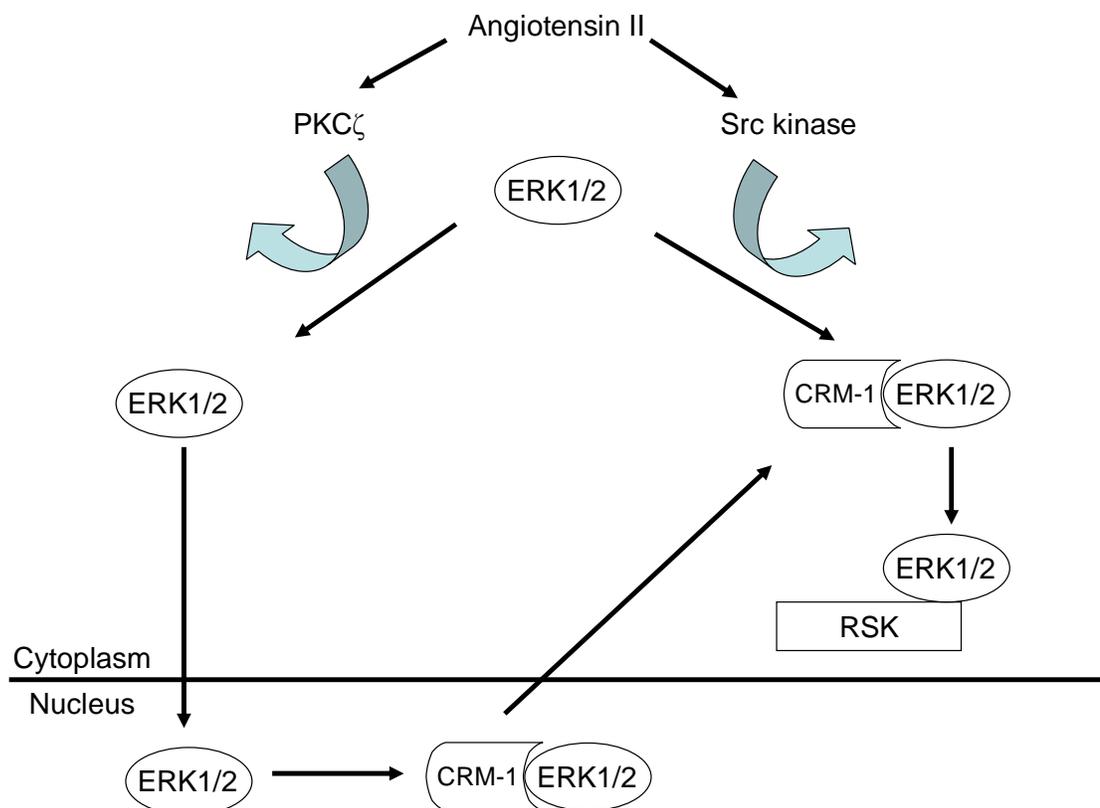


Figure 6-1. Possible CRM-1-dependent mechanism influencing ERK1/2 localization. ERK1/2 is activated in a Src kinase or PKC ζ -dependent manner in response to Ang II. ERK1/2 activated by PKC ζ translocates into the nucleus. When active, CRM-1 shuttles ERK1/2 back into the cytoplasm, where it sequesters ERK1/2 phosphorylated by Src kinases. Src kinase activated ERK is then free to bind RSK. A portion of CRM-1 activity would be shut-off by heterotrimeric G protein/PKC ζ signaling, allowing some ERK1/2 to remain in the nucleus.

Murphy and colleagues have shown that mutations in the DEF-domain binding pocket prevent activation of DEF-domain-containing effectors but not RSK, which contains a D domain (27). Conversely, mutation of the ERK2 CD domain, which interacts with D domains, prevents RSK activation but not DEF-domain signaling proteins. Additionally, ERK1/2 DEF mutants were deficient in elk1/TCF activity. As

such, perhaps when ERK1/2 is activated within the cytoplasm, a portion these phosphorylated proteins are sequestered by RSK through binding of the ERK1/2 CD domain to the

