Molecular Pharmacology Fast Forward. Published on April 22, 2015 as DOI: 10.1124/mol.115.097857
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GPCR and RhoA-stimulated transcriptional responses: links to inflammation, differentiation and cell proliferation

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Molecular Pharmacology Fast Forward. Published on April 22, 2015 as DOI: 10.1124/mol.115.097857
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Running Title: GPCR and RhoA-stimulated transcriptional responses

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Text pages: 27
Figures: 1
References: 40
Abstract word count: 194
Introduction word count: 876
Discussion word count: 5300

Alpha SMA – Alpha smooth muscle actin; AMOT – Angiomotin; AP-1 – Activator protein 1; ApoE – Apolipoprotein E; ATF-2 – Activating transcription factor 2; Bcl10 - B-cell lymphoma/leukemia 10 ; CARMA - caspase recruitment domain family member ; c-Fos – FBJ osteosarcoma virus oncogene; c-Jun – JUN proto-oncogene; CCN1/ Cyr61 - Cysteine-rich, angiogenic inducer, 61; CCN2 / CTGF – Connective tissue growth factor; ChIP – Chromatin immunoprecipitation; COX2 – Cyclooxygenase 2; fMLP - N-formyl-methionine-leucine-phenylalanine; GDP-Guanosine-5'-diphosphate; GEF - Guanine Nucleotide Exchange Factor; GPCR – G-protein coupled receptor; GTP -Guanosine-5'-triphosphate; GTPase – Guanosine-5'-triphosphate hydrolase; HEK293 – Human embryonic kidney cells 293; IkB - Inhibitor of kB; IKK – Inhibitor of kB kinase; JNK – c-Jun N-terminal kinase; Lats 1/2 - Large tumor suppressor kinase 1 and 2; LPA – Lysophosphatidic acid; mAChR - Muscarinic acetylcholine receptor; MALT1 - Mucosa-associated lymphoid tissue lymphoma translocation protein 1; MAPK - Mitogen activated kinase-like protein; mDia-1 – diaphanous-related formin1; MEF2 – Myocyte enhancer factor 2; MI – Myocardial infarction; MRTF-A – Myocardin-related transcription factor-A; MRTF-B – Myocardin-related transcription factor-B; NFkB - Nuclear factor kappa-light-chain-enhancer of activated B cells; PAR1 – Protease activated receptor 1; PI3K – Phosphoinositide 3 kinase; PiK3Cb - Phosphatidylinositol 4, 5-bisphosphate 3-kinase catalytic subunit beta isoform; PIP3 - Phosphatidylinositol (3,4,5)-trisphosphate; PKD – Protein kinase D; PLCε– Phospholipase C epsilon; Ras – Rat sarcoma virus; RBD – Rho binding domain; RelA - Nuclear factor NFkB p65 subunit; RhoA - Ras homolog family member A; RhoGEF – Ras homolog family member guanine nucleotide exchange factor; RNA-seq – Ribonucleic acid sequencing; ROCK – Rho kinase; RunX2 - Runt-related transcription factor 2; S1P – Sphingosine-1-phosphate; SAPK - Stress-activated protein kinase; SM-MHC – Smooth muscle myosin heavy chain; SM22 alpha – Smooth muscle 22 alpha; SRE – Serum response element; SRF – Serum response factor; TCF – Ternary complex factor; TEA – Transcriptional enhancer factor domain; TEAD/ TEF – Transcriptional enhancer factor; TXA2 – Thromboxane A2; VEGFR2 – Vascular endothelial growth factor receptor 2; Wnt – Wnt proto-oncogene; YAP – Yes associated protein

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ABSTRACT

The low molecular weight G-protein RhoA serves as a node for transducing signals through G-protein coupled receptors (GPCRs). Activation of RhoA occurs through coupling of G-proteins, most prominently $G_{12/13,}$ to Rho guanine nucleotide exchange factors. The GPCR ligands that are most efficacious for RhoA activation include thrombin, lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P) and thromboxane A2 (TXA2). These ligands also stimulate proliferation, differentiation and inflammation in a variety of cell and tissues types. Most of these pleiotropic effects of GPCRs and RhoA can be dissociated from the cytoskeletal changes classically associated with RhoA signaling. Instead, the molecular events underlying these responses are the activation of transcription factors, transcriptional co-activators and downstream gene programs. This review describes the pathways leading from GPCRs and RhoA to the regulation of activator protein-1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), myocardin-related transcription factor-A (MRTF-A) and Yes associated protein (YAP). We also focus on the importance of two prominent downstream transcriptional gene targets, the inflammatory mediator cyclooxygenase-2 (COX-2) and the matricellular protein cysteine-rich angiogenic inducer 61 (CCN-1/Cyr61). Finally we describe the importance of GPCR induced activation of these pathways in the pathophysiology of cancer, fibrosis, and cardiovascular disease.

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INTRODUCTION

RhoA is a member of the Ras family of low molecular weight GTPases. It is activated through exchange of GDP for GTP catalyzed by specific guanine nucleotide exchange factors (GEFs). RhoA activation was initially shown to occur in response to serum stimulation (Ridley and Hall, 1992). It was subsequently established that ligands for G-protein coupled receptors can also activate RhoA. Amongst the earliest studies were those demonstrating RhoA activation in neutrophils in response to the chemoattractant fMet-Leu-Phe (fMLP) (Huang et al., 2001). Further progress in this area was enabled by development of an assay to measure active (GTP-ligand) RhoA, taking advantage of its specific binding to the Rho binding domain (RBD) of one of its target proteins, rhotekin (Ren et al., 1999). Thrombin, thromboxane A2, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are GPCR ligands that have been well established as efficacious activators of RhoA (Djellas et al., 1999; Ishii et al., 2001; Moers et al., 2003; Nobes et al., 1995; Post et al., 1996; Siehler et al., 2001; Walsh et al., 2008a; Zhao et al., 2014).

