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MINI-REVIEW

Crosstalk between WIP and Rho family GTPases

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ABSTRACT

Through actin-binding proteins such as the neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP-interacting protein (WIP), the Rho family GTPases RhoA, Rac1 and Cdc42 are major modulators of the cytoskeleton. (N-)WASP and WIP control Rho GTPase activity in various cell types, either by direct WIP/(N-)WASP/Cdc42 or potential WIP/RhoA binding, or through secondary links that regulate GTPase distribution and/or transcription levels. WIP helps to regulate filopodium generation and participates in the Rac1-mediated ruffle formation that determines cell motility. In neurons, lack of WIP increases dendritic spine size and filamentous actin content in a RhoA-dependent manner. In contrast, WIP deficiency in an adenocarcinoma cell line significantly reduces RhoA levels. These data support a role for WIP in the GTPase-mediated regulation of numerous actin-related cell functions; we discuss the possibility that this WIP effect is linked to cell proliferative status.

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Introduction

In this review, we will summarize some of the most recent data that describe the link between WIP (WASP-interacting protein) and some classical GTPases and how they regulate several general cell processes such as migration, proliferation and differentiation.

Rho GTPase family members

From the pioneer report by Ridley and Hall, who demonstrated that active Rho stimulates stress fiber formation[1], the Rho GTPase family has expanded to 20 members. They are usually classified in two major groups, the canonical (Rho, Rac, Cdc42, RhoD/F) and the atypical (Rnd, RhoU/V, RhoH, RhoBTB). Many reports describe the activation of canonical Rho-family members through membrane receptors and cell adhesion molecules which leads to activation of the Arp2/3 (actin-related protein) complex and actin assembly. Studies on less well characterized atypical members of the Rho-family support new levels of complexity and inter-connectivity in Rho-family GTPase signaling during cell migration.[2,3] In response to extra- and intracellular stimuli that modulate their many roles, including regulation of the actin cytoskeleton and cell signaling, most Rho family canonical GTPases and some atypical members cycle between an active GTP-bound form and an inactive GDP-bound form. This itinerant status is governed by three sets of proteins, the activating guanine nucleotide-exchange factors (GEF), the inhibitory GTPase-activating proteins (GAP), and the inhibitory guanine nucleotide-dissociation inhibitors (GDI).[4] When bound to GTP, the GTPases can interact with and activate downstream effector proteins, and thus stimulate a plethora of cell processes (migration, division and adhesion, neuron development, final organ morphogenesis). This capacity can affect cytoskeletal structures, modifying not only actin-based activities but also microtubule dynamics, in almost all cell lineages. An essential set of information arose from analysis of the canonical elements Rho, Rac, Cdc42, and their direct control of filamentous actin (F-actin) regulation.[5] Cdc42 has a conserved role in regulating cell polarity and stimulating filopodium induction, whereas Rac proteins generate lamellipodia that regulate membrane ruffle formation and induce membrane extension. In contrast, the three homologous Rho isoforms — RhoA, RhoB and RhoC— all induce stress fiber formation when overexpressed in fibroblasts.

The atypical GTPases Rnd1, Rnd2 and Rnd3 are always bound to GTP and, therefore, are not regulated by the same kind of effectors than the canonical ones. Some reports have provided important insights into the mechanisms that control the function of Rnd proteins; rather

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than by the GDP/GTP switch, their activity is regulated by their expression, localization and phosphorylation. Interestingly, there are common players between canonical and atypical forms as Rnd1 and Rnd3 antagonize RhoA-mediated actin remodeling during cell migration through localized recruitment of p190RhoGAP.[6]

The generation of GTPase-regulated cytoskeletal structures usually requires the action of several actin-related proteins that provide the final link between the GTPases and the regulation of F-actin formation. The active/inactive status of each GTPase can control some central elements of polymerization, such as the Arp2/3 complex, as well as a broader group of proteins that transduce overall signals into specific actions or territories; some of these are GTPase-specific, such as cofilin, rhotekin, NADPH oxidase, citron kinase, profilin, and Par3/Par6. Alternatively they can trigger both actin polymerization and force generation through activation of the formin mDia.[2]

Wiskott-Aldrich syndrome protein (WASP) family proteins

Among the best-characterized GTPase effectors, two of the six mammalian WASP family members stand out; these are WASP and neural WASP (N-WASP), whose main function is to act as nucleation-promoting factors (NPF) for actin polymerization.[7] (N-)WASP-mediated activation of the Arp2/3 complex allows 70° angle nucleation over an existing "mother" actin filament. This generates a branched cytoskeletal mesh that supports cell protrusions such as the lamellipodium, which is involved in processes such as cell adhesion or migration.