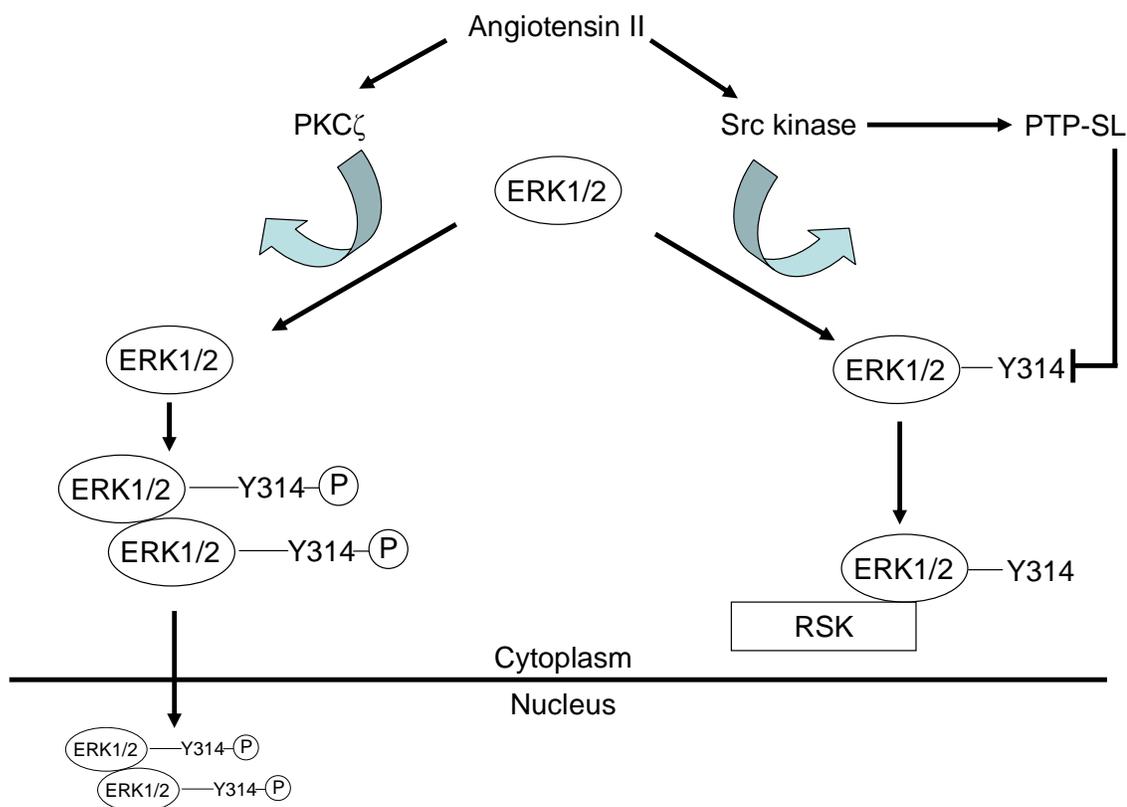


Figure 6-2. Possible mechanism whereby phosphorylation of tyrosine 314 affects ERK1/2 subcellular localization. ERK1/2 activation occurs via PKC ζ and Src kinase-dependent signaling. Once activated, ERK1/2 is phosphorylated at tyrosine 314 through an undetermined mechanism, causing ERK1/2 to dimerize and translocate into the nucleus. Src kinases phosphorylate PTP-SL, which dephosphorylates nearby ERK1/2 at tyrosine 314. This disables ERK1/2 from dimerizing, and sequesters it to the cytoplasm. Cytoplasmic ERK1/2 then binds and activates RSK.

D domain of RSK. This would prevent a portion of ERK1/2 from directly translocating into the nucleus. There appears to be much more ERK1/2 than RSK inside the cell, as

