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REVIEW

Rho GTPases, their post-translational modifications, disease-associated mutations and pharmacological inhibitors

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ABSTRACT

The 20 members of the Rho GTPase family are key regulators of a wide-variety of biological activities. In response to activation, they signal via downstream effector proteins to induce dynamic alterations in the organization of the actomyosin cytoskeleton. In this review, post-translational modifications, mechanisms of dysregulation identified in human pathological conditions, and the ways that Rho GTPases might be targeted for chemotherapy will be discussed.

Introduction

The Rho GTPases were initially discovered on the basis of their homology to the Ras GTPases,[1] and they were quickly discovered to have significant effects on cell morphology and actin cytoskeleton organization. RhoA activation leads to the formation of filamentous actin (F-actin) bundles called stress fibers,[2,3] Rac1 promotes the generation of F-actin-rich lamellipodia,[4] while CDC42 induces the extension of bundled F-actin projections called filopodia[5,6] (Fig. 1). Since their initial discovery over 30 years ago, Rho GTPases have emerged as critically important and central regulators of the signaling pathways that influence the actomyosin cytoskeleton in processes including adhesion, migration and cell division,[7-9] as well as proliferation, differentiation and gene transcription.[10-12] This review will focus on the Rho proteins themselves, including recent findings regarding their post-translational modifications, and how this knowledge informs our understanding of disease-associated mutations and contributes to small molecule inhibitor development.

The Rho GTPase family

The Rho GTPase family is comprised of 20 members (Table 1), which act as molecular switch proteins that undergo cycles of activation and inactivation in response to GDP/GTP exchange and GTP hydrolysis (Fig. 2).

Guanine nucleotide exchange factors (GEFs) promote the release of GDP, allowing GTP to associate with the Rho protein and induce conformational changes for downstream signal transduction. GTPase accelerating proteins (GAPs) increase the catalytic activity of Rho proteins to hydrolyse GTP to GDP by providing an "arginine finger" that enables catalysis.[13,14]

An amino acid identity matrix is shown in Figure 3a that indicates the percentage relatedness of each Rho GTPase protein to the other family members. In agreement with previous alignment studies,[15] grouping by hierarchical clustering[16] indicated that there are 4 main protein clusters: Rac (red typeface) Rho (purple), RhoH (brown) and RhoBTB (green). The relationship between Rho GTPases is shown in Figure 3b by a phylogenetic tree where the separation between proteins is proportional to the calculated evolutionary distances.[16] Although clustering was based on amino acid identity, many of the proteins within clusters share overlapping biological functions. Figure 4a shows the amino acid conservation greater than 50% for the amino acids within the most well conserved segment of the 18 most related proteins, corresponding to residues 4 to 177 for the Rac1 reference sequence.

The most conserved regions of the Rho GTPases are the 5 G-boxes (Fig. 4a; G1-G5) that fold in the 3-dimensional protein to form the nucleotide binding region as shown for the Rac2-GDP structure (PDB ID: 2W2T[17]) depicted in Figure 4b (green). The Switch 1
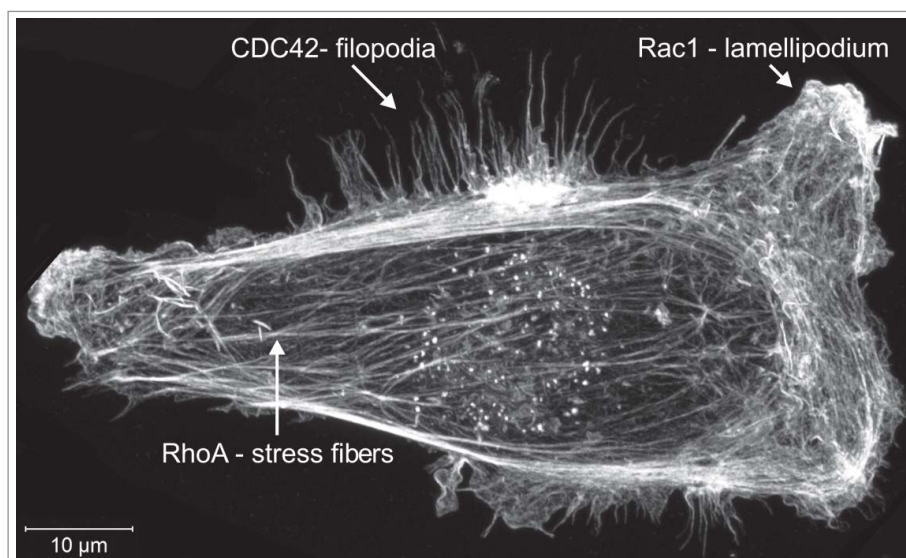
Figure 1. Filamentous actin structures. MDA MB 231 human breast cancer cells were fixed and stained with fluorescently labeled phalloidin to visualize filamentous actin structures. The image shows a maximum-projection assembled from 22 Z-plane images acquired with a Zeiss LSM 880 Airyscan microscope.

