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MINI REVIEW

Regulation of RhoA GTPase and novel target proteins for ROCK

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ABSTRACT

Rho GTPases play significant roles in cellular function and their activity is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), providing activation and inactivation of these GTPases, respectively. Active GTP-bound form of RhoA activates its effector proteins while the inactive GDP-bound form of RhoA exists in a RhoA-RhoGDI (guanine nucleotide dissociation inhibitor) complex in the cytosol. In particular, IκB kinase γ IKKγ/NF-κB essential modulator (NEMO) plays a role as a GDI displacement factor (GDF) for RhoA activation through binding to RhoA-RhoGDI complex. Meanwhile, prion protein inactivates RhoA despite RhoA/RhoGDI association. Novel target proteins for Rho-associated kinase (ROCK) such as glycogen synthase kinase (GSK)-3β and IKKβ are recently discovered. Here, we elaborate on a post-translationally modified version of RhoA, phosphorylated at Tyr42 and oxidized at Cys16/20. This form of RhoA dissociates from RhoA-RhoGDI complex and activates IKKβ on IKKγ/NEMO, thus providing possibly a critical role for tumourigenesis.

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Introduction

Rho GTPases belong to the family of Ras-related small GTP binding proteins that includes RhoA, Cdc42 and Rac1/2 and play several important roles in cellular function including cytoskeletal rearrangement, reactive oxygen species (ROS) production along with regulation of cell morphology, cell movement, and transcription. A dysregulation of Rho GTPases is also linked a variety of diseases including various cancer types. Rho GTPases are activated by association with GTP, catalysed by guanine nucleotide exchange factors (GEFs). The reverse of this reaction involves GTP hydrolysis, a step catalysed by GTPase activating proteins (GAPs), leading to inactivation of Rho GTPases.[1-3] For various cellular processes, specific GEFs and GAPs are regulated by a set of stimulants in order to elicit defined states for Rho activity. Rho GTPases are also covalently modified with a lipid moiety that include a prenyl group attached to a defined cysteine residue in their terminal region as part of a CAAX motif (C, cysteine; A, any aliphatic amino acid; X, any amino acid). Inactive GDP-bound Rho exists in the cytosol in a complex with RhoGDI (guanine nucleotide dissociation inhibitor) while GTP-bound Rho is associated with the cell membrane through its prenyl group. For Rho GTPases to be activated, they need to be dissociated from RhoGDI, made possible by GDI displacement factor (GDF), as GEF cannot directly act on the Rho GTPase-RhoGDI complex.

Activated Rho GTPases bind to effector proteins that transmit the Rho-mediated signal to downstream target proteins. These typically include two Rho-associated coiled coil kinases (ROCKs) 1/2 that are activated by RhoA and six p21-activated protein kinases (PAKs) 1–6 that are activated by Cdc42 and Rac1. For RhoA, ROCK then phosphorylates myosin phosphatase, myosin light chain kinase and LIM kinase2 (LIMK2), leading to formation of actin filaments and increased actin-myosin interactions.[4] Meanwhile, LIMK1 is activated by PAK1.[5] In this mini-review, we focus on the description of novel targets for RhoA and ROCK. We also introduce the prion protein as an additional regulatory protein of RhoA and discuss the post-translational modifications of RhoA involving Cys16/20 oxidation and Ty42 phosphorylation that are likely to be critical for cell tumourigenesis.

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Novel target proteins of ROCK identified