A seminal discovery regarding the mechanism by which GPCR signaling activates RhoA was published from the Sternweis lab in 1998 (Hart et al., 1998). These investigators demonstrated that the alpha subunit of $G_{13,}$ a member of the $G_{12/13}$ family of G-proteins, was able to bind to and activate the p115RhoGEF, a guanine nucleotide exchange factor for RhoA. Additional work expanded the concept to demonstrate that other RhoGEFs were also regulated by $G_{12/13}$ and, accordingly, GPCRs that coupled efficiently to $G_{12/13}$ proteins were those that activated RhoA (Tanabe et al., 2004). It is now known that the alpha subunit of G_q can also bind to and activate some RhoGEFs,

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resulting in RhoA activation (Chikumi et al., 2002; Shankaranarayanan et al., 2010; Vaque et al., 2013) in addition to its better known and more dedicated effect on phospholipase C beta (PLCβ).

Much of the early work on GPCRs and RhoA signaling focused on how activation of RhoA regulated cell shape, migration, and contraction (Hall, 1998; Kaibuchi et al., 1999). Many of the cytoskeletal effects of RhoA signaling are mediated through Rho kinase (ROCK) which binds RhoA and catalyzes phosphorylation of its substrates including mDia1 (diaphanous-related formin 1) (Narumiya et al., 2009; Thumkeo et al., 2013). Amongst the best studied and physiologically important contractile targets of RhoA and ROCK is the myosin binding subunit of a phosphatase which regulates myosin light chain phosphorylation and thereby alters calcium sensitivity and contractility of smooth muscle (Kitazawa et al., 1991).

Treatment of fibroblasts with serum or LPA not only affects cell morphology but also induces cell proliferation and gene expression. The effects of RhoA on gene expression were established in early papers examining increases in the immediate early gene, c-Fos, through serum response factor (SRF) (Hill et al., 1995; Wang et al., 1998). RhoA mediated c-Fos gene expression was determined not to be regulated through the previously described transcriptional coactivator, ternary complex factor (TCF) (Hill et al., 1995). The transcriptional co-activator downstream of RhoA was identified as a member of the myocardin family of proteins, myocardin related transcription factor A (MRTF-A) (Cen et al., 2003; Miralles et al., 2003). MRTF-A and MRTF-B regulate genes involved in vascular smooth muscle differentiation (Wang et al., 2003) and this gene program can be stimulated through S1P and RhoA activation (Lockman et al., 2004).

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Mechanisms of RhoA mediated MRTF-A activation will be detailed in the review that follows.

GPCR ligands that signal through $G_{12/13}$ and RhoA are efficacious mitogens, mimicking the effects of receptor tyrosine kinases such as EGF and those of serum (of which LPA and S1P are major components). One potential mechanism for the growth promoting effects of GPCRs is cross talk with or transactivation of EGF or other growth factor receptors. This type of mechanism has been convincingly demonstrated for LPA, ET-1, and thrombin (Arora et al., 2008; Daub et al., 1996). Independent of cross talk, however, we have demonstrated that thrombin stimulates the proliferation of human glioblastoma cells through activation of $G_{12/13}$, RhoA, and subsequent regulation of the transcription factor, activating protein-1 (AP-1) and its target genes (Aragay et al., 1995; Majumdar et al., 1998; Post et al., 1996; Trejo et al., 1992; Walsh et al., 2008a). Our work and other studies examining regulation of AP-1 through RhoA are discussed in this review.

The Rozengurt group first established that cyclooxygenase-2 (COX-2) is regulated by GPCR stimulation with gastrin-inducing peptide and linked this to RhoA signaling and activation of the transcription factor NFkB (Slice et al., 1999). The mechanism by which RhoA regulates NFkB was shown to involve signaling through protein kinase D (PKD). Studies exploring the molecular mechanisms by which RhoA signaling engages the NFkB pathway are detailed in this review and underscore the role for GPCR and RhoA mediated gene expression in inflammation.

The most recent addition to the GPCR and RhoA mediated transcriptional network is Yes associated protein (YAP). YAP is a transcriptional co-activator that has been

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implicated in regulation of cell size, proliferation and stem cell biology (Mo et al., 2014). Studies showing that LPA, S1P, thrombin, and carbachol activate YAP through RhoA have exciting implications regarding the role of GPCRs in cell fate determination as well as in cancer cell proliferation.

GPCR and RHOA SIGNALING TO AP-1

AP-1 activation

A seminal early discovery in recognition that hormones working outside the cell could regulate gene expression came from studies of genes induced in response to phorbol esters and serum. These genes were determined to contain a short response element in their 5' promoter that bound a transcription factor called AP-1 (Angel et al., 1987). This led to the important concept that signal transduction pathways involving second messengers and protein kinases have profound effects on gene expression. AP-1 is a transcription factor composed of hetero or homodimers of various members of the Fos and Jun family (Angel et al., 1987; Lee et al., 1987). Addition of serum or other growth promoting stimuli to HeLa cells results in induction of Jun and Fos family members, their dimerization to form AP-1, and AP-1 dependent gene transcription. Subsequent studies demonstrated that mitogen activated kinases (MAP kinases) could phosphorylate and regulate these transcription factors (e.g. that Jun kinase (JNK) phosphorylated c-Jun and enhanced its transcriptional activity) (Karin, 1995). c-Fos and c-Jun are required for cell cycle progression as well as for transformation by a variety of oncogenes (Pandey and Wang, 1995; Wisdom et al., 1999), implicating AP-1 and its regulators in growth control.