(Fig. 4a and b, yellow) and Switch 2 regions (Fig. 4a and b, magenta) change their conformations when GDP is exchanged for GTP to signal downstream to effector

proteins. By mapping amino acid identity of the 18 most related Rho GTPases (Fig. 4a) onto the 3-dimensional Rac2-GDP structure, the conservation of the guanine nucleotide binding region as well as Switch 2 are clear (Fig. 4c, red), while distal regions are less conserved (Fig. 4c, white to blue). The conservation of Switch 1 amino acids is more variable, with conserved and divergent residues. The amino acid diversity in Switch 1 allows each Rho protein to associate with different effectors to induce distinct responses and behaviors.

Table 1. Rho GTPases, post-translational modifications and mutations.

Rho GTPase	Alternative name	Post-translational modifications	Mutations
Rac1		AMP-Y32;[23] pY64;[24] pS71;[27] Ub-K147;[43,45] Ub-K166;[46]	P29S;[63-66] N92I;[66]
Rac2			P29L;[63,66] P29Q;[66] D57N;[54-56] W56X;[5757]
Rac3		Ub-K166[42]	
RhoG			
CDC42	G25K	AMP-Y32;[23] pY32;[20] pY64;[25] pS71;[28]	
RhoJ	TCL		
RhoQ	TC10	pT189;[114]	
RhoU	Wrch1	pY254;[115]	
RhoV	Chp/Wrch2		
RhoA		Ub-K6, Ub-K7;[39] pS26;[26] AMP-Y32;[23] nY32;[21] pY66;[19] Ub-K135;[40]	G17V;[72-74] R5Q;[72,75,76] R5W, G17E, L22R, V38G, Y42C, E54K, W58S, R68P, L69R, Y74D;[76] C16R, G17E, T19I, D120Y;[74] I23R, L22H, Y42F, Y42S, L69R, D76V;[75] E40Q, Y42I;[65] A161E;[73]
RhoB			
RhoC			
RhoD			
RhoF	Rif		
RhoE	Rnd3		
RhoN	Rnd2		
RhoS	Rnd1		
RhoH	TTF		Y38X;[59]
RhoBTB1			
RhoBTB2	DBC2		

pY = phospho-tyrosine, pS = phospho-serine, pT = phospho-threonine, nY = nitrated tyrosine, AMP-Y = AMPylated tyrosine, Ub-K = ubiquitylated lysine.

Regulatory post-translational modifications

In addition to the carboxyl-terminal modifications that have been reviewed elsewhere[18] which direct membrane localization, including prenylation (geranylgeranylation or farnesylation), endoproteolytic removal of the terminal 3 amino acids and carboxy-methylation, as well as palmitoylation of some family members, various Rho GTPases undergo further post-translational modifications that influence their activity and functions. Given the extensive homology within the Rho GTPase family, it is likely that comparable post-translational modifications may occur at conserved sites (Table 1 and Fig. 5). Phosphorylations (purple) and ubiquitylations (brown) from the literature and from PhosphoSite (www.phosphosite.org) have been plotted on the amino acid identity grid in Figure 5a. In addition, phosphorylations (purple) and ubiquitylations (brown) identified for Rac2 have been mapped onto the front (Fig. 5b) and reverse (Fig. 5c) views of the 3-dimensional structure.
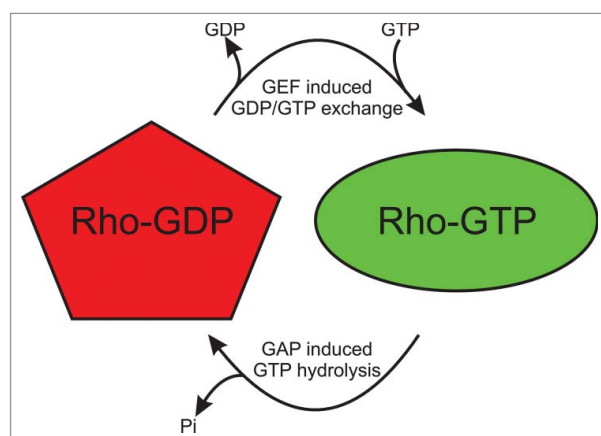
Figure 2. Rho GTPases are molecular switches. When associated GEF molecules promote GDP release, GTP is bound which results in conformational changes in the Switch 1 and Switch 2 regions that enable interactions with downstream effector proteins and consequent signal transduction. Upon association with GAPs, GTP is hydrolysed to GDP and Pi is released to inactivate the Rho protein.