ROCK phosphorylates various target proteins in addition to regulatory proteins that control actin filament dynamics.[6,7] For example, nuclear ROCK2 phosphorylates p300 acetyltransferase, leading to an increase in overall acetyltransferase activity.[8] Also, during tumour cell migration, FilGAP, a Rac GTPase activating protein, is phosphorylated by ROCK, leading to increased tumour invasion.[9] For ROCK1, it directly interacts with and phosphorylates c-Myc at Thr58 and/or Ser62, resulting in stabilization of c-Myc and activation of its transcriptional activity.[10] In leukemic cells, activated RhoA/ROCK1 binds to Erk1/2, leading to suppression of Erk1/2 activity.[11] Also in the context of the insulin binding to its receptor at the cell surface, ROCK subsequently phosphorylates insulin receptor substrate (IRS)-1 Ser632/635, leading to increased glucose uptake by the cell.[12] By using GST-ROCK fusion protein as a bait, several additional candidate proteins have been identified to interact with ROCK; these include amyloid precursor protein (APP), receptor-type tyrosine protein phosphatase delta (PTPRD), AP180 and doublecortin (DCX).[13] ROCK also binds to and phosphorylates 3-phosphoinositide-dependent kinase 1 (PDK1).[14]

We also identified ROCK phosphorylating glycogen synthase kinase (GSK)-3β in response to Wnt3A and active ROCK1 was shown to directly phosphorylate GSK-3β at Ser9 *in vitro*. Although p-Ser9 GSK-3β has been reported not to be related to β-catenin stabilization,[15] RhoA/ROCK stabilizes β-catenin through an unknown mechanism(s), leading to increased expression of c-Myc and cyclin D1 and stimulating cell proliferation upon Wnt3A administration.[16] RhoA/ROCK-mediated β-catenin activation in response to Wnt3A also induces expression of a chemokine, MIP-1α, leading to an increase in cell migration.[16] We also revealed that ROCK1 phosphorylates IKKβ at Ser 177/181 in response to cell treatment with TGF-β1 and in the *in vitro* setting, active ROCK leads to nuclear factor-κB (NF-κB) activation (Fig. 1).[17] As ROCK phosphorylates its various targets, this may also determine the destination of ROCK, localizing in the cell[7] (Fig. 1 and Table 1).

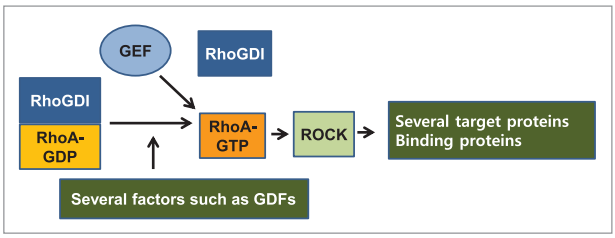

Figure 1. Factors for RhoA-RhoGDI dissociation and ROCK target proteins. RhoA-GDP/RhoGDI complex existing in cytosol needs to be dissociated by several factors such as GDFs (GDI displacement factors) before RhoA can be activated by GEFs. Activated RhoA then binds to ROCK, which then phosphorylates a variety of target proteins or binds to certain proteins, leading to regulation of downstream proteins. Detailed factors and proteins are described in Table 1 and Table 2.

Table 1. Target proteins for ROCK.

Target proteins	Function or feature	Reference
P300 acetyltransferase	Increases of acetyltransferase activity	8
FilGAP	Rac GAP, Tumour invasion	9
C-Myc	Stabilization of C-Myc, Increase of transcriptional activity	10
ERK1/2	Binding to ERK1/2, Suppression of ERK1/2 activity	11
IRS-1	Glucose uptake	12
IKKβ	Activation	17
GSK-3β	Inactivation by Ser9 phosphorylation	16
APP	Binding	13
PTPRD	Binding	13
AP180	Binding	13
Doublecortin (DCX)	Binding	13
PDK1	Binding	14

Abbreviations: APP, amyloid precursor protein; IRS, insulin receptor substrate; PTPRD, Receptor-type tyrosine protein phosphatase dela; DCX, Doublecortin; PDK, 3-phosphinositide-dependent kinase.