The multidomain protein structure of (N-)WASP is ideal for integrating multiple input signals to coordinate appropriate final changes in the overall actin cytoskeleton.[8] In several cell systems, the interaction of active Cdc42-GTP with the (N-)WASP GTPase binding domain (GBD) promotes 'opening' of the (N-)WASP structure and increases its nucleation activity.[9-11] An in vitro pyrene-actin polymerization assay using recombinant proteins demonstrated that (N-)WASP activation by Cdc42 is also regulated by WIP binding, which promotes the (N-)WASP inactive conformation.[12] Together with cell membrane-bound Cdc42-GTP, WIP is also involved in (N-)WASP recruitment to specific subcellular locations.[13-15] These interactions recruit (N-)WASP to the cell region at which actin must polymerize, and contribute to the indispensable spatial and temporal control of this process. WIP also mediates actin tail formation for vaccinia virus motility; after its Nck-dependent recruitment, (N-)WASP engages with the Cdc42 GEF intersectin-1 to activate the GTPase and maintain sustained actin polymerization.[16,17] The WIP/(N-)WASP role is conserved in evolution, as reported for D-WIP (*Drosophila*-WIP); the Arp2/3 WASP/WIP complex, located at the actin caplets during spermatogenesis in flies, is needed to ensure correct spermatid release from the head cyst cell.[18] In addition to Cdc42 and the cortical actin cytoskeleton, (N-)WASP also binds curved anionic membranes in lipid rafts, thought to be induced by glycosylphosphatidylinositol-anchored proteins.[19]

WASP-interacting protein (WIP)

WIP was first identified as a partner of the hematopoietic-specific WASP in lymphocytes,[20,21] and then as an N-WASP partner in many other cell types (e.g., fibroblasts, neurons, epithelial cells), as both WIP and N-WASP are expressed ubiquitously.[22] WIP can also exert its activity independently of its (N-)WASP interaction.[23,24]

-WIP relation with Rho GTPases

Twenty years ago, WIP was shown to bind the WASP N-terminal region at a site distinct from the GBD, which interacts with Cdc42, weakly with Rac, and not with Rho.[9-11,20] From the outset, WIP was therefore linked indirectly to GTPases, and the lack of any detectable GBD in its sequence suggested that direct WIP/GTPase interaction is unlikely. A recent publication reports a pull-down experiment using mixed lysates containing tagged overexpressed proteins (HA-RhoA or WIP-His) and speculate on direct WIP binding to Rho.[25] In vitro assays using purified recombinant proteins (full-length and deletion mutants), alone or with (N-)WASP, would nonetheless be needed to determine the precise nature of this WIP-RhoA interaction. Of great interest for GTPase/cytoskeletal studies would also be the confirmation of endogenous WIP and Rho protein interaction in physiological resting and/or stimulation conditions.

WIP and small GTPases are also linked through the direct, WASP-independent binding of GEF DOCK8 (dedicator of cytokinesis 8) to WIP.[24] DOCK8 associates constitutively with the WIP/WASP complex in resting primary T cells and this multi-complex persists after T cell receptor (TCR)-mediated stimulation. The spatial proximity of DOCK8, WASP, and actin in this complex ensures that Cdc42 (activated by DOCK8 following TCR binding) drives WASP-mediated actin polymerization. This control of the subcortical actin cytoskeleton regulates immune synapse formation, mechanotransduction, T cell transendothelial migration, and homing to lymph nodes, all of which also depend on WASP.

There is much less information on the connection between GTPases and other verprolin/WIP family

members such as CR16 (corticosteroids and regional expression 16) or WICH/WIRE (WIP-CR16 homologous/WIP-related).[26] The few available studies on these proteins report that Cdc42 regulates IRSp53-WIRE interaction as well as localization of this complex to the plasma membrane to generate filopodia,[27] whereas Toca-1 (transducer of Cdc42-dependent actin assembly) promotes actin nucleation by activating the (N-)WASP-WIP/CR16 complex.[28]

-WIP as a regulator of actin-rich cellular components

At the functional level, WIP activity is associated with the generation of specific actin-rich structures such as filopodia, lamellipodia and stress fibers, as a result of actin cytoskeleton reorganization mediated by Cdc42, Rac, and Rho activation, respectively. In 3T3 murine fibroblasts, WIP regulates N-WASP-induced actin nucleation and contributes significantly to the formation of actin-containing microspikes promoted by bradykinin and Cdc42-GTP; WIP microinjection in murine fibroblasts induces Cdc42-GTP/N-WASP-dependent filopodia, whereas anti-WIP microinjection prevents their generation.[12]

In this same cell system, WIP also participates in Rac-mediated actin reorganization and dorsal/circular ruffle formation induced by PDGF (platelet-derived growth factor), a chemotactic factor for fibroblasts.[29] WIP overexpression enhances dorsal/circular ruffle formation in response to PDGF and, conversely, microinjection of anti-WIP antibody (or lack of WIP in knocked-down primary murine fibroblasts) leads to decreased ruffle formation in response to PDGF. In this setting, the WIP effect depends on its ability to interact directly with actin, as overexpression of a shortened WIP form that lacks the actin-binding site prevents PDGF-induced membrane ruffling.