The tyrosine analogous to RhoA Y34 (identical in 20/20 Rho proteins) in the Switch1 region is phosphorylated in RhoA[19] and CDC42.[20] Nitration of RhoA Y34 in response to lipopolysaccharide treatment results in reduced GDP affinity to promote GDP/GTP exchange

and consequent RhoA activation,[21] which suggests that phosphorylation at this conserved site might also be a mechanism of activation. Interestingly, AMPylation of RhoA Y34, Rac1 Y32 or CDC42 Y32, which could be catalyzed by the human HYPE protein,[22] blocked interactions with downstream effector proteins to inhibit signaling,[23] suggesting that modifications at this site could be a general mechanism for inactivation as well as activation. Phosphorylation of the tyrosine in the Switch 2 region analogous to RhoA Y66 (15/20 identical) occurs in RhoA,[19] Rac1,[24] and CDC42.[25] Rac1 Y64 phosphorylation inhibited cell spreading and lamellipodia formation, likely due to increased association with RhoGDIs and decreased association with GEFs and effectors,[24] while CDC42 Y64 phosphorylation also increased RhoGDI binding.[25] The conservation of these tyrosine residues, their accessibility on the Switch 1 and Switch 2 sites and the impact of their modifications on protein function suggest that they may be commonly targeted for post-translational modification as a regulatory mechanism.

RhoA can also be inhibited by Mst3-mediated phosphorylation on S26[26] which is serine or threonine at this analogous position in 12/20 Rho GTPases. Rac1 is
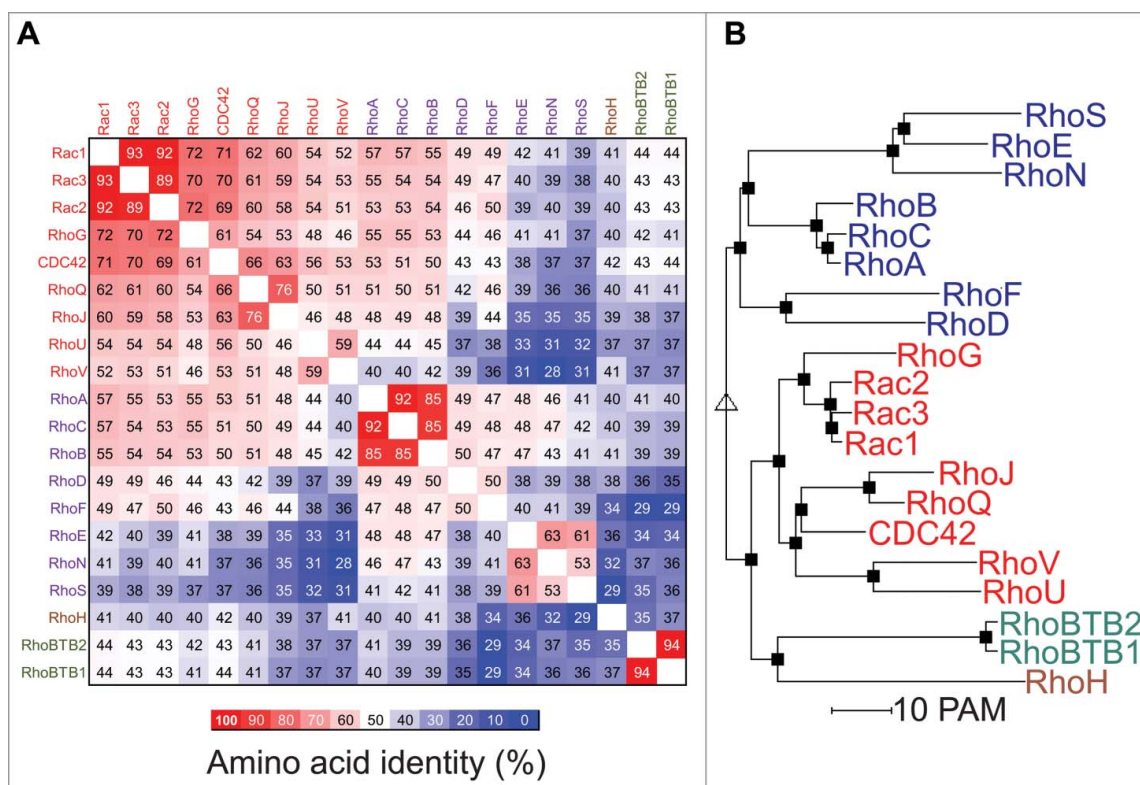