Factors affecting the dissociation of Rho GTPases-RhoGDI complex

Agents acting as GDFs and dissociating the Rho-RhoGDI complex: Ezrin/radixin/moesin (ERM) directly interacts with RhoGDI, reducing the activity of RhoGDI and initiating Rho activation. It is then proposed that ERM acts as a GDF.[18] Also, the neurotrophin receptor p75NTR directly interacts with RhoGDI and initiates RhoA activation by releasing RhoA from RhoGDI as RhoA inactivation is closely related to neurite outgrowth. Nerve growth factor (NGF), which induces neurite outgrowth, interferes with binding between p75NTR and RhoGDI, whereas Nogo and MAG (myelin-associated glycoprotein), which inhibit neurite outgrowth, strengthen p75NTR-RhoGDI complex formation. Thus, p75NTR acts as a GDF that dissociates the RhoA-RhoGDI complex to activate RhoA (Fig. 1).[19]

Modifications of RhoGDI regulating its affinity to Rho GTPases: Serine/threonine-protein kinase Pak1 binds to and phosphorylates RhoGDI at Ser101 and Ser174, which causes dissociation of Rac1-RhoGDI, but not RhoA-RhoGDI complexes.[20] Also, tyrosine kinase Src phosphorylates RhoGDI at Tyr156 residue, which causes a dramatic decrease in the ability of RhoGDI to form a complex with RhoA, Rac1 and Cdc42.[21] Recently, it was reported that RhoGDIα is acetylated at Lys127 and Lys141, which decreases the RhoGDI affinity toward RhoA, leading to facilitation of RhoA activation.[22] Furthermore, Lys acetylation of RhoGDI increases GEF-catalysed GTP binding to RhoA. Hence,

it is proposed that acetylation of RhoGDI functions as a GDF event.[23]

Very recently, we found that factors related to the reactive oxygen species (ROS) generation may also affect RhoGDI activity. It is shown that oxidation of RhoGDI by hydrogen peroxide abolishes its complex formation with RhoA.[24] As such, RhoGDI oxidation is likely to be a prerequisite for RhoA activation. RhoGDI has only one cysteine residue (Cys79) and yet in the presence of hydrogen peroxide, the RhoGDI Cys79Ala mutant, which is supposed to be an oxidation-resistant form, also does not bind to RhoA *in vitro* (unpublished data) (Fig. 1). This result suggests that RhoGDI is oxidized at residue(s) other than Cys79. Candidates may include unidentified methionine residue(s) oxidized by hydrogen peroxide for RhoGDI, as sulphur-containing amino acids such as methionine as well as cysteines are particularly vulnerable to oxidation[25] (Fig. 1 and Table 2).

Prion protein: The cellular prion protein (PrP^C), a cell-surface glycosylphosphatidylinositol (GPI)-anchored glycoprotein, has also been implicated in neuritogenesis and neuronal differentiation by modulating the RhoA-ROCK-LIMK-cofilin signalling pathway.[26,27] Neurite outgrowth by NGF, bFGF, and cAMP in PC12 cells requires the inactivation of RhoA and the mechanism involved has been described.[28-30] Contrary to a general notion that proteins binding to RhoGDI activate RhoA through the dissociation of RhoA-RhoGDI complex, the prion protein inactivates RhoA although prion protein binds to both RhoA and RhoGDI (data not shown). Although the function of RhoGDI binding to prion protein remains elusive, we speculate that prion protein provides RhoGDI to the inactivated RhoA. Prion protein induces the phosphorylation of RhoA Ser188 by protein kinase A (PKA), leading to complex formation with RhoGDI.[31] P190RhoA, Rap-dependent RhoGAP (ARAP3) and RhoA phosphorylated at Ser188 by PKA attribute to RhoA inactivation in response to NGF, bFGF and cAMP, leading to enhanced complex formation with RhoGDI and neurite outgrowth from PC12 cells.[28-30] Notably, PrP^C recruits and links RhoA and p190 RhoGAP, resulting in RhoA inactivation in response to NGF stimulation in PC12 cells with PrP^C playing a role as a signalling and/or enhancer molecule of neurite outgrowth.[27] Interestingly, pathogenic prions (PrP^{Sc}) impair both RhoA inactivation and neurite outgrowth in NGF-stimulated PC12 cells (Fig. 2).