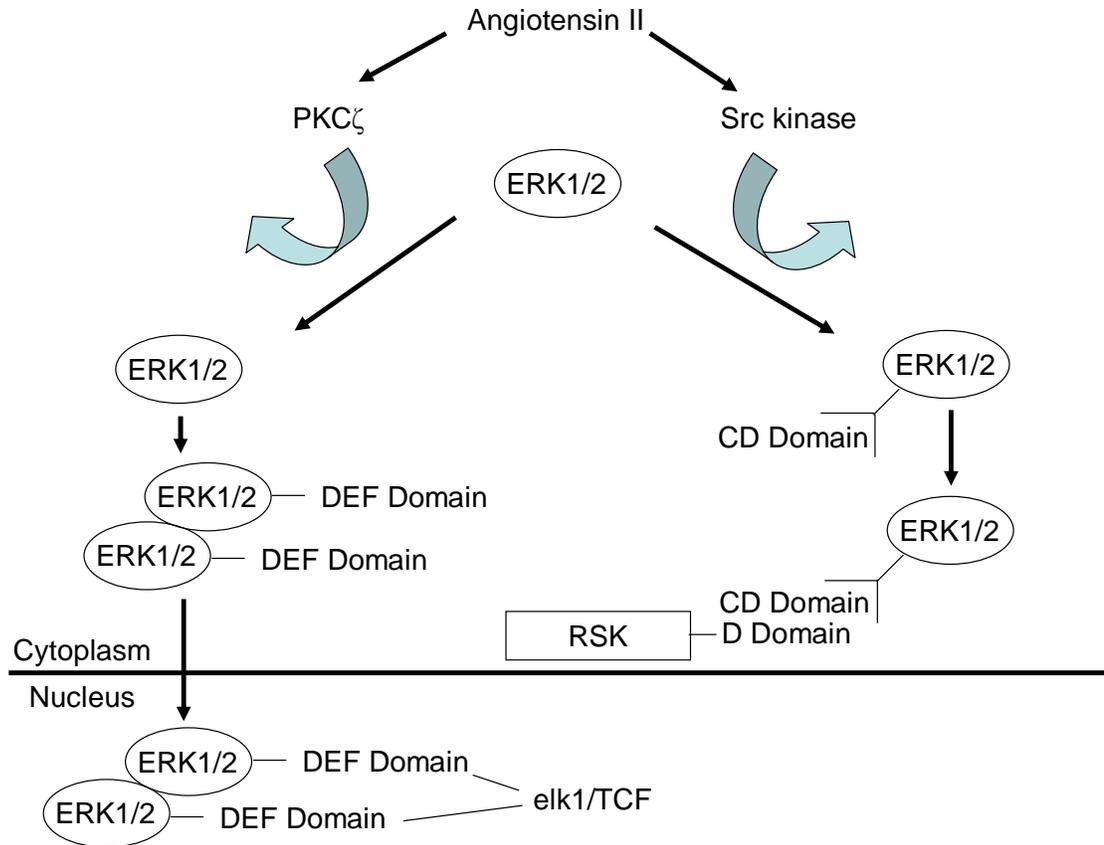


Figure 6-3. Proposed mechanism whereby substrate recognition influences ERK1/2 subcellular localization. ERK1/2 activation occurs via PKC ζ and Src kinase-dependent signaling. ERK1/2 activation via PKC ζ signaling causes receptor dimerization, which sequesters the CD domain but exposes residues important for DEF domain recognition (DEF domain). Dimerized ERK1/2 translocates into the nucleus, and activates elk1/TCF via DEF domain interactions. ERK1/2 in the cytoplasm recognizes RSK via D domain interaction.

was observed by Western blot as well as immunofluorescence in Chapters 2-4. This observation is in line with previous findings that show only about 50% of ERK1/2 co-localizes with RSK in *Xenopus* oocytes (52). Therefore, it is likely that some ERK1/2 does not come in contact with RSK and forms dimers, while at the same time a portion of ERK1/2 is binding to RSK. Dimerization may block the ability of ERK1/2 to interact

with the D domains of RSK in the cytoplasm, while at the same time creating a favorable tertiary structure for DEF domain recognition. Perhaps Src kinase and PKC ζ upstream signaling affect the processes mentioned in the proceeding two paragraphs, which ultimately contributes to favored substrate recognition and ultimately an ERK1/2 effect within the nucleus or cytoplasm. These events are illustrated in Figure 6-3.

It is likely that heterotrimeric G protein/PKC ζ signaling and Src kinase-dependent signaling influence whether or not activated ERK1/2 enters the nucleus or remains in the cytoplasm through a combination of the mechanisms just listed. From preliminary data generated by our group, it appears that not all ERK1/2 and RSK co-localized within the cell in the absence of ligand treatment (Figure 6-4). Therefore, it is highly likely that differences in ERK1/2 subcellular distribution affect whether or not ERK1/2 is activated by either Src kinase-dependent signaling or heterotrimeric G protein/PKC ζ -dependent signaling. More work will need to be done in order to determine exactly how ERK1/2 is targeted to the nucleus and cytoplasm.