Figure 3. The Rho GTPase family. (A) An amino acid identity matrix was generated by comparing the primary sequence of 20 human Rho proteins. Grouping into Rac (red), Rho (purple), RhoH (brown) and RhoBTB (green) groups was by hierarchical clustering using the Multalin multiple sequence alignment program.[16] (B) A rooted phylogenetic tree of the 20 human Rho GTPases was generated by hierarchical clusing using the Multalin multiple sequence alignment program.[16] Distances between proteins are proportional to their PAM (point accepted mutation) score of molecular evolution.
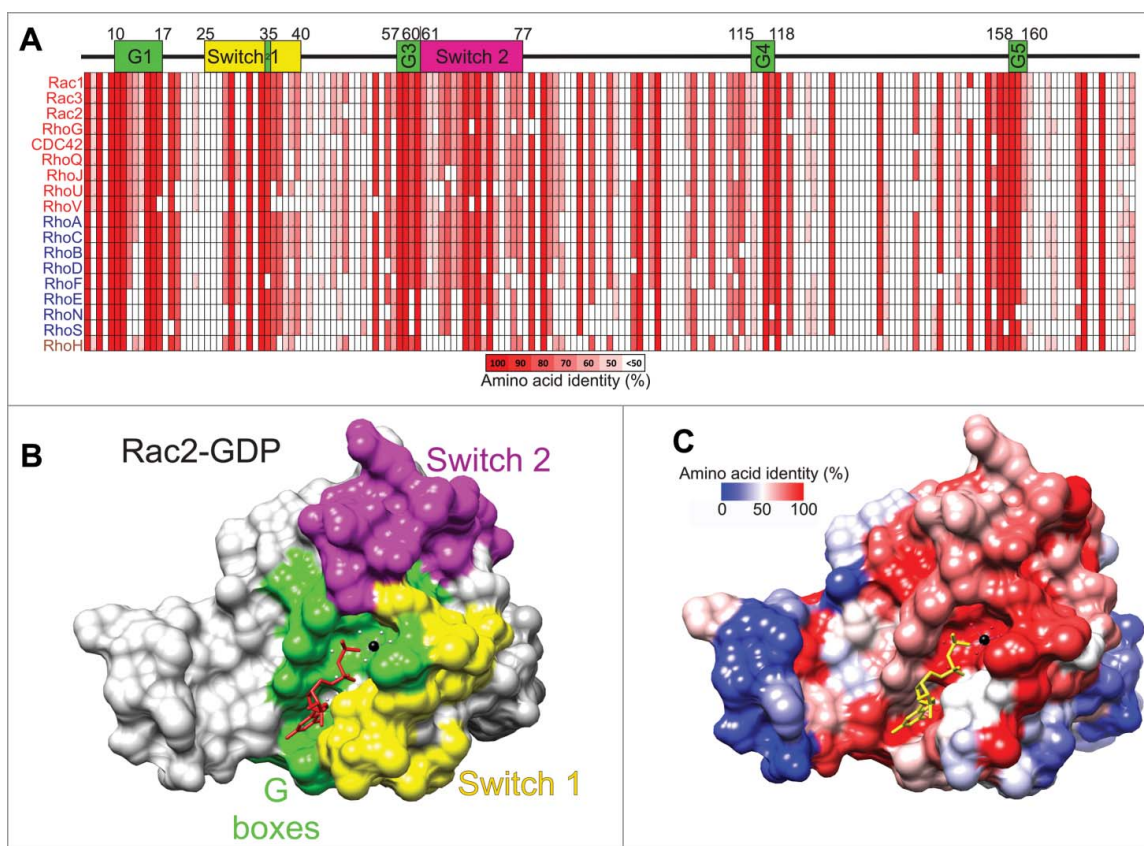
Figure 4. Amino acid conservation in the Rho GTPases. (A) The core conserved segments, with important features numbered by their positions within amino acids 4–177 of Rac1, of the 18 most related Rho GTPases are depicted with percentages of amino acid identity ranging from 100% (red) to less than 50% (white). Guanine nucleotide binding regions are shown as G1 to G5 boxes (green). Switch 1 (yellow) and Switch 2 (magenta) regions are also depicted. (B) Rac2-GDP (PDB ID: 2W2T) structure was rendered with Chimera,[112] with G boxes (green), Switch 1 (yellow) and Switch 2 (magenta) regions indicated. (C) Amino acid identity for 18 Rho GTPases was determined with Clustal Omega [113] and mapped onto Rac2-GDP with Chimera. Colors depicting amino acid identity from 100% (red) to 0% (blue) as indicated.

phosphorylated on S71 (13/20 identical) by Akt to inhibit GTP binding,[27] similar to the CDC42 S71 phosphorylation[28] that is PI-3 kinase inhibitor sensitive.[29] The extensive conservation of many of the phosphorylation sites suggests that they may be general targets for post-translational modifications to provide additional levels of regulation. How these modifications may interact with disease-associated mutations (discussed below) is currently unknown, nor is it clear how these modifications would impact the efficacy of small molecule inhibitors. The consequence of these modifications on Rho GTPase activity and function illustrate the complexity and multi-layered nature of the regulatory mechanisms.

Ubiquitylation and regulation of protein stability

In addition to regulation through modulation of inactivation/activation cycling and subcellular localization, Rho GTPase signaling may also be influenced by varying protein levels. Expression of RhoB,[30] RhoC,[31] Rac3,[32] RhoG,[33] RhoU,[34] RhoE,[31,35] RhoS[36] and RhoH[37] can be induced by various stimuli. Conversely, ubiquitylation and subsequent proteasome-mediated degradation act to limit signaling and consequent effects on the actin cytoskeleton. RhoA is modified by ubiquitin ligase complexes directed by the E3 targeting proteins Smurf1 on K6 and K7,[38,39] by FBXL19 on K135,[40] and BACURD family proteins.[41] Rac3 is ubiquitylated on K166 by the E3 protein FBXL19,[42] while Rac1 is ubiquitylated by HACE on K147,[43,44] XIAP and c-IAP1 on K147,[45] and by FBXL19 on K166 dependent on S71 phosphorylation.[46] Unlike the conservation of several tyrosine residues that undergo phosphorylation in multiple Rho GTPases, the positions of ubiquitylated lysines are not conserved (Fig. 5a), and most identified ubiquitylation events occur in a "Ubiquitylation region" on the opposite side of the protein from the GTP-binding and switch regions (Fig. 5b and c). Many additional Rho GTPases have lysines scattered around this surface that are potentially ubiquitylated to regulate protein stability.