Rac-regulated circular ruffling is commonly associated with macropinocytosis (internalization of solutes and membrane components), a process that takes place prior to cell movement, as transient ruffling contributes to establishment of polarity in motile cells. The reduced ability of WIP-deficient murine fibroblasts to form circular ruffles suggested a role for WIP in fibroblast movement, which was later confirmed by demonstration that both mesenchymal and amoeboid motility depend on WIP levels.[30] The use of lentivirally reconstituted WIP-deficient murine fibroblasts broadened our knowledge of the requirement for WIP interaction with N-WASP and the adaptor Nck for efficient dorsal ruffle formation; it also identified the need for WIP-Nck binding for efficient fibroblast chemotaxis to PDGF-AA but not to stimuli such as lysophosphatidic acid, epidermal growth factor or fibroblast growth factor. In addition, WIP participates in the amoeboid form of B lymphocyte motility in response to the B cell-specific chemokine CXCL13 (C-X-C motif chemokine 13) by controlling lamellipodium formation and cell polarization. In both types of migration, mesenchymal and amoeboid, WIP regulates the directional persistence of cell movement, whereas cell speed is only affected in amoeboid B lymphocytes.

-WIP participates in invasiveness and oncogenic activities

A recent functional connection was identified between the oncogenic effect of WIP and Rac activity in astrocytes and glioblastoma (GB) cells.[31] As WIP overexpression in cultured primary human astrocytes increases cell survival by stabilizing the transcriptional co-activators YAP/TAZ (Yes-associated protein/transcriptional coactivator with PDZ- binding motif), screening of molecules that produce cell death in this system allows identification of pathway components able to impair WIP-mediated survival. Incubation of cells with the Rac inhibitor NSC23766 reversed WIP ability to stabilize YAP/TAZ, which was unaffected by Cdc42 (casin) or RhoA (Y16) inhibitors in astrocytes and GB. Conversely, YAP/TAZ stability, which is notably reduced in WIP knocked-down cells, was unaffected by the presence of a constitutive active Rac mutant (Rac-V12), which suggests a downstream GTPase effect. WIP knockdown in GB also reduced phosphorylation of PAK, a Rac substrate in many processes. These findings support strong dependence on Rac/PAK activation for the WIP oncogenic effect in astrocytes, as well as in breast cancer cells.

Several recent reports highlight WIP as an important regulator of cell invasion, proliferation and anchorage-independent growth in various tumor types. WIP expression is upregulated in human GB explants and in invasive breast cancer cell lines,[31] in ameloblastoma,[32] in highly metastatic A5-RT3 cells (Ras-transformed keratinocytes) vs. non-metastatic parental HaCaT cells, in cancerous A549 cells vs. non-cancerous human small airway epithelial cells (H-SAEC),[25] and in human papillary thyroid tumors.[33]

In ameloblastoma, the most commonly diagnosed odontogenic epithelial neoplasm, WIP is upregulated significantly along the tumor invasive front compared to tumor centers; it is the most widely expressed invadopodial protein in this tumor.[32] Invadopodia are F-actin-rich membrane protrusions that concentrate and secrete metalloproteases, which facilitate extracellular matrix (ECM) degradation.[34] Invadopodia attach to the ECM via their ring-shaped adhesion domain, which confines the actin core and contains GTPases. After the adhesion stage and the receptor-mediated signaling event, cytoskeleton activators are recruited to the membrane, which leads to

actin polymerization in invadopodium cores mediated by a Rho-family small GTPase-regulated process.[35] This mainly Cdc42-dependent mechanism can activate the (N-)WASP/WIP complex directly and produce a sustained invasive protuberance and promotes its penetration of the ECM. Rac1 involvement in invadopodium formation was recently identified in melanoma cells; decreased expression of wild-type Rac1 reduces invadopodium-dependent matrix degradation, in contrast to decreased expression of a hyperactive Rac1 mutant that enhances invadopodium function.[35] GTPases have a central role not only in invadopodium generation and maturation, but also in forming functional podosomes, degradative structures closely related to invadopodia, in which Cdc42 and RhoA control actin reorganization.[36]

WIP localizes to the tip of the invadopodium; its expression is necessary for invadopodium formation and ECM degradation by basal breast cancer cells, but is not sufficient to induce invasiveness in luminal cells.[37,38] The identification of WIP as a potential biomarker that correlates directly with tumor aggressiveness in ameloblastoma, GB, breast cancer and thyroid cancer is of considerable clinical relevance. Due to its differential distribution at the invasive front in ameloblastoma, WIP might also be a promising target for the development of patient-tailored treatment strategies.

- WIP exerts opposite roles in neuronal differentiation and glioma proliferation

The lack of WIP in hippocampal neurons co-cultured with astrocytes leads to increased dendritic spine size and F-actin levels.[39,40] Identification of the molecular mechanism underlying this phenotype showed direct involvement of increasing amounts of the RhoA GTPase but not of Cdc42 or Rac1, whose levels appear to be normal. Absence of WIP produces a three-fold increase in RhoA levels as well as consistently higher RhoA activity and membrane-associated distribution. Levels of the two RhoA downstream effectors ROCK (Rho-associated protein kinase) and profilin IIa are thus increased three-fold in WIP-deficient synaptosomes compared to controls. In this way, WIP deficiency facilitates activation of the RhoA-ROCK-profilin IIa pathway and contributes to increased F-actin levels in dendritic spines that lack WIP. