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MINIREVIEW—50th ANNIVERSARY SPECIAL ISSUE

G Protein–Coupled Receptor and RhoA-Stimulated Transcriptional Responses: Links to Inflammation, Differentiation, and Cell Proliferation

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ABSTRACT

The low molecular weight G protein RhoA (rat sarcoma virus homolog family member A) 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, sphingosine-1-phosphate, and thromboxane A2. These ligands also stimulate proliferation, differentiation, and inflammation in a variety of cell and tissues types. The molecular events underlying these responses are the activation of transcription factors, transcriptional coactivators, and downstream gene programs. This review describes the pathways leading from GPCRs and RhoA to the regulation of activator protein-1, NFκB (nuclear factor κ-light-chain-enhancer of activated B cells), myocardin-related transcription factor A, and Yes-associated protein. We also focus on the importance of two prominent downstream transcriptional gene targets, the inflammatory mediator cyclooxygenase 2, and the matricellular protein cysteine-rich angiogenic inducer 61 (CCN1). Finally, we describe the importance of GPCR-induced activation of these pathways in the pathophysiology of cancer, fibrosis, and cardiovascular disease.

Introduction

Rat sarcoma virus (Ras) homolog family member A (RhoA) is a member of the Ras family of low molecular weight guanosine-5′-triphosphate hydrolases. It is activated through the exchange of guanosine-5′-diphosphate for guanosine-5′-triphosphate catalyzed by specific guanine nucleotide exchange factors. 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 (GPCRs) can also activate RhoA. Among the earliest studies were those demonstrating RhoA activation in neutrophils in response to the chemoattractant *N*-formyl-methionine-leucine-phenylalanine (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 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 (Nobes et al., 1995; Post et al., 1996; Djellas et al., 1999; Ishii et al., 2001; Siehler et al., 2001; Moers et al., 2003; Walsh et al., 2008a; Zhao et al., 2014).

A seminal discovery regarding the mechanism by which GPCR signaling activates RhoA was published by the Sternweis laboratory in 1998 (Hart et al., 1998). The investigators demonstrated that the α subunit of G_{13}, a member of the $G_{12/13}$ family of G proteins, was able to bind to and activate the Rho guanine nucleotide exchange factor (RhoGEF), 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 α subunit of

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ABBREVIATIONS: AMOT, angiomotin; CCG-1423, *N*-[2-[4(4-chlorophenyl)amino]-1-methyl-2-oxoethoxy]-3,5-bis(trifluoromethyl)-benzamide; fMLP, *N*-formyl-methionine-leucine-phenylalanine; GPCR, G protein–coupled receptor; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; JNK, Jun kinase; LPA, lysophosphatidic acid; mAChR, muscarinic acetylcholine receptor; MI, myocardial infarction; PKC, protein kinase C; PKD, protein kinase D; Ras, rat sarcoma virus; ROCK, Rho kinase; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; TEAD, transcriptional enhancer factor domain.

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G_q can also bind to and activate some RhoGEFs, 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 β (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 diaphanous-related formin 1 (Narumiya et al., 2009; Thumkeo et al., 2013). Among 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 (FBJ osteosarcoma virua oncogene), 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 coactivator 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). 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 epidermal growth factor 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 epidermal growth factor or other growth factor receptors. This type of mechanism has been convincingly demonstrated for LPA, ET-1, and thrombin (Daub et al., 1996; Arora et al., 2008). Independent of cross-talk, however, we have demonstrated that thrombin stimulates the proliferation of human glioblastoma cells through activation of $G_{12/13}$ and RhoA and subsequent regulation of the transcription factor, activating protein-1 (AP-1) and its target genes (Trejo et al., 1992; Aragay et al., 1995; Post et al., 1996; Majumdar et al., 1998; Walsh et al., 2008a). Our work and other studies examining the 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-releasing peptide and linked this to RhoA signaling and activation of the transcription factor NFκB (Slice et al., 1999). The mechanism by which RhoA regulates NFκB was shown to involve signaling through protein kinase D (PKD). Studies exploring the molecular mechanisms by which RhoA signaling engages the NFκB 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 the Yes-associated protein (YAP). YAP is a transcriptional coactivator that has been implicated in the regulation of organ size, cell 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 cancer cell proliferation.

GPCR and RhoA Signaling to AP-1

AP-1 Activation. A seminal early discovery in the 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 heterodimers or homodimers of various members of the Fos and Jun family (Angel et al., 1987; Lee et al., 1987). The 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 kinase–like protein (MAPK) 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 transformation by a variety of oncogenes (Pandey and Wang, 1995; Wisdom et al., 1999), implicating AP-1 and its regulators in growth control.

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 protease activated receptor 1 (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 acetylcholine receptor (mAChR) by carbachol did not elicit AP-1–mediated gene expression, which required prolonged (4–12 hours) 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; Post et al., 1996; Majumdar et al., 1998; Walsh et al., 2008a). In contrast, activation of the endogenous 1321N1 cell mAChRs that couple to G_q and PLCβ (Masters et al., 1984; Evans et al., 1985) did not activate RhoA, significantly increase c-Jun, induce CCN1 expression, or efficiently stimulate cell proliferation (Trejo et al., 1992; Post et al., 1996; Walsh et al., 2008a).

Although 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

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stimulation, as was the AP-1 reporter gene, and that AP-1 activation occurred through MEF2 (myocyte enhancer factor 2) (Coso et al., 1995, 1997; Collins et al., 1996). 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 a 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 cysteine-rich, angiogenic inducer, 61 (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 include not only PAR1, but also receptors for LPA and S1P (Walsh et al., 2008a,b; 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; Young et al., 2009; Kim et al., 2013). 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 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 was shown to involve transcriptional regulation through an AP-1 site, as indicated by mutagenesis of the CCN1 promoter. Mechanistically, actin dynamics and p38 MAPK 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 (Walsh et al., 2008b; Jun and Lau, 2011). Remarkably many of these are downstream targets of RhoA signaling (AP-1, NFκB, MRTF-A, and 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 [epidermal growth factor receptor] activation) for GPCRs to engage and use parallel growth factor pathways.

GPCR and RhoA Signaling to NFκB

NFκB Regulation by RhoA. Another transcriptional regulatory pathway shown to be regulated through RhoA signaling is that for NFκB. NFκB exists as a complex of two subunits (p50 and p65) and a third protein, inhibitor of κB (IκB), which prevents the dimer from translocating to the nucleus to activate gene expression (Verma et al., 1995). When IκB is phosphorylated by its upstream regulator IκB kinase (IKK), it dissociates from the complex and is targeted for proteasomal degradation, promoting nuclear localization of NFκB p50/p65 and transcriptional responses.

Constitutively activated RhoA was shown to robustly increase the transcriptional activity of NFκB though increased phosphorylation of IκB (Perona et al., 1997). Others have suggested that RhoA signaling to NFκB is not mediated through the actions of the canonical upstream activator IKK (Cammarano and Minden, 2001). Some Rho GTP hydrolases (Rac1 and Cdc42) regulate NFκB activation through a pathway involving the JNK/stress-activated protein kinase members of the MAPK family, but this was determined not to be the mechanism by which RhoA signals to NFκB (Montaner et al., 1999). In some systems the RhoA effector implicated in RhoA-mediated NFκB activation is ROCK (Benitah et al., 2003; Segain et al., 2003); however, the molecular link between ROCK and NFκB activation is not clear.

NFκB Regulation by GPCRs. Agonists shown to activate NFκB 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 (Kang et al., 2005; Kawanami et al., 2011; Dusaban et al., 2013; Leonard et al., 2013), S1P (Siehler et al., 2001), and LPA (Hwang et al., 2006).

S1P activates NFκB in HEK293 cells through collaborative effects of RhoA and protein kinase C (PKC) 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 NFκB signaling. In contrast, the $S1P_1$ receptor, which couples exclusively to G_i, does not activate RhoA or NFκB signaling in most cells (Siehler et al., 2001; Mutoh et al., 2012). PKC, which is activated downstream of phospholipase C, has been demonstrated to enhance NFκB activation in T cells through the formation of a complex involving a scaffold of proteins, including CARMA (caspase recruitment domain family member), Bcl10 (B cell lymphoma/leukemia 10), and MALT1 (mucosa associated lymphoid tissue lymphoma translocation protein 1). A series of studies using a Bcl10 dominant negative proteins, and small interfering RNA (siRNA) demonstrated that these scaffolding proteins were also required for NFκB activation in response to 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 NFκB activation through RhoA-mediated phosphorylation of the NFκB subunit RelA/p65, as observed for angiotensin II in vascular smooth

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muscle cells (Cui et al., 2006) and thrombin in endothelial cells (Anwar et al., 2004).

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 that involves stimulation of PI3 kinase (phosphoinositide 3 kinase) and effects of its product, PIP3 (phosphatidylinositol 3,4,5 triphosphate), on a RhoA guanine nucleotide exchange factor (Huang et al., 2001). Thus, in these cells, NFκB is activated by fMLP through G_i but 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 NFκB nuclear signaling. Whether this occurs through RhoA-mediated increases in the phosphorylation of IKK or RelA/p65 and whether it involves PKC or ROCK or other kinases remains to be clarified.

NFκB Activation through Protein Kinase D. Another mechanism that could support the cooperative effects of RhoA and G_q or G_i signaling pathways on NFκB is through regulation of PKD. PKD is a serine/threonine kinase that is activated by both diacylglycerol and PKC (Yuan et al., 2003; Fu and Rubin, 2011). PKD 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 PKC) (Yuan et al., 2001). PKD has been implicated in LPA-induced NFκB activation in human colonic epithelial cells (Chiu et al., 2007) and in NFκB activation in response to thrombin and S1P in astrocytes (Dusaban et al., 2013). Moreover, we identified novel phospholipase C ε (PLCε) as the mediator through which activation of RhoA leads to sustained PKD activation (Dusaban et al., 2013; Xiang et al., 2013). How PKD activation regulates NFκB and whether it is involved in the phosphorylation of IKK or RelA/p65 has not been examined to our knowledge.

Phospholipase C ε in NFκB and Cyclooxygenase-2 Regulation. 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 proinflammatory signals (Kujubu et al., 1991). S1P and thrombin effectively induce COX-2 through NFκB signaling (Syeda et al., 2006; Ki et al., 2007; Dusaban et al., 2013). 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 were found to mediate 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 PKC and subsequent PKD activation (Yuan et al., 2002, 2003). A link between GPCR signaling to $G_{12/13}$ and RhoA and the subsequent activation of PKD, NFκB, 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 phosphatidylinositol 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 (Sorensen et al., 2003; Nicole et al., 2005; Dusaban et al., 2013), and we 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 NFκB and its downstream inflammatory targets, including COX-2 and various cytokines. We also demonstrated that a stab wound injury 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/NFκB 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 (Smrcka et al., 2012; Zhang et al., 2013; Dusaban and Brown, 2015). 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 (Smrcka et al., 2012; Dusaban and Brown, 2015). 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 NFκB activation in endothelial cells (Leonard et 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 NFκB.

GPCR and RhoA Regulation of MRTF-A

MRTF-A Activation. Serum response factor 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 coactivators. TCF is the transcriptional coactivator first determined to bind SRF and contribute to c-Fos regulation by ligands that activate MAPK 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). MRTF-A is part of the myocardin family of transcriptional coactivators that 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 coactivator responding to serum-stimulated RhoA activation and is 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-actin to form F-actin filaments, freeing MRTF-A to translocate to the

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nucleus (Miralles et al., 2003; Cen et al., 2004; Guettler et al., 2008). 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 (Vartiainen et al., 2007; Baarlink et al., 2013; Staus et al., 2014).

GPCR Activation of MRTF-A. A convenient method of assessing MRTF-A activation in cells is by using a TCF-independent SRE 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 TCF-independent SRE luciferase construct to carry out chemical screens identified CCG-1423 [*N*-[2-[4(4-chlorophenyl)amino]-1-methyl-2-oxoethoxy]-3,5-bis(trifluoromethyl)-benzamide], a compound that potently and selectively inhibits MRTF-A signaling (Evelyn et al., 2007). This inhibitor and another subsequently identified inhibitor with 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 coactivator that functions to mediate CCN1 gene expression in response 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 chromatin immunoprecipitation analysis (Esnault et al., 2014). Of additional interest, many of the MRTF-SRF dependent genes (i.e., connective tissue growth factor and CCN1) overlap with genes regulated through the transcriptional coactivator YAP, as discussed below.

RhoA Signaling to YAP

YAP Activation. The most recent addition to the RhoA-regulated transcriptional activation pathway is 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 Lats 1/2 (large tumor suppressor kinase 1 and 2). 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 (Zhao et al., 2007; Hao et al., 2008).

Much like MRTF-A, YAP is a transcriptional coactivator that 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 (Yagi et al., 1999; Vassilev et al., 2001), but the transcriptional enhancer factor domain (TEAD) family of TEAD–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 [runt-related transcription factor 2], or ErbB4), the most robust activation was that 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 et al. 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 an 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 (Dupont et al., 2011; Aragona et al., 2013). 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). These data suggested that other interventions that activate RhoA might also lead to YAP activation.

YAP Activation by RhoA and GPCRs. The discovery that YAP activation was RhoA mediated was extended by work from the Guan laboratory showing that YAP activation could be regulated through GPCRs and their receptor-specific ligands (Mo et al., 2012; Yu et al., 2012). Specifically, stimulation with S1P, LPA, and thrombin, all of which are 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 small hairpin RNA. Expression of dominant negative RhoA effectively blocked YAP activation, whereas 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 RhoGEF 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

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YAP dephosphorylation, nuclear accumulation, and increased cell proliferation.

What remains to be determined is how RhoA activation leads to YAP activation. The downstream Rho kinase ROCK has been implicated in MRTF-A activation (Parmacek, 2007; Olson and Nordheim, 2010), but its role in RhoA-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 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 proto-oncogene ligand blocked YAP cytoplasmic sequestration by this complex, leading to its nuclear translocation as well as β-catenin stabilization (Imajo et al., 2012; Azzolin et al., 2014). Although 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, LPA, and 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/coactivators discussed above (AP-1, NFκB, MRTF-A, and 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, which is part of the process generally referred to as cardiac remodeling, results from induction of a myofibroblast phenotype associated with transcriptional upregulation 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 a 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 laboratory demonstrated a role for MRTF-A in fibrosis associated with a bleomycin-induced skin injury (Haak et al., 2014). In this model, MRTF-A also mediates increases in α-SMA expression, which is consistent with its role in mediating a fibroblast to myofibroblast transition.

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 α (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 (Lockman et al., 2004; Hinson et al., 2007; Mack, 2011). In the in vivo setting, pathologic 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 apolipoprotein E (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). Although 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 the development of in vivo retinal angiogenesis, which is mediated through effects of NFκB and subsequent expression of vascular endothelial growth factor receptor 2 (Sivaraj et al., 2013).

It is interesting to note that the CCN1 gene is a target for all of the transcriptional regulators discussed in this review: AP-1, MRTF-A, NFκB, and YAP. CCN1 in turn serves multiple functions. Microvessel growth in ischemic muscle was linked to increased expression of CCN1 and its proangiogenic properties (Hinkel et al., 2014). CCN1 is also upregulated in a liver injury model 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 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

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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 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).