The significance of Rac1 regulation via ubiquitylation was revealed by the discovery of HACE1 ubiquitin ligase
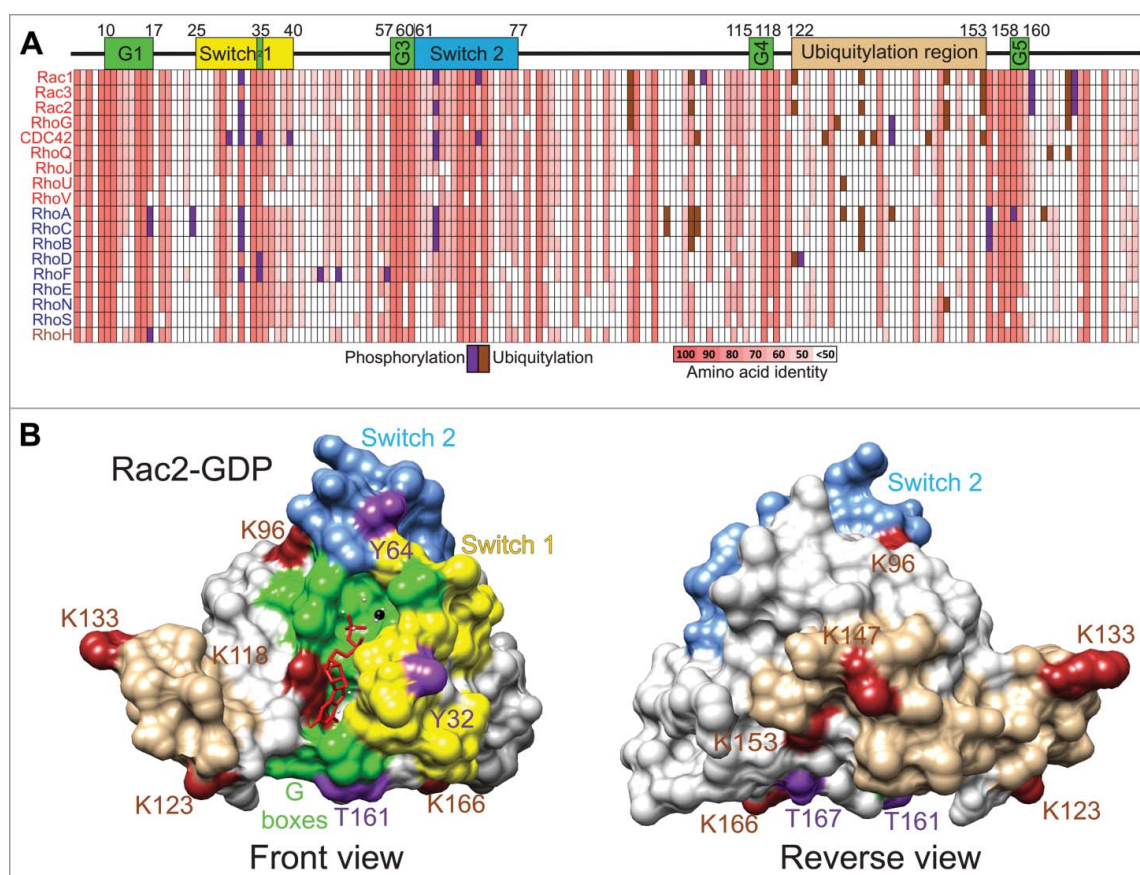
Figure 5. Phosphorylation and ubiquitylation of Rho GTPases. (A) Phosphorylation (purple) and ubiquitylation (brown) events reported in the literature or PhosphoSite (www.phosphosite.org) were mapped onto the amino acid identity grid depicting the core conserved segments, analogous to amino acids 4–177 of Rac1, of the 18 most related Rho GTPases, ranging from 100% identity (salmon) to less than 50% (white). (B) The phosphorylations (purple) and ubiquitylations (brown) of Rac2 were mapped onto the 3-dimensional structure, with G boxes (green), Switch 1 (yellow) and Switch 2 (magenta) regions depicted. (C) View rotated 180° of Rac2 phosphorylations and ubiquitylations.

as a tumor suppressor in Wilm's tumor.[47] Subsequent investigations found HACE1 expression to be reduced in multiple cancers by several mechanisms including chromosomal translocation, deletions or loss-of-heterozygosity, as well as by epigenetic suppression.[47,48] Although reduced HACE1 expression would be predicted to have pleiotropic consequences due to its multiple targets, a key effect of HACE1 loss is increased Rac1 activity that induces cell migration and reactive oxygen species generation[43,44,49] that likely contribute to Rac-mediated tumor progression.[50]

Rho GTPase mutations

Almost concurrent with their discovery, it was recognized that *HRas, KRas* and *NRas* GTPases had high frequencies of activating mutations in human cancers that work by reducing intrinsic and/or GAP-induced GTPase activity, thereby locking the protein in the on-state.[51] Similar GTPase deficient mutations have not been detected for *Rho* GTPases, and for many years it was

believed that Rho proteins were not mutated in pathological conditions. However, the advent of large-scale gene sequencing has revealed both gain-of-function and loss-of-function mutations in several *Rho* GTPases. These findings have implications for how these proteins might be targeted by therapeutic agents, and suggest potential adverse effects that such treatments might evoke.

Immunodeficiency syndromes

Although infrequent, inactivating *Rac2* mutations are associated with human immunodeficiency syndromes.[52] Rac2 has important roles in regulating the NADPH oxidase complex that generates superoxide in phagocytic cells of the immune system.[53] In addition, Rac2 also contributes to the chemotactic and phagocytic activities of immune cells such as neutrophils.[52] A *Rac2* D57N mutation was identified in a human neutrophil immunodeficiency syndrome patient; the effect of this mutation was to decrease Rac2 GTP-binding, resulting in a dominant-

negative acting protein that repressed endogenous Rac function.[54,55] *Rac2* D57N was also identified in an additional patient screened for T-cell lymphopenia.[56] Homozygous *Rac2* nonsense mutations at codon 56 (W56X) were identified in siblings with common variable immunodeficiency.[57] Unlike the manifestation of neutrophil dysfunction in patients bearing D57N mutations within weeks after birth, patients with W56X mutations did not present severe neonatal abnormalities. Instead, symptoms including recurrent infections did not emerge until the patients reached 6 months and 2 y of age,[57] suggesting that the effect of Rac2 protein absence was less potent than the dominant-inhibitory action of Rac2 D57N on endogenous wild-type Rac1.[54,55]

RhoH is predominantly expressed in haematopoietic cells,[52] and is GTPase defective due to 2 differences at conserved sites analogous to Rac1 G12 and Q61 (similar to differences in RhoE, RhoN, RhoS, RhoBTB1 and RhoBTB2 at these positions) that would affect attacking water and GAP arginine finger co-ordination such that it remains constitutively GTP-bound.[37] *RhoH* deletion in mice revealed essential roles in T cell receptor signaling that are required for thymocyte selection and maturation.[58] Two adult human siblings with T cell defects that made them susceptible to infections by *β*-papilloma viruses were found to have homozygous nonsense *RhoH* mutations in codon 38 (Y38X) that resulted in loss of protein expression.[59] Consistent with the effects observed in $RhoH^{-/-}$ mice,[58,59] T cell receptor function was impaired and there were reduced numbers of tissue-homing integrin $β_7$-positive T cells that would likely contribute to the observed susceptibility to *β*-papilloma viral infections.

Cancer

Although many of the first DBL-homology-containing guanine nucleotide exchange factors (GEFs) were discovered on the basis of their ability to oncogenically transform murine fibroblasts, including the prototype GEF Dbl,[60] GTPase-defective Rho proteins are inefficient oncogenes. Their poor transforming activity was attributed to a requirement for rapid GDP/GTP cycling, akin to the effect of activated GEFs, rather than persistent activation induced by GTPase-inactivating mutations. Consistent with this hypothesis, the rapid-cycling CDC42 F28L mutation, in which nucleotide-binding site and hydrogen bonding network disorder enhance spontaneous GDP/GTP exchange,[61] had potent transforming activity.[62]

Recent high-throughput sequencing has led to the discovery of several significantly occurring *Rac1* mutations (Table 1). In sun-exposed melanomas, *Rac1* P29S substitutions were identified[63,64] that were proposed to alter Switch 1 conformation to destabilize the GDP-bound state and stabilize the GTP-bound form.[63,64] The *Rac1* P29S mutation was also detected in a case of head and neck squamous cell carcinoma.[65] Analogous *Rac2* P29L[63,66] and P29Q mutations[66] have been identified, reinforcing the importance of this Proline residue for normal Switch I region function. Additional activating *Rac1* mutations were identified in various cancer cell lines,[66] each of which were found to increase spontaneous GDP release to allow rapid GDP/GTP cycling that increases signal output.[66] Similarly, there is elevated expression of the rapidly GDP/GTP exchanging Rac1B splice variant in colorectal,[67] breast,[68] lung,[69] thyroid,[70] and pancreatic[71] cancers. These findings indicate that increased Rac signaling contributes to processes that promote tumorigenesis.