Since MAP kinase signaling has been implicated in so many processes, many groups have already begun to recognize the importance of identifying the mechanisms underlying ERK1/2 signaling processes in order to selectively inhibit certain signaling events without disrupting ERK1/2 signaling as a whole. For example, selective pharmacological inhibitors have already been generated which block ERK1/2 binding to either the DEF or D domains within ERK1/2 substrates (27). These compounds, along with other targeting strategies, will be important for discerning ERK1/2 function as it relates to angiotensin II signaling, as well as signaling through other receptors.

What is clear from the studies contained in Chapters 2-4 is that Src kinase signaling and heterotrimeric G protein signaling are not separate events leading to different,

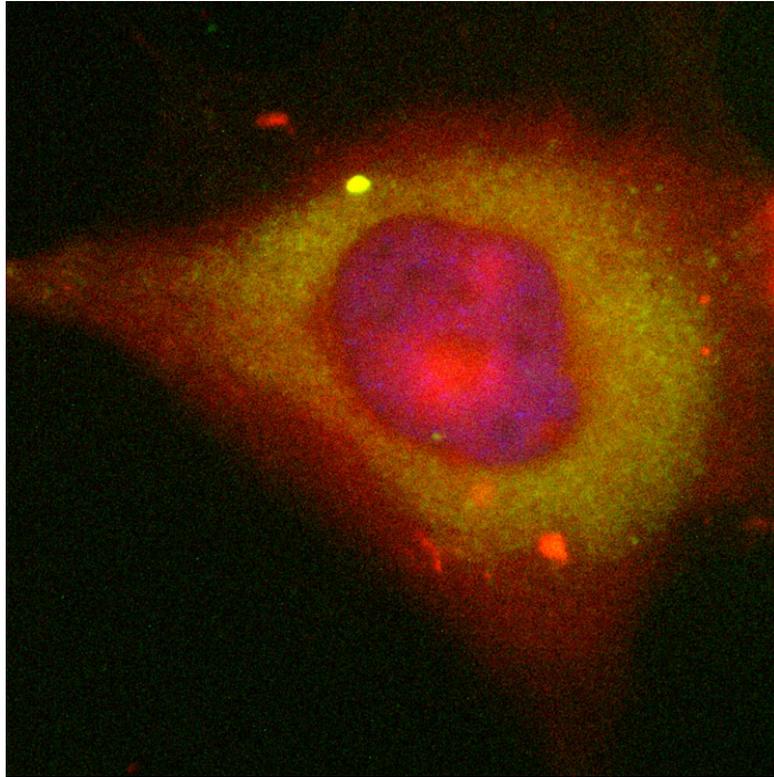


Figure 6-4. Preliminary data supporting the hypothesis that ERK1/2 subcellular distribution effects the mechanism of upstream activation. The immunofluorescent image is of an unstimulated WT/AT₁ cell. ERK2 is shown in red, RSK2 in green. DAPI staining (blue) shows the position of the nucleus. RSK2 fluorescence does not overlap entirely with ERK2 fluorescence, indicating that some ERK1/2 may not be positioned to interact with RSK in the cytoplasm.

distinct cellular outcomes. Both Src kinase-dependent ERK1/2 activation and heterotrimeric G protein/PKC ζ -dependent ERK1/2 activation regulate a portion of Ang II-induced cell proliferation, albeit through different mechanisms. These two mechanisms seem to converge at the level of *c-fos*, and mediate a portion of *c-fos* protein expression. Thus, the angiotensin II signaling paradigm is not as simplistic as once thought, with heterotrimeric G protein signaling mediating all signaling associated with

the pressor response and tyrosine kinase signaling mediating cell proliferation. Clearly, these two types of signaling cascades can act in concert with one another in order to achieve a common outcome. This is illustrated in other examples as well. For instance, calcium and heterotrimeric G protein/PKC-dependent activation of Pyk2 has been demonstrated to mediate Src activation by GPCRs (26, 108). In addition, *Gαq*/PLCβ signaling through GPCRs affects the activity of tyrosine kinases, including Src, focal adhesion kinase, PYK2 and epidermal growth factor receptor (30, 32, 67, 144, 152). Third, intracellular calcium release—a process necessary for muscle contraction—has been shown to be dependent upon both Jak2 tyrosine kinase (150) as well as heterotrimeric G protein signaling (118). Whether through direct activation of one another or coordinate (but separate) signaling events, heterotrimeric G protein and tyrosine kinase signaling often work together. As such, a paradigm shift is beginning to emerge in the field of angiotensin II signaling, causing researchers to rethink the older model of angiotensin signaling.

A question specifically regarding AT₁ receptor-induced cell proliferation does arise as it relates to the rethinking of this paradigm: why does the cell utilize two independent pathways to achieve the same effect. These data presented in Chapters 2-4 suggest that heterotrimeric G protein/PKCζ signaling and Src kinase signaling have an additive effect on Ang II-induced ERK1/2 activation and cell proliferation. Therefore, one explanation is that these pathways may be somewhat redundant with one another in that ERK1/2 activation and cell proliferation can persist (albeit at reduced amounts) when one pathway is disrupted. Perhaps this redundancy exists as an evolutionary conserved mechanism to protect the cell's ability to divide and proliferate. If a spontaneous mutation suddenly

arises in say the gene encoding PKC ζ for example, the cell ultimately has another mechanism capable of initiating cell proliferation in a PKC ζ -independent manner.

However, angiotensin II-induced cell proliferation has to date only been implicated in aberrant cell proliferation during disease states, including both cardiovascular disease and cancer (24, 31, 49, 56, 60, 72, 89, 104, 112, 120, 137). Therefore, another explanation for why the cell may use two pathways to achieve the same effect may be due to the role of angiotensin II in the progression of disease. Many studies have shown that the magnitude and duration of ERK1/2 activation determines the outcome of ERK1/2 activation (101, 102, 120). Perhaps a more sustained, robust and simultaneous Ang II-induced activation of ERK1/2 by two independent signaling pathways results in abnormal cell proliferation, whereas activation of ERK1/2 at comparatively reduced levels by one pathway may help maintain normal levels of cell proliferation in cells expressing the AT₁ receptor. Work by Lenormand and colleagues suggests that the magnitude and temporal pattern of ERK1/2 activation is carefully controlled by interactions with different scaffolding proteins, which effectively insulate proteins from pathways which could otherwise influence their activity (101). Perhaps protein scaffolding determines whether ERK1/2 is activated by one pathway or two. As such, the simultaneous activation of ERK1/2 by two-independent signaling cascades may be key to causing the Ang II-induced cell proliferation commonly associated with many disease states.

It should be noted that the mechanisms of Ang II-induced ERK1/2 activation described in the preceding chapters describe ERK1/2 activation as it relates to cellular proliferation. Angiotensin II also binds to receptors in cells which do not proliferate, including neurons and cardiac myocytes. Therefore, these cellular proliferation pathways

may only be applicable to cells which are capable of proliferating, including vascular smooth muscle cells and fibroblasts. These cells likely possess the right combination of proteins in order for angiotensin II to elicit cellular proliferation during pathological conditions (i.e. cardiovascular diseases and cancer). ERK1/2 is also phosphorylated in other non-proliferative AT₁ receptor-expressing cells, but may lead to different effects. For example, Ang II-induced ERK1/2 activation in cardiac myocytes may primarily trigger hypertrophy of these cells instead of cellular proliferation while in neurons, ERK1/2 may modulate ion channel activity.

The role of SHP-2 in angiotensin II, Jak2/STAT signaling

Phosphatases are often thought of as “the off switch” for many different signaling events through dephosphorylation at tyrosine residues. This is sometimes the case during Jak2 signaling. For example, MAP kinase phosphatase 1 (MKP-1) has been shown to be activated in a Jak2-dependent manner, and effectively turns off Ang II-induced ERK2 activation through dephosphorylation of ERK2 (111). However, it is demonstrated in Chapter 5 that SHP-2, a classic example of a phosphatase, mediates angiotensin II, Jak2/STAT signaling by facilitating Jak2 co-association with the AT₁ receptor. Thus, in the case of angiotensin II, SHP-2 serves initially as an adaptor molecule necessary to positively mediate Jak2/STAT signaling. As such, SHP-2 is not acting to “turn off” Jak2/STAT signaling through dephosphorylation of specific tyrosine residues.