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 underlie 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., 2015). The other is the finding that the p110 catalytic subunit of phosphoinositide 3-kinase β (PiK3CB) is a transcriptional target of YAP, which, through its transcriptional upregulation, enhances activation of the well known cardioprotective signaling molecule Akt. There are as yet no data relating GPCR and RhoA signaling to YAP-mediated protection in the heart. Notably, however, there is extensive literature showing that S1P and RhoA signaling protect cardiomyocytes and the isolated perfused heart against ischemic injury (Del Re et al., 2007; Means et al., 2007; Karliner, 2009; Xiang et al., 2011, 2013), making involvement of YAP activation in this context an area that is 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. Although signals from RhoA may be intended to serve adaptive functions and protect cells

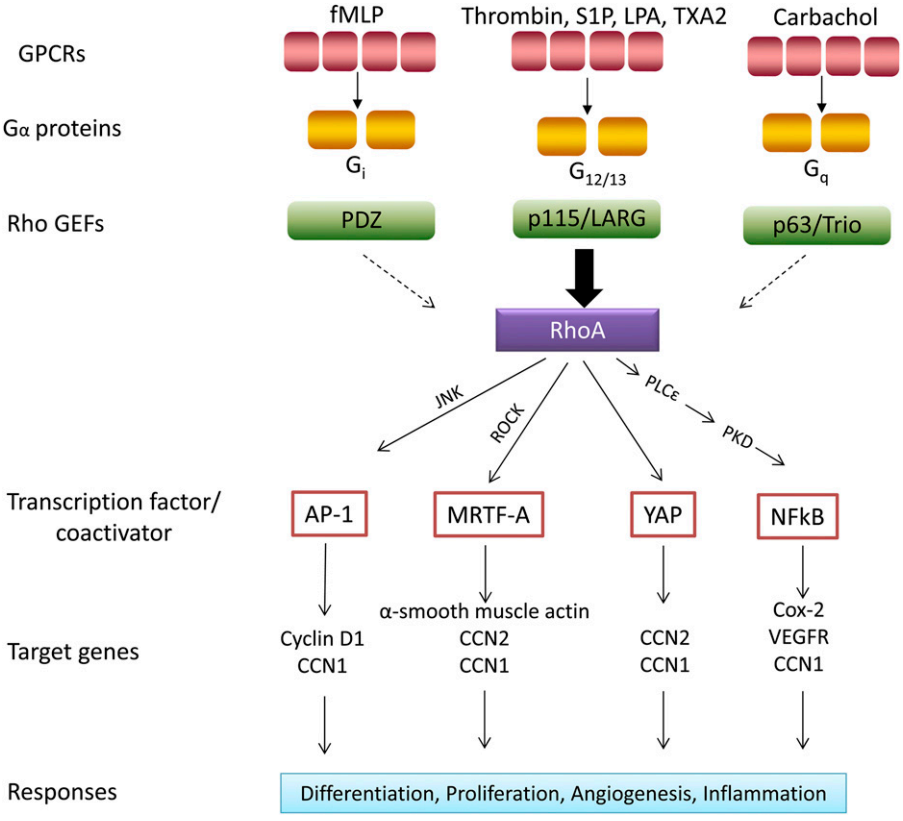

Fig. 1. Schematic of GPCR pathways involved in RhoA activation and gene expression.

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from injury, chronic stimulation of these GPCRs turns on a plethora of transcriptional responses (Fig. 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, NFκB, 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 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/PKC and Ras/MAPK 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 (Violin and Lefkowitz, 2007; Soh and Trejo, 2011; Hollenberg et al., 2014). 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 early seminal papers, which showed that RhoA was required for and cooperates with Ras to mediate cell transformation (Qiu et al., 1995; Olson et al., 1998).

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 Fig. 1 and review), which is not only regulated by AP-1 and NFκB 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.

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 coactivators/transcription factors discussed in this review. Interrogating the extent to which these transcription factors drive physiologic versus pathophysiologic gene programs will also inform future consideration regarding the therapeutic value of targeting these transcription factors or their upstream regulators.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Brown, Yu.

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