In contrast to the significant occurrence of Rac1 activation in cancer, frequent inactivating *RhoA* G17V mutations have been detected in T cell lymphomas.[72-74] The substitution of Valine for Glycine in the nucleotide binding pocket was predicted to introduce a bulky side-chain[72] that would result in reduced GTP binding.[73,74] In addition, RhoA G17V more effectively bound RhoGEFs than wild-type RhoA, and acted as a dominant-negative protein in cells to inhibit endogenous RhoA functions.[73,74] Sequencing RhoA in Burkitt lymphomas revealed additional mutations (Table 1) that were predicted to reduce GEF binding and consequent GDP/GTP exchange.[75] In diffuse-type gastric cancer, further *RhoA* mutations were identified and found to confer growth promoting effects that wild-type RhoA did not.[76] Two additional mutations were found in *RhoA* in head and neck squamous cell carcinoma that mapped to the Switch 1 region.[65] Given that the mutations often clustered in regions important for GTP binding or effector interaction (including recurrent Y42 mutations),[77] these alterations may act as loss-of-function mutations that exert dominant-negative actions. The distribution of apparently inactivating *RhoA* mutations at varying amino acids also suggests that loss-of-function mutations could act via different mechanisms to achieve the same outcome. Since Rho signaling antagonizes Rac activity,[78,79] one possibility is that the effect of reduced RhoA signaling on tumorigenesis is mediated, at least in part, by enabling Rac functions.

In addition to the coding mutation described above, the *RhoH* gene is frequently altered by mutations in 5' untranslated regions and by chromosomal translocations.[80] In fact, the intronless *RhoH* gene was first detected as part of a translocation between chromosomes 3 and 4 with the BCL6 gene in a non-Hodgkin lymphoma cell line, and was initially called TTF for

translocation three four.[81] The *RhoH* gene was found to have undergone aberrant somatic hypermutation in germinal center-derived diffuse large-cell lymophomas, akin to the hypermutation of immunoglobulin variable region genes that normally takes place in B cell centroblasts to increase antibody diversity.[82] Since its initial discovery, *RhoH* gene mutations have been identified in lymphomas, mature B-cell neoplasms, multiple myeloma and lymphoproliferative disorders linked to immunodeficiencies associated with viral infections.[80,83] In addition, reduced RhoH expression has been described in hairy cell leukemia[84] and acute myeloid leukemia,[85] although the causes for the low expression has not been determined. These alterations consistently lead to decreased RhoH expression, however the link between low RhoH protein levels and haematopoietic tumourigenesis is unclear. Although *RhoH*$^{-/-}$ mice have impaired T cell receptor signaling and defective thymocyte selection and maturation that lead to T cell deficiency, they did not develop lymphomas.[58,86] It was noted that basal Rac1 activity was higher in *RhoH*$^{-/-}$ mice,[86] while RhoH was also found to inhibit signaling downstream of active Rac1.[37] Therefore, one possibility is that, like inactivating RhoA mutants, reduced RhoH signaling may contribute to tumourigenesis by enhancing Rac functions.

In addition to their Rho GTPase related N-terminal region, RhoBTB1 and RhoBTB2 have a large C-terminal extension that includes a Proline-rich region, 2 BTB (broad-complex, tramtrack, bric à brac) domains and a conserved C-terminal region.[87] The additional domains associate with a number of proteins, several of which are components of ubiquitin ligase complexes that regulate the degradation of proteins involved with cell cycle progression and vesicle transport, as well as the influencing the stability of the RhoBTB proteins themselves. RhoBTB2 was identified as a putative tumor suppressor gene (Deleted in Breast Cancer 2; *DBC2*) that is located on chromosome 8p21 which is deleted or mutated in breast cancer.[88] Subsequently, it has been found that there is significant loss of heterozygosity (LOH) of the *RhoBTB2* gene in bladder[89] and gastric cancers,[90] and LOH of the *RhoBTB1* gene in head and neck squamous cell carcinoma.[91] In addition, rare somatic mutations have been identified in the *RhoBTB1* and *RhoBTB2* genes in these studies. However, more frequent are observations of reduced RhoBTB1 and RhoBTB2 expression, likely due to epigenetic factors including promoter methylation.[92] The tumor suppressor functions of RhoBTB1 and RhoBTB2, which may be lost as a result of gene deletion, mutation or reduced gene expression, are believed to be related to actions of the protein-associating C-terminal extension and not the Rho-homologous N-termini.[87] Although expression of RhoBTB1 or RhoBTB2 did not significantly affect actin cytoskeleton structures,[93] it has been suggested that the Rho-homologous region may interact with the BTB domain containing region to regulate associations with additional protein partners.[94] Therefore, it remains to be determined whether the rare mutations detected in the Rho-homolgous region (http://cancer.sanger.ac.uk/cosmic) may affect the tumor suppressive function of RhoBTB1 and RhoBTB2 by interfering with the formation of protein complexes with the C-terminal regions.