According to a recent report,[14] over-activation of ROCK by prion infection leads to an impairment of neurite sprouting through ROCK-induced phosphorylation and activation of 3-phosphoinositide-dependent kinase 1 (PDK1). Subsequent PDK1 overstimulation, which precludes α-cleavage of PrP^C by TACE α-secretase, induces PrP^{Sc} production.[14] Thus, PrP^C acts as a signalling module by engaging with RhoA and p190 RhoGAP, leading to efficient RhoA inactivation, followed by controlling neuronal polarity.[27,32]

Table 2. Factors to regulate the formation of RhoA-RhoGDI complex.

Factor	Function or feature	Reference
Ezrin/radixin/moesin	GDF	18
p75NTR	GDF	19
Phosphorylation of RhoGDI by Pak1	Decrease of RhoGDI activity to bind to Rac1	20
Phosphorylation of RhoGDI by Src	Decrease of RhoGDI activity to bind to RhoA, Rac1 and Cdc42	21
Acetylation of RhoGDI	Decrease of RhoGDI activity to bind to RhoA	22
IKKγ/NEMO	GDF	17
Oxidation of RhoGDI	GDF	24
Oxidation of RhoA	GDF	24
Ser188 phosphorylation of RhoA by PKA, PKG, AMPK, ERK	Increase of RhoA binding to RhoGDI, Inactivation of RhoA	31, 34-36
Tyr42 phosphorylation of RhoA	GDF	37
Prion	Inactivation of RhoA	27

Abbreviations: GDF, GDI displacement factor; NTR, neurtrophin receptor; Pak, p21-activated kinase; IKKγ, IκB kinase γ; GDI, guanine nucleotide dissociation inhibitor; NEMO, NF-κB essential modulator; PKA, protein kinase A; PKG, protein kinase G, AMPK, AMP-dependent kinase.

Modifications of Rho GTPases regulating their mode of action

Phosphorylations of RhoA GTPase: Rho GTPases undergo post-translational modifications that include phosphorylation, ubiquitination and AMPylation, affecting their function.[33] For example, phosphorylation of RhoA Ser188 by protein kinase A (PKA) increases its affinity to RhoGDI.[31] Cyclic GMP-dependent protein kinase also phosphorylates RhoA at Ser188 in vascular myocytes, leading to inhibition of RhoA.[34] Furthermore, AMP kinase α1 (AMPKα1) phosphorylates RhoA at Ser188 in vascular smooth muscle cells in response to estradiol, also leading to RhoA inhibition.[35] In addition, upon EGF stimulation of the cell, ERK phosphorylates RhoA at Ser88 and Thr100, which then upregulate the activity of RhoA.[36] It is noteworthy that Tyr42 phosphorylation of RhoA is critical for tumourigenesis,[37] also discussed in the later section.

Cys16/20 oxidation and Tyr42 phosphorylation of RhoA: It has been well established that NADPH oxidase (NOX) regulates ROS production. Rac1/2 is involved in activation of $p67^{PHOX}$, a component of NADPH oxidase, leading to superoxide generation.[38] In addition, RhoA has also been reported to regulate superoxide production through indirect activation of NADPH oxidase.[39,40] Furthermore, Rap1 and RhoA reveal complementary additive functions for superoxide production in response to IgG-oposonized particles.[41]