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Studies carried out in our laboratory in 1992 were among the first to demonstrate that AP-1 was controlled by GPCR agonists. In particular we found that thrombin, which activates PAR1 in human 1321N1 glioblastoma cells, is an efficacious inducer of c-Jun and AP-1 mediated gene expression (Trejo et al., 1992). We observed approximately 7-fold increases in AP-1 regulated luciferase gene expression in cells treated with thrombin. Notably, activation of the muscarinic receptor (mAChR) by carbachol did not elicit AP-1 mediated gene expression, which required prolonged (4-12hr), rather than transient increases in c-Jun mRNA, JunB protein expression, and AP-1 DNA binding (Trejo et al., 1992). In subsequent studies we demonstrated that the mitogenic response of 1321N1 cells to thrombin was mediated through activation of $G_{12/13}$, RhoA, and the AP-1 mediated target gene CCN1 (Aragay et al., 1995; Majumdar et al., 1998; Post et al., 1996; Walsh et al., 2008a). In contrast, activation of the endogenous 1321N1 cell mAChRs which couple to G_q and PLCβ (Evans et al., 1985; Masters et al., 1984) did not activate RhoA, significantly increase c-Jun, induce CCN1 expression, or efficiently stimulate cell proliferation (Post et al., 1996; Trejo et al., 1992; Walsh et al., 2008a).

While RhoA activation through G_q signaling is not as ubiquitous or dedicated a response as activation of RhoA through $G_{12/13}$, G_q can couple to specific RhoGEFs and RhoA in some cancer cells or when overexpressed. Early work from the Gutkind laboratory using NIH3T3 cells overexpressing high levels of M_1 muscarinic receptors (now known to couple to G_q) demonstrated the basic principle that GPCR signaling can induce cell proliferation (Gutkind et al., 1991). Further studies revealed that JNK was activated through mAChR stimulation as was the AP-1 reporter gene and that AP-1 activation occurred through MEF2 (Collins et al., 1996; Coso et al., 1995; Coso et al., 1997).

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Gutkind's group subsequently established a mechanism by which RhoA regulates c-Jun expression and AP-1 induction (Marinissen et al., 2004). The pathway examined in cells stimulated with LPA involved activation of JNK and phosphorylation of c-Jun and ATF2 resulting in increases in c-Jun expression.

CCN1 as an AP-1 target

We subsequently carried out microarray analysis to uncover genes that were selectively regulated in response to AP-1 activation and could contribute to the mitogenic effects of thrombin. We looked for genes that were induced by thrombin but not carbachol and found, at the top of the list, a gene called Cyr61 (Walsh et al., 2008a). This protein, now called CCN1, is the founding member of the CCN gene family. CCN1 is highly and rapidly induced in 1321N1 glioblastoma cells stimulated through a subset of GPCRs. These GPCRs are distinguished by their ability to activate RhoA, and including not only PAR1, but also receptors for LPA and S1P (Walsh et al., 2008a; Walsh et al., 2008b; Zhao et al., 2014). Work from other laboratories supports the concept that activation of RhoA signaling in response to stretch or receptor ligands induces CCN1 gene expression (Han et al., 2003; Kim et al., 2013; Young et al., 2009). CCN1 is a matricellular protein that is secreted and resides in the extracellular matrix where it regulates cellular responses through interactions with integrins (Lau, 2011). We demonstrated that CCN1 expression and resulting integrin activation are required for thrombin stimulated proliferation of 1321N1 glioblastoma cells (Walsh et al., 2008a) and that induction of CCN1 contributes to S1P and RhoA mediated protection of cardiomyocytes against ischemic injury (Zhao et al., 2014). The importance of CCN1 as a target gene is indicated by these findings as well as by a wealth of evidence

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implicating CCN1 in cancer cell proliferation, survival, and invasion (Jun and Lau, 2011; Lau, 2011).

Early studies on CCN1 induction by GPCRs demonstrated its regulation by AP-1 (Han et al., 2003). S1P treatment led to rapid and robust increases in CCN1 expression in primary cultures of bovine smooth muscle cells. This response was dependent on Rho and ROCK signaling and shown to involve transcriptional regulation through an AP-1 site as indicated by mutagenesis of the CCN1 promoter. Mechanistically, actin dynamics and p38 MAP kinase signaling were also implicated in the transcriptional response (Han et al., 2003). Other studies using vascular smooth muscle cells confirmed that S1P induced CCN1 through AP-1, and showed that this occurred through RhoA and $G_{12/13}$ but not G_q or G_i signaling (Kim et al., 2011). Our studies in glioblastoma cells also implicated AP-1 in CCN1 induction by GPCRs and RhoA (Walsh et al., 2008a). Thus, signaling from GPCRs to RhoA and c-Jun increases AP-1 activity to regulate gene expression, with CCN1 as a prominent example. Notably, however, CCN1 is highly regulated and its promoter is enriched in binding sites for a great number of transcription factors in addition to AP-1 (Jun and Lau, 2011; Walsh et al., 2008b). Remarkably many of these are downstream targets of RhoA signaling (AP-1, NFkB, MRTF-A, YAP) thus, as discussed further, CCN1 induction may play an integrative role in responding to transcriptional signals from GPCR mediated activation of RhoA. In addition, since secreted CCN1 activates integrins and integrins signal to tyrosine kinases (Walsh et al., 2008a), this provides another mechanism (similar to EGFR activation) for GPCRs to engage and utilize parallel growth factor pathways.

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GPCR AND RHOA SIGNALING TO NFkB

NFkB regulation by RhoA

Another transcriptional regulatory pathway shown to be regulated through RhoA signaling is that for nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB). NFkB exists as a complex of two subunits (p50 and p65) and a third protein, IkB which prevents the dimer from translocating to the nucleus to activate gene expression (Verma et al., 1995). When IkB is phosphorylated by its upstream regulator IkB kinase (IKK) it dissociates from the complex and is targeted for proteasomal degradation, promoting nuclear localization of NFkB p50/p65 and transcriptional responses.

Constitutively activated RhoA was shown to robustly increase the transcriptional activity of NFkB though increased phosphorylation of IkB (Perona et al., 1997). Others have suggested that RhoA signaling to NFkB is not mediated through the actions of the canonical upstream activator IKK (Cammarano and Minden, 2001). Some Rho GTPases (Rac1, Cdc42) regulate NFkB activation through a pathway involving the JNK/SAPK members of the MAP kinase family, but this was determined not to be the mechanism by which RhoA signals to NFkB (Montaner et al., 1999). One downstream RhoA effector that has been implicated in RhoA mediated NFkB activation is Rho kinase (ROCK) (Benitah et al., 2003; Segain et al., 2003) however the molecular link between ROCK and NFkB activation is not clear.

NFkB regulation by GPCRs

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Agonists shown to activate NFkB through RhoA signaling include neurotensin (Zhao et al., 2003), bradykinin (Pan et al., 1998), gastrin releasing peptide (Slice et al., 2003), angiotensin (Cui et al., 2006), fMLP (Huang et al., 2001), thrombin (Dusaban et al., 2013; Kang et al., 2005; Kawanami et al., 2011; Leonard et al., 2013), S1P (Siehler et al., 2001) and LPA (Hwang et al., 2006).

S1P activates NFkB in human embryonic kidney (HEK293) cells through collaborative effects of RhoA and protein kinase C activation (Siehler et al., 2001). Collaborative signaling mechanisms are likely to be quite common since many receptors coupled to $G_{12/13}$ and RhoA also couple to G_q and phospholipase C. Thus the $S1P_2$ and $S1P_3$ receptors, which can couple either to $G_{12/13}$ and Rho activation, or to G_q with subsequent phospholipase C activation, were found to be effectively linked to NFkB signaling; in contrast the $S1P_1$ receptor, which couples exclusively to G_i, does not activate RhoA or NFkB signaling in most cells (Mutoh et al., 2012; Siehler et al., 2001). Protein kinase C, which is activated downstream of phospholipase C, has been demonstrated to enhance NFkB activation in T-cells through formation of a complex involving a scaffold of proteins including CARMA, Bcl10 and MALT1. A series of studies using a Bcl10 gene deletion, dominant negative proteins and siRNA demonstrated that these scaffolding proteins were also required for NFkB activation in response LPA, angiotensin II and endothelin-1 (Klemm et al., 2007; McAllister-Lucas et al., 2007; Wang et al., 2007). There is also some evidence that GPCRs lead to NFkB activation through RhoA mediated phosphorylation of the NFkB subunit, RelA/p65, as observed for angiotensin II in vascular smooth muscle cells (Cui et al., 2006) and thrombin in endothelial cells (Anwar et al., 2004).

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The ability of GPCRs to activate RhoA through enhanced G_q signaling was mentioned earlier. In addition, hematopoietic cells have pathways for activation of RhoA through G_i. In human peripheral blood leukocytes the GPCR ligand fMLP activates RhoA through a pertussis toxin dependent (G_i) pathway which involves stimulation of PI3 kinase and effects of its product, PIP_3, on a RhoA guanine nucleotide exchange factor (Huang et al., 2001). Thus in these cells NFkB is activated by fMLP through G_i but it is, as in the other systems discussed, blocked by functional inactivation of RhoA with its inhibitor C3 toxin (Huang et al., 2001). The fundamental message is that multiple routes of GPCR induced RhoA activation in native cells can stimulate NFkB nuclear signaling. Whether this occurs through RhoA mediated increases in phosphorylation of IKK or of RelA/p65, and whether it involves protein kinase C or ROCK or other kinases remains to be clarified.

NFkB activation through protein kinase D

Another mechanism that could support the cooperative effects of RhoA and G_q or G_i signaling pathways on NFkB is through regulation of protein kinase D (PKD). PKD is a serine/threonine kinase that is activated by both diacylglycerol and protein kinase C (Fu and Rubin, 2011; Yuan et al., 2003). Protein kinase D can be regulated through RhoA activation in response to thrombin and S1P (Dusaban et al., 2013; Xiang et al., 2013). The GPCR agonist bombesin was also shown to activate PKD through G_{13} (to RhoA) and G_q (to protein kinase C) (Yuan et al., 2001). PKD has been implicated in LPA induced NFkB activation in human colonic epithelial cells (Chiu et al., 2007) and in NFkB activation in response to thrombin and S1P in astrocytes (Dusaban et al., 2013). Moreover we identified the novel phospholipase C epsilon (PLCε) as the mediator

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through which activation of RhoA leads to sustained PKD activation (Dusaban et al., 2013; Xiang et al., 2013). How PKD activation regulates NFkB and whether it is involved in phosphorylation of IKK or RelA/p65 has not to our knowledge been examined.

Phospholipase C epsilon in NFkB and cyclooxygenase-2 regulation

Cyclooxygenase (COX-2), which converts arachidonic acid to prostaglandins and other eicosanoids that mediate inflammation, is rapidly induced as an immediate early gene in response to pro-inflammatory signals (Kujubu et al., 1991). S1P and thrombin effectively induce COX-2 through NFkB signaling (Dusaban et al., 2013; Ki et al., 2007; Syeda et al., 2006). Earlier studies also demonstrated COX-2 induction in response to expression of RhoA and its upstream regulators G_{13} and G_q (Slice et al., 1999). Neither cytoskeletal effects of RhoA nor tyrosine kinase activation mediated this response. COX-2 induction by RhoA was also shown to be independent of Ras and Rac activation (Slice et al., 2000). As mentioned above, these same investigators linked RhoA and $G_{12/13}$ signaling to protein kinase C and subsequent protein kinase D activation (Yuan et al., 2002; Yuan et al., 2003). A link between GPCR signaling to $G_{12/13}$ and RhoA, and the subsequent activation of PKD, NFkB, and COX-2 was elucidated by recent work from our laboratory (Dusaban et al., 2013).