Large scale sequencing efforts have identified sporadic mutations in all 20 Rho GTPases in a large variety of cancers (http://cancer.sanger.ac.uk/cosmic). Some substitutions may be so conservative that they have little to no effect on protein function, while others may be adventitious mutations that are merely by-products of high mutation rates. Additional research will reveal whether specific mutations are passengers or drivers that contribute substantively to tumor initiation, growth and progression.

Small molecule inhibitor development

Given the initial associations of Rho GTPase signaling with cancer metastasis[95] and later discovery of activating mutations in tumors, there has been interest in developing small molecule inhibitors to inhibit Rho GTPase signaling.[96] One strategy employed has been to inhibit the interactions between GTPase and cognate GEF. The most widely-used GEF blocking compound is NSC23766, which was originally identified by *in silico* molecule docking and then validated as impairing the interaction of Rac1 with Trio and TIAM1 GEFs.[97] Although the potency of this compound is relatively low, limiting its potential for further development as a clinical candidate, it has been very useful as a tool compound and for providing proof-of-concept data. The inhibitor EHT1864,[98] which binds Rac1, Rac1b, Rac2 and Rac3, induces nucleotide release and can block TIAM1 interaction leading to inhibition of signaling[99] Additional small molecule Rac1/RhoG inhibitors that target GEF interactions have been identified[100,101] on the basis of blocking Trio GEF interactions, as well as Y16[102] and Rhosin[103] that were discovered based on interference with LARG-RhoA binding. Cell-based screening for inhibitors of PIP$_2$-induced actin polymerization in *Xenopus laevis* egg cytoplasmic extracts led to the identification of a series of Pirl1-related compounds that were found to block GEF-induced CDC42 activation,[104] latterly the Pirl1 related compound 2 was named CASIN.[105]

An alternative strategy has been to directly target the GTPase. Compound library screening based on competition with fluorescent-GTP for bead-immobilized GTPase binding identified both broad-specificity[106] and CDC42-selective molecules.[107] *In silico* docking and validation by

surface-plasmon resonance resulted in the identification of RhoA inhibitors that act by GTP-competition.[108] A similar virtual screen led to the discovery of Rhosin,[103] however in this case binding occurs in a shallow groove that forms part of the interface with GEFs including LARG, DBL and LBC.

Although these results validate the feasibility of discovering Rho GTPase inhibitors, there are several challenges that must be met if compounds are to be developed toward the ultimate goal of clinical use. GTP-competitive molecules would require very high affinities to overcome high cellular GTP concentrations.[109] Given that many of the activating mutations identified for Rac1 enable GEF-independent GDP/GTP exchange[63,64] as does the Rac1B splice variant often elevated in cancers,[67-71,110] direct GTPase inhibition, including through GTP-competition, may be the most effective means to reduce signal output. Target selectivity is a significant issue, particularly since examining the selectivity of protein-protein interaction inhibitors is technically challenging due to the complexity of potential on-target and off-target protein binding partners. Indeed, by testing NSC23766 and EHT1864 in homozygous *Rac1* knockout platelets, it was determined that both compounds have Rac1-independent effects including direct inhibition of the Rac1 effectors PAK1 and PAK2.[111] Many protein-protein inhibitors bind to surface pockets at the interaction interface, rather than deep pockets such as the nucleotide binding region. Although there may be sufficient differences in these relatively shallow surface pockets to confer selectivity, it is may be difficult for structure-activity relationship (SAR) medicinal chemistry to simultaneously increase potency and maintain selectivity.

The human mutation data suggests that selectivity would indeed be important for compounds to be used clinically. While Rac1 inhibition appears to be a potentially efficacious strategy, concomitant RhoA inhibition could have pro-tumorigenic effects.[65,72-74,76] In addition, the immunodeficiencies observed in patients with *Rac2* mutations[54-56] suggest that a Rac1 inhibitor might be immunosuppressive due to the likely difficulty in avoiding simultaneous inhibition of the highly-related (92% identity) Rac2 protein. If Rac1 inhibitors also blocked RhoH function, there might also be a risk of immunosuppression[59] that could lead to susceptibility to viral infection and an increased risk of haematopoietic cancers.[80,83-85]

Future directions

Despite the relative maturity of the Rho GTPase field, novel discoveries are continually made. Greatest attention has been paid to RhoA, Rac1 and CDC42, leaving considerable opportunity for details regarding the regulation and function of the remaining family members to be uncovered.

Post-translational modifications at highly-conserved sites are suggestive that they may occur broadly. The occurrence of genetic mutations in the coding sequences of every Rho GTPase also suggests that there may be functional consequences for each protein. The recent emergence of gene-editing technologies will enable studies on the effect of these mutations in cellular contexts, and using *in vivo* models. Conditional genetically-modified *in vivo* models will continue to uncover the biological functions of the Rho GTPases in physiologically relevant environments as well as their contributions to pathological conditions, and may provide important clues about the potential utility, as well as adverse effects, of pharmacological inhibitors. Ultimately, the benefits and risks of potential Rho GTPase targeted therapeutics will be elucidated following rigorous testing in appropriate model systems.

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