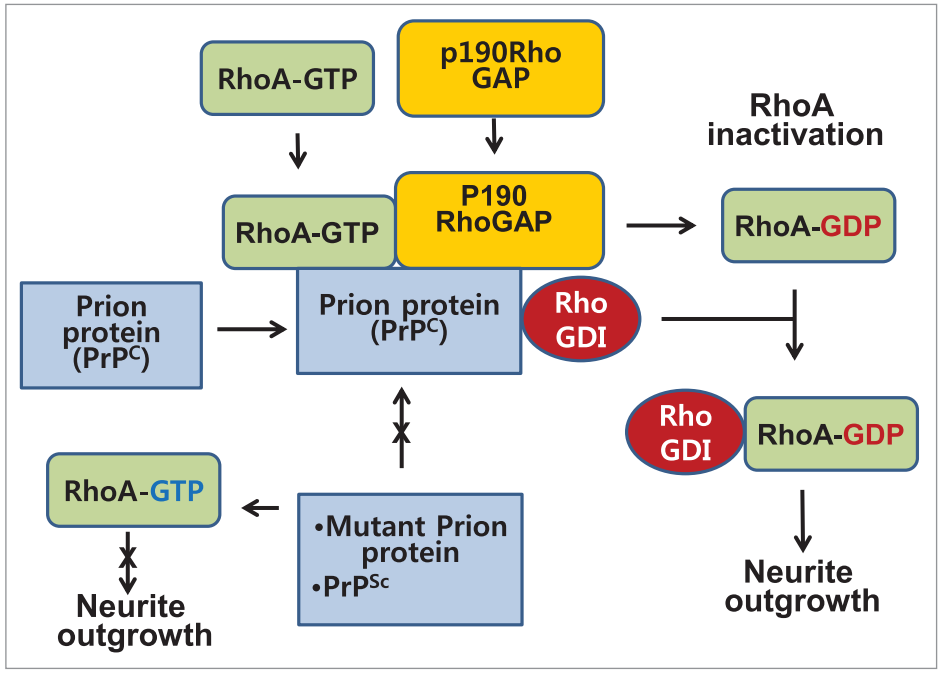

Figure 2. Prion protein as a linker of RhoA and p190RhoGAP, thereby facilitating RhoA inactivation. Prion protein recruits and links RhoA and p190RhoA together, thereby accelerating RhoA inactivation by p190RhoGAP. Subsequently RhoGDI binding with prion protein is likely to be provided to inactivated RhoA. Thus prion protein contributes to neurite outgrowth through RhoA inactivation, whereas mutant prion proteins and pathogenic PrP^{Sc} cannot facilitate RhoA inactivation, resulting in high RhoA activity and suppression of neurite outgrowth.

There is also a report of ROS inhibiting RhoA by p190RhoGAP activation, as ROS inhibits low-molecular weight protein tyrosine phosphatase (LMW-PTP), leading to tyrosine phosphorylation and activation of p190RhoGAP. Here Rac is involved in superoxide production, indicating that Rac downregulates RhoA through p190RhoGAP activation.[42] Also as noted above, a low concentration of hydrogen peroxide activates RhoA via RhoA oxidation and Vav2 activation.[24]

Recently, there has also been an evidence of Rho GTPases being regulated by the redox-state of the cell and in turn Rho GTPases regulating the redox-state by controlling enzymes that produce ROS and reactive nitrogen species (RNS).[43] Of note, Cys16 and Cys20 in RhoA can be oxidized to form a disulphide bond,[44] affecting its activity.

Although there are many reports of RhoA being involved in NF-κB activation,[45] its mechanism has not been clearly elucidated. Recently, we proposed a detailed mechanism by which RhoA activates NF-κB. It involves RhoA-GDP/RhoGDI complex binding to IKKγ/NEMO (NF-κB essential modulator). RhoA binds to the N-terminal domain (amino acids 1–43) of IKKγ/NEMO and RhoGDI binds to its large C-terminal domain (amino acids 100–419). Active Dbl, a GEF, slightly increases GTP binding to RhoA in the presence of RhoGDI. Furthermore, IKKγ/NEMO significantly enhances GTP binding to RhoA via active Dbl in the presence of RhoGDI. Therein, we have proposed that IKKγ/NEMO plays a role as a GDF to dissociate the RhoA-RhoGDI complex before GEF action in the TGF-β1 signalling pathway (Fig. 3A).[17]