Previous work has generated discrepancies as far as the role of SHP-2 in Jak2-dependent signaling, which have often been attributed to the cell type and ligand utilized. In COS7 cells expressing the GH receptor, SHP-2 appears to positively regulate Jak2 signaling (61). Likewise, in Chapter 5, SHP-2 appears to positively mediate Jak2/STAT signaling in response to angiotensin II. However, SHP-2 appears to negatively regulate

Jak2 signaling in fibroblasts treated with interferons (160). Perhaps SHP-2 signaling positively regulates Jak2 signaling in the context of growth promoting signals, but negatively regulates Jak2/STAT signaling when activated by other factors. In the context of GPCRs like the AT₁ receptor, it is clear that SHP-2 helps recruit Jak2 to the receptor and facilitate downstream signaling. In the case of interferons, SHP-2 dephosphorylates phospho-tyrosine residues in order to shut off Jak2 signaling. It is unclear how SHP-2 positively regulates Jak2/STAT signaling in the case of the GH receptor, since this receptor already contains intrinsically bound Jak2 proteins which become phosphorylated upon receptor dimerization. Perhaps SHP-2 is playing a role as an adaptor protein here as well, helping to facilitate the formation of the Jak2/STAT signaling complex at the receptor. More work will need to determine exactly why SHP-2 serves as both a positive and negative regulator of Jak2/STAT signaling.

Angiotensin II signaling and disease

As demonstrated in the dissertation, angiotensin II signaling is complex. This is illustrated by the fact that multiple signaling cascades can influence the same processes, such as angiotensin II-induced cell growth and proliferation. Additionally, some proteins can have more than one function inside the cell. For example, SHP-2 is a phosphatase which can also serve to positively regulate Jak2/STAT signaling in response to angiotensin II. Current AT₁ receptor antagonists, such as losartan or candesartan, are excellent AT₁ receptor blockers and effective blood pressure reducers when administered *in vivo* (56, 104, 136, 146). Simply blocking the receptor may not always be the most effective means to achieve a desired cellular outcome, though, since a number of downstream signaling events will be effected in addition to the activity of a few desired proteins. For example, administering an AT₁ receptor antagonist *in vivo* could be

beneficial for inhibiting aberrant cell proliferation, but could also cause an undesired decrease in blood pressure.

Understanding the complexity of angiotensin II signaling may aid in designing a more tailored approach for treating Ang II-associated diseases. From these studies, it has been observed that RSK inhibition via SL0101 is an effective means for lowering Ang II-induced cell proliferation. In combination with a PKC ζ inhibitor, one could theoretically completely attenuate Ang II-induced cell proliferation as demonstrated in the cell culture systems utilized in this work. Thus, perhaps dual PKC ζ inhibition and RSK inhibition, achieved via administering two compounds, could effectively reduce Ang II-induced cell growth and proliferation during disease states. Theoretically, these compounds would have little to no effect on vascular tone since this process is thought to occur via different signaling mechanisms (118). As such, this dissertation provides valuable insight into the underlying signaling associated with AT₁ receptor activation and ultimately into the treatment of Ang II-associated diseases.

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BIOGRAPHICAL SKETCH

Mr. Godeny was born on October 10, 1979, in Flemington, New Jersey. His love for the life sciences was sparked early in his childhood as he enjoyed collecting rocks, reading about animals and learning about space. In the 7th Grade, Mr Godeny decided that he wanted to become a cardiologist while writing an essay about the heart. In June of 1998, Mr. Godeny graduated with honors from Freedom High School in Bethlehem, Pennsylvania. He later enrolled as a freshman at The Pennsylvania State University in University Park, Pennsylvania, where he started as a premedicine major. In the spring semester of his freshmen year, Mr. Godeny worked as a work-study in the laboratory of Dr. Regina Vasilatos-Younken in the Department of Poultry Science. There, he gained his first exposure to scientific research. In 2000, Mr. Godeny was accepted into the Ronald E. McNair Scholars program at Penn State. As part of the requirements, Mr. Godeny spent two semesters and summers doing research in the laboratory of Dr. Richard Ordway studying the mechanisms of synaptic transmission in *Drosophila melanogaster*. Mr. Godeny graduated from Penn State in May of 2002 with a bachelor's degree in biology. In August of 2002, he enrolled as a graduate student at the University of Florida in Gainesville, Florida. There, Mr. Godeny worked as a graduate student in the laboratory of Dr. Peter Sayeski studying angiotensin II signaling. Mr. Godeny will attain a Ph.D. in biomedical science in August of 2006, and plans to work as a Postdoctoral Research Fellow at St. Jude Children's Research Hospital in Memphis, Tennessee.