RhoA is a direct activator of PLCε (Seifert et al., 2004; Wing et al., 2003). We previously demonstrated that PLCε is required for thrombin, S1P, and LPA induced PI hydrolysis as well as thrombin mediated proliferation in murine astrocytes (Citro et al., 2007). Astrocytes are major mediators of neuroinflammation induced by thrombin, LPA and S1P (Dusaban et al., 2013; Nicole et al., 2005; Sorensen et al., 2003) and we

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hypothesized that RhoA signaling to PLCε contributes to inflammatory signals in astrocytes. We tested this using astrocytes from PLCε knockout mice with COX-2 as a primary readout for inflammation. Our studies demonstrated that thrombin induced COX-2 expression requires PLCε . Furthermore we showed that PLCε is required for activation of PKC and prolonged activation of PKD in response to thrombin (Dusaban et al., 2013). Using inhibitors and siRNA to PKD we demonstrated that PKD mediates activation of NFkB and its downstream inflammatory targets including COX-2 and various cytokines. We also demonstrated that stab wound injury *in vivo,* and the associated upregulation of inflammatory cytokines and astrogliotic markers, are attenuated in the absence of PLCε (Dusaban et al., 2013). It is likely that released cytokines or eicosanoids formed through the RhoA/NFkB pathway also feedback and contribute to sustained inflammation *in vivo* and *in vitro*.

PLCε contains unique regulatory domains not found in other PLC subtypes, most importantly a CDC25 Rap exchange domain. We propose that activated Rap feeds back on the enzyme's RA2 domain to lead to further PLCε activation and sustained signaling. This feedback is additionally enabled by localization of PLCε to the Golgi (Dusaban and Brown, 2015; Smrcka et al., 2012; Zhang et al., 2013). Sustained activation, and thus, sustained generation of diacylglycerol and activation of its regulated kinases, PKC and PKD appears to be critical for mediating inflammatory gene expression (Dusaban and Brown, 2015; Smrcka et al., 2012). We also demonstrated that PLCε mediates RhoA signaling to PKD in cardiomyocytes (Xiang et al., 2013). This leads to phosphorylation and inactivation of the cofilin phosphatase, slingshot. Cofilin phosphorylation was recently implicated in thrombin induced NFkB activation in endothelial cells (Leonard et

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al., 2013). Since slingshot is regulated by PKD phosphorylation (Fu and Rubin, 2011; Xiang et al., 2013), PKD effects on cofilin mediated responses could play a role in the activation of NFkB.

GPCR AND RHOA REGULATION OF MRTF-A

MRTF-A Activation

Serum response factor (SRF) was identified more than 25 years ago as the transcription factor through which serum regulates the c-Fos gene (Norman et al., 1988). SRF is considered to be constitutively bound to the serum response element (SRE) on its target genes and control of its activity is through transcriptional co-activators. Ternary complex factor or TCF is the transcriptional co-activator first determined to bind SRF and contribute to c-Fos regulation by ligands that activate MAP kinase signaling (Shaw et al., 1989). In contrast when SRF dependent gene regulation was examined in response to LPA, a major component of serum, it was shown to occur independently of activation of TCF (Hill et al., 1995; Sahai et al., 1998). Myocardin-related transcription factor A (MRTF-A, also known as MKL and MAL) is part of the myocardin family of transcriptional coactivators which includes MRTF-A, MRTF-B, and myocardin (Cen et al., 2004). MRTF-A was identified, through studies using MRTF-A knockdown, as the alternate transcriptional co-activator responding to serum-stimulated RhoA activation and responsible for c-Fos induction (Cen et al., 2003; Miralles et al., 2003).

Under basal conditions MRTF-A is largely sequestered in the cytoplasm where it binds free G-actin. Activation of RhoA by serum or other ligands induces polymerization of G-

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actin to form F-actin filaments, freeing MRTF-A to translocate to the nucleus (Cen et al., 2004; Guettler et al., 2008; Miralles et al., 2003). Not only is this interaction observed in response to changes in cytosolic actin polymerization, but there is also polymerization of nuclear actin which directly regulates the amount of free MRTF-A and its association with SRF in the nucleus to control MRTF-A dependent smooth-muscle cell transcription (Baarlink et al., 2013; Staus et al., 2014; Vartiainen et al., 2007).

GPCR Activation of MRTF-A

A convenient method of assessing MRTF-A activation in cells is by using a SRE.L (TCF-independent serum response element) luciferase construct. This reporter gene shows low activity when SRF is constitutively bound but increased activity in response to LPA and constitutively active G_{12} and G_{13} ,which effectively activate RhoA to increase nuclear MRTF-A. Recent studies using LPA and the SRE.L construct to carry out chemical screens identified CCG-1423, a compound that potently and selectively inhibits MRTF-A signaling (Evelyn et al., 2007). This inhibitor and another subsequently identified as higher potency have been used to block and thereby identify downstream MRTF-A mediated cellular responses (Haak et al., 2014; Zhao et al., 2014).

As mentioned above, we determined that CCN1, the founding member of the CCN gene family of matricellular proteins, is highly and rapidly induced by thrombin in 1321N1 glioblastoma cells. This gene is induced in response to GPCRs that activate RhoA including the PAR1, LPA and S1P receptors, as well as in response to stretch (Han et al., 2003; Walsh et al., 2008a; Young et al., 2009). MRTF-A was identified as the transcriptional co-activator that functions to mediate CCN1 gene expression in response

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to stretch in smooth muscle cells (Hanna et al., 2009). We also recently demonstrated that RhoA mediated increases in nuclear MRTF-A are required for CCN1 induction in cardiomyocytes (Zhao et al., 2014).

An important observation emerged from recent studies of gene regulation in fibroblasts treated with serum (Esnault et al., 2014). This work used genome wide analysis to demonstrate that early transcriptional responses of fibroblasts to serum are predominantly regulated by MRTF rather than TCF binding to SRF (Esnault et al., 2014). Indeed RNA sequencing analysis defined an SRF target gene set of 960 serum-inducible genes of which more than 70% were MRTF targets, and this was confirmed with MRTF ChIP analysis (Esnault et al., 2014). Of additional interest, many of the MRTF-SRF dependent genes (i.e. CTGF and CCN1) overlap with genes regulated through the transcriptional coactivator Yes associated protein (YAP), discussed below.

RHOA SIGNALING TO YAP

YAP activation

The most recent addition to the RhoA regulated transcriptional activation pathway is Yes-associated protein (YAP). YAP was originally discovered in Drosophila as a transcriptional coactivator that promotes cell proliferation and inhibits apoptosis (Huang et al., 2005). It is functionally inhibited in the Hippo kinase cascade by phosphorylation through the Lats 1/2 kinases. Phosphorylated YAP is unable to translocate to the nucleus and remains sequestered in the cytoplasm. Thus, YAP dependent genes involved in proliferation and cell survival are kept in check until appropriate stimulation leads to YAP dephosphorylation and activation (Hao et al., 2008; Zhao et al., 2007).

Molecular Pharmacology Fast Forward. Published on April 22, 2015 as DOI: 10.1124/mol.115.097857
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Much like MRTF-A, YAP is a transcriptional coactivator which does not contain a DNA binding domain but binds to transcription factors to induce gene transcription. A number of transcription factors have been reported to interact with YAP (Vassilev et al., 2001; Yagi et al., 1999), but the TEAD family of TEA-domain containing transcription factors appear to be the major target (Zhao et al., 2008). When constitutively active YAP was expressed with luciferase constructs containing response elements for various YAP associated transcription factors (TEAD, RunX2, or ErbB4) the most robust activation was of TEAD. In addition knockdown of YAP attenuated cell growth and expression of TEAD regulated genes, and knockdown of TEAD with siRNA abolished YAP stimulated gene expression and cell growth (Zhao et al., 2008).

The YAP pathway was first shown to be regulated by cell/matrix interactions and changes in cell density. Guan and colleagues pioneered the concept that maintenance of YAP phosphorylation underlies contact inhibition, signaling cultured cells to stop dividing when they become confluent (Zhao et al., 2007). The regulation of YAP by cell/cell and cell/matrix contact suggested involvement in mechanotransduction which involves sensing the stiffness of the extracellular matrix. Indeed, YAP nuclear accumulation was demonstrated to occur in cells exposed to stretch, or increased stiffness of the surrounding extracellular matrix (Aragona et al., 2013; Dupont et al., 2011). Importantly, studies examining mechanotransduction demonstrated that treatment of mammary epithelial cells with C3, an inhibitor of Rho function, abolished YAP activation by stretch (Dupont et al., 2011). This data suggested that other interventions that activate RhoA might also lead to YAP activation.

YAP activation by RhoA and GPCRs

Molecular Pharmacology Fast Forward. Published on April 22, 2015 as DOI: 10.1124/mol.115.097857
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The discovery that YAP activation was RhoA mediated was extended by work from the Guan laboratory showing that YAP activation could be regulated through G-protein coupled receptors (GPCRs) and their receptor-specific activating ligands (Mo et al., 2012; Yu et al., 2012). Specifically, stimulation with S1P, LPA and thrombin, all agonists that activate the $G_{12/13}$ pathway and RhoA, caused a Rho-dependent translocation of YAP to the nucleus and YAP dephosphorylation, as assessed by phos-tag gels (Mo et al., 2012; Yu et al., 2012). LPA also increased cell proliferation which was inhibited by knockdown of YAP using shRNA. Expression of dominant negative RhoA effectively blocked YAP activation while the constitutively active form of RhoA caused YAP to translocate to the nucleus (Yu et al., 2012).

As indicated above, Rho activation is not always dependent on GPCR activation of the $G_{12/13}$ pathways but can occur through high levels of G_q signaling (Chikumi et al., 2002). Uveal melanomas often have activating mutations in either the G_q or G_{11} genes and these mutations were recently shown to lead to RhoA mediated activation of YAP, resulting in increased cancer cell proliferation and tumor progression (Feng et al., 2014; Yu et al., 2014). The Rho GEF Trio was identified as the guanine nucleotide exchange factor responsible for connecting G_q signaling to Rho and eventually YAP activation in these cells (Vaque et al., 2013), but as described above any route to RhoA could theoretically lead to YAP dephosphorylation, nuclear accumulation, and increased cell growth.

What remains to be determined is how RhoA and its effect on the actin cytoskeleton lead to YAP activation. The downstream Rho kinase ROCK has been implicated in MRTF-A activation (Olson and Nordheim, 2010; Parmacek, 2007) but its role in RhoA

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mediated YAP activation is uncertain. In some settings YAP activation is independent of ROCK (e.g. pharmacological inhibition of ROCK does not affect YAP activation by LPA or thrombin) (Mo et al., 2012; Yu et al., 2012). Inhibition of ROCK did, however, abolish cytoskeletal tension and eliminate stretch induced YAP activation (Aragona et al., 2013). Another potential regulator of YAP activation was identified through the discovery of a direct interaction of YAP with angiomotin (AMOT) family proteins, which appear to maintain YAP in its phosphorylated and inhibited state (Zhao et al., 2011). It was further established that F-actin polymerization prevents AMOT association with YAP, freeing it to enter the nucleus, whereas inhibition of actin polymerization increases YAP cytosolic sequestration with AMOT (Feng et al., 2014)**.** Of particular interest, YAP was reported to be sequestered in the cytoplasm as part of the destruction complex responsible for phosphorylating and eliminating β-catenin. Stimulation of Frizzled using a Wnt ligand blocked YAP cytoplasmic sequestration by this complex, leading to its nuclear translocation as well as β-catenin stabilization (Azzolin et al., 2014; Imajo et al., 2012). While no direct relationship between GPCR mediated YAP activation and β-catenin has been established, it is notable that the $G_{12/13}$ proteins bind β-catenin and that RhoA activation has been linked to β-catenin signaling (Krakstad et al., 2004; Rossol-Allison et al., 2009).

PATHOPHYSIOLOGICAL CONSEQUENCES OF GPCR AND RHOA MEDIATED TRANSCRIPTIONAL RESPONSES.

Two fundamental characteristics of the GPCRs and transcriptional pathways delineated above underscore their potential importance in disease progression and treatment. First, known ligands for the GPCRs that most effectively couple to RhoA (e.g. thrombin,

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LPA, S1P) are formed or delivered directly to sites of cell injury and inflammation, and are thus available to turn on GPCR signaling. Second, the gene expression programs elicited by the transcription factors/co-activators discussed above (AP-1, NFkB, MRTF-A, YAP) have been extensively linked to pathophysiological processes including cancer cell growth, angiogenesis, inflammation and fibrosis. There is at present a gap, and concomitantly considerable future promise, in linking GPCR signaling through these transcriptional events to diseases and potential treatments. We conclude by discussing some newer findings that begin to bridge the gap between GPCR and RhoA mediated transcriptional events and disease.

The primary roles for MRTF-A signaling appear to be in regulating cell fate and differentiation. Studies using MRTF-A null mice demonstrated that they were protected against scar formation induced by myocardial infarction or chronic infusion of the GPCR agonist, angiotensin II (Small et al., 2010). Formation of scar tissue, part of the process generally referred to as cardiac remodeling, results from induction of a myofibroblast phenotype associated with transcriptional up regulation of genes such as α- smooth muscle actin (α-SMA). Subsequent fibrosis is characterized by increased collagen production and both of these events are attenuated in the MRTF-A knockout mouse heart (Small et al., 2010). The implication that MRTF-A activation drives fibrosis in the heart is complemented by work in other systems. Studies using the MRTF-A inhibitor developed in the Neubig lab demonstrated a role for MRTF-A in fibrosis associated with bleomycin induced skin injury (Haak et al., 2014). In this model, MRTF-A also mediates increases in α-SMA expression, consistent with its role in mediating a fibroblast to myofibroblast transition.

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MRTF-A has an established role as a regulator of vascular smooth muscle differentiation. Thus treatment with S1P induced α-SMA and other markers of differentiation including smooth muscle 22 alpha (SM22α) and smooth muscle myosin heavy chain (SM-MHC) in vascular smooth muscle cells and these responses were inhibited by a dominant negative form of MRTF-A (Hinson et al., 2007; Lockman et al., 2004; Mack, 2011). In the *in vivo* setting, pathological stress induces a switch in which smooth muscle cells become less contractile and better poised for proliferative and migratory responses that contribute to vascular remodeling. Vascular remodeling induced by femoral artery wire injury or ApoE deletion were shown to depend on MRTF-A using MRTF-A knockout mice and CCG-1423 treatment, and were associated with regulation of SRF target genes, including α-SMA, vinculin, integrin β1 and MMP-9 (Minami et al., 2012). S1P signaling through $G_{12/13}$ has also been linked to vascular injury mediated through AP-1 and its effect on its downstream target CCN1 (Kim et al., 2011).

Vascular remodeling is also induced by ischemia. Neovascularization in the murine ischemic hindlimb model has been shown to be mediated through MRTF-A (Hinkel et al., 2014) as has retinal vascularization in the postnatal mouse eye (Weinl et al., 2013). While these MRTF-A-mediated responses have not been linked to GPCR signaling, GPCR signaling through G_{13} was recently demonstrated to play a critical role in development of *in vivo* retinal angiogenesis, mediated through effects of NFkB and subsequent expression of VEGF receptor 2 (Sivaraj et al., 2013).

It is interesting to note that the CCN1 gene is a target for all the transcriptional regulators discussed in this review: AP-1, MRTF-A, NFkB and YAP. CCN1 in turn

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serves multiple functions. Microvessel growth in ischemic muscle was linked to increased expression of CCN1 and its pro-angiogenic properties (Hinkel et al., 2014). CCN1 is also upregulated in liver injury along with myofibroblast markers such as α-SMA (Kim et al., 2013). Surprisingly loss of CCN1 exacerbates rather than attenuates liver fibrosis as a result of the ability of CCN1 to induce myofibroblast senescence, but whether senescence develops due to alterations in MRTF-A and SRF signaling is not yet known. Our recent studies linked MRTF-A activation to CCN1 induction in response to GPCR and RhoA signaling in cardiac myocytes and demonstrated that CCN1 mediates protection against ischemic injury in myocytes and in the isolated perfused heart (Zhao et al., 2014).

The importance of CCN1 signaling in cancer progression is indicated by a wealth of evidence implicating CCN1 dysregulation in cancer cell proliferation, survival and invasion (Jun and Lau, 2011; Lau, 2011). Increased YAP signaling to induce CCN1 expression has been linked to lung cancer and basal cell carcinoma progression (Hsu et al., 2014; Quan et al., 2014). We demonstrated that CCN1 expression and resulting integrin activation were required for thrombin stimulated proliferation of 1321N1 glioblastoma cells (Walsh et al., 2008a). Since GPCR and RhoA signaling can increase CCN1 induction through multiple transcriptional pathways this matricellular protein may serve as a global integrator and effector of aberrant cell growth responses initiated by enhanced GPCR signaling.

The Hippo/YAP pathway has been widely implicated in cancer. Specifically, YAP was found to be one of the primary genes overexpressed in a myriad of cancers (Overholtzer et al., 2006). High levels of YAP expression in colorectal cancer resulted in

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increased proliferation and dysplasia which was recapitulated in a mouse model in which YAP overexpression in hepatocytes lead to extensive liver growth culminating in liver cancer (Camargo et al., 2007). Interestingly transgenic mouse lines in which the LPA1 or LPA2 receptors were overexpressed in mammary epithelial cells were shown to have increased nuclear YAP staining and a greater tendency to develop mammary hyperplasias due to tissue overgrowth (Yu et al., 2012). Two recent papers demonstrate that uveal melanomas, which harbor activating mutations in the gene encoding G_q, signal through RhoA to activate YAP and induce YAP target genes. Importantly YAP knockdown was shown to decrease growth and proliferation of uveal melanoma cells both *in vitro* and in an *in vivo* xenograft model (Feng et al., 2014; Yu et al., 2014).

A growing literature concerns the role of the Hippo/YAP pathway in cardiac growth responses. The heart is a terminally differentiated organ so proliferation of cardiomyocytes in the adult heart is extremely limited. Cardiomyocyte death, and the inability to replace these cells, underlies the development of heart failure following myocardial infarction (MI). Mice with cardiomyocyte-specific inactivation of YAP were shown to have increased infarct size and apoptosis after MI, and this was related to loss of the ability of YAP to stimulate cardiomyocyte proliferation (Del Re et al., 2013). Subsequent studies using both cardiac-specific YAP knockout and YAP transgenic mice subjected to MI demonstrated that the YAP pathway is necessary and sufficient for cardiomyocyte proliferation and regeneration of the neonatal heart (Xin et al., 2013). Two additional findings relevant to understanding YAP activation and YAP target genes in the heart have recently emerged. One is a role for α-catenins, components of cardiomyocyte intercellular junctions, as upstream regulators of YAP activation (Li et al.,

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2015). The other is the finding that the p110 catalytic subunit of phosphoinositide 3-kinase (encoded by the Pik3cb gene) is a transcriptional target of YAP, which through its transcriptional upregulation, enhances activation of the well-known cardioprotective signaling molecule, Akt. There is as yet no data relating GPCR and RhoA signaling to YAP-mediated protection in the heart. Notably, however, there is an extensive literature showing that S1P and RhoA signaling protect cardiomyocytes and the isolated perfused heart against ischemic injury (Del Re et al., 2007; Karliner, 2009; Means et al., 2007; Xiang et al., 2013; Xiang et al.), making involvement of YAP activation in this context an area ripe for further investigation.

CONCLUSIONS

Activated RhoA is the major "effector" mediating responses to GPCRs that couple to $G_{12/13}$. Ligands for these receptors are generated or delivered in response to cell injury and clearly play a role in cell physiology. While signals from RhoA may be intended to serve adaptive functions and protect cells from injury, chronic stimulation of these GPCRs turns on a plethora of transcriptional responses (Figure 1). The transcriptional programs are clearly initiated by RhoA activation, and some of the molecular events allowing the transcription factor or cofactor to become active have been elucidated. Many involve cytosolic phosphorylation or dephosphorylation events, but there is as yet little consensus on what these events are, except perhaps in particular cell types.

It seems likely that AP-1, NFkB, MRTF-A and YAP are regulated through divergent molecular interactions downstream of RhoA and thus differ in their regulatory control by feedback and other cellular signals. Thus RhoA activation would not necessarily turn on

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all of these transcriptional programs and cellular responses simultaneously, or in all cells. Notably, GPCRs that couple to $G_{12/13}$ and RhoA are typically able to also couple to G_q and G_i, albeit to different extents and in a cell type dependent manner. Coincident activation of these other G-protein signaling pathways would be expected to result in stimulation of PLCβ/protein kinase C and Ras/MAP kinase signaling cascades along with activation of RhoA. The extent to which each of these occurs would vary not only on cell type, but also on the ligand, since biased signaling through GPCRs could favor activation of RhoA versus activation of pathways that lead to G_q or β-arrestin signaling (Hollenberg et al., 2014; Soh and Trejo, 2011; Violin and Lefkowitz, 2007). With regard to the notion that the effects of RhoA on gene expression work in concert with other signaling pathways, this is indeed what was observed in the early seminal papers which showed that RhoA was required for and cooperates with Ras to mediate cell transformation (Olson et al., 1998; Qiu et al., 1995).

There is, without a doubt, system and ligand based divergence in the extent to which one would observe activation of each of the RhoA transcriptional signals covered in this review. On the other hand there are clearly some intriguingly common gene targets for all of these transcription factors. A prime example is CCN1 (see Figure 1 and review) which is not only regulated by AP-1 and NFkB but appears, from our recent as yet unpublished studies, to require coordinate activation by MRTF-A and YAP. Regulation by multiple RhoA transcriptional activators could be the paradigm for genes that are central to the ability of RhoA to activate pathophysiological cellular programs but only gets the green light to do so when several simultaneous signals are received.

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We are currently analyzing the gene expression profiles associated with GPCR and RhoA signaling with the goal of understanding the extent to which specific versus distinct programs are mediated through the transcriptional co-activators/ transcription factors discussed in this review. Interrogating the extent to which these transcription factors drive physiological versus pathophysiologic gene programs will also inform future consideration regarding the therapeutic value of targeting these transcription factors or their upstream regulators.

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AUTHOR CONTRIBUTIONS

Wrote or contributed to the writing of the manuscript: Brown, J.H., Yu, O.M.

Molecular Pharmacology Fast Forward. Published on April 22, 2015 as DOI: 10.1124/mol.115.097857
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Footnotes

This work was supported by the Cellular and Molecular Pharmacology Training Grant [T32GM007752]; the Endocrinology and Metabolism Training Grant [T32DK007541]; and National Institutes of Health grants [HL028143, GM036927].

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Molecular Pharmacology Fast Forward. Published on April 22, 2015 as DOI: 10.1124/mol.115.097857
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FIGURE LEGEND

Figure 1. Schematic of GPCR pathways involved in RhoA activation and gene expression.

Molecular Pharmacology Fast Forward. Published on April 22, 2015 as DOI: 10.1124/mol.115.097857
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Figure 1