Although RhoA-GTP rarely binds to IKKγ/NEMO,[17] RhoA-GTP that is oxidized at Cys16/20 and phosphorylated at Tyr42 readily binds to the region composed of amino acids 100–200 in IKKγ/NEMO and on which, RhoA-GTP stimulates IKKβ, which is juxtaposed with the RhoA. As such, IKKβ directly activated by RhoA oxidized at Cys16/20 and phosphorylated at Tyr42 then phosphorylates IκB, leading to IκB degradation and subsequent NF-κB activation. This in turn leads to expression of C-myc and cyclin D1 and consequent cell proliferation (Fig. 2B).[24,37] Also, RhoA oxidized at Cys16/20 and phosphorylated at Tyr42 reduces its affinity to RhoGDI (Fig. 1).[24,37] We also speculate that endogenous ROS sources may be in the tumour microenvironment as a co-culture of breast cancer and macrophage cells produced ROS.[24,37]

Tyr42 phosphorylation of RhoA is brought about by Src tyrosine kinase and ROS contributes to Src activation through hyperoxidization of Src Cys245 and Cys487 residues, leading to Tyr416 phosphorylation of Src.[46] P-Tyr42 of RhoA then serves as a binding site for Vav2, which activates RhoA. Thus, RhoA that is oxidized at Cys16/20 and phosphorylated at Tyr42 induces expression of c-Myc and cyclin D1, both essential for cell

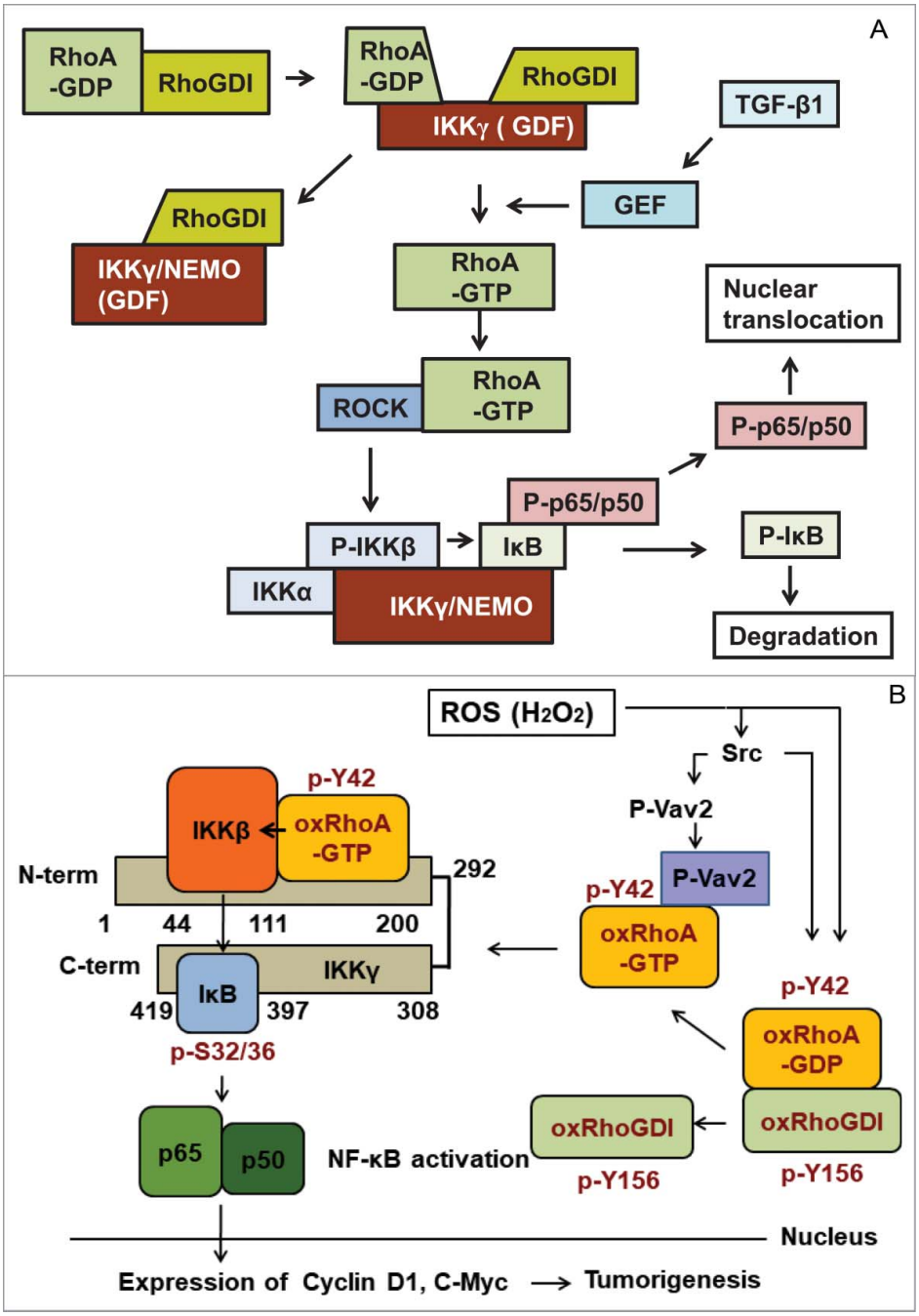

Figure 3. IKKγ/NEMO is required for activation of NF-κB through RhoA GTPase. (A) IKKγ/NEMO facilitates RhoA activation through dissociation of the RhoA-RhoGDI complex. Activated RhoA is first released from IKKγ/NEMO and then activates ROCK, which then phosphorylates IKKβ, leading to IκB phosphorylation and NF-κB activation. (B) Oxidation of Cys16/20 and phosphorylation of Tyr42 of RhoA results in the dissociation of the RhoA-RhoGDI complex. Oxidized/phosphorylated RhoA can be activated by Vav2 GEF and then binds to IKKγ/NEMO, where RhoA directly activates IKKβ juxtaposed to RhoA on IKKγ/NEMO. IKKβ phosphorylates IκB, leading to IκB degradation and NF-κB activation. From this chain of events, ROS induces tumourigenesis through the signalling pathway.

proliferation. In support of this hypothesis, p-Tyr42 Rho has been detected in patient breast cancer samples, and presence of p-Tyr42 Rho, p-Tyr416 Src and p-Ser527 p65/RelA positively correlate.[37] Thereby, we speculate that RhoA Tyr42 phosphorylation and Cys16/20 oxidation are likely to be critical for tumourigenesis in response to ROS (Fig. 3B and Table 2).

Conclusion

Activation of RhoA and ROCK has been known to regulate dynamics of actin filament formation. Novel target proteins and novel functions of RhoA and ROCK have also been discovered. In particular, IKKγ/NEMO functions as a GDF to activate RhoA

through binding with RhoA-RhoGDI complex. However, prion protein inactivates RhoA despite RhoA/RhoGDI association; Instead, prion protein plays a role as a platform protein to recruit and link p190RhoA and RhoA, leading to efficient RhoA inactivation and neurite outgrowth. We also focused post-translational modification of RhoA including Tyr42 phosphorylation and Cys16/20 oxidation. The oxidized/phosphorylated RhoA directly activates IKKβ, leading to NF-κB activation and consequently expression of cyclin D1 and C-Myc upon exposure to hydrogen peroxide and as part of events that are closely relevant to tumourigenesis. P-Tyr42 RhoA may also play multiple roles in a variety of unidentified cellular functions.

Abbreviations

APP	amyloid precursor protein
DCX	doublecortin
EGF	epithelial growth factor
FAK	focal adhesion kinase
GAP	GTPase activating protein
GDF	GDI displacement factor
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GSK	glycogen synthase
IKK	IκB kinase
IκB	NF-κB inhibitor
IRS	insulin receptor substrate
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-κB
NGF	nerve growth factor
NOX	NADPH oxidase
NTR	neurotrophin receptor
PAK	p21-activated kinase
PDK	phosphatidylinositol-dependent kinase
PDK1	3-phosphoinositide-dependent kinase 1
PKA	protein kinase A
PrP	prion protein
PTPRD	receptor-type tyrosine proteinphosphatase delta
ROCK	Rho-associated coiled coil kinase
ROS	reactive oxygen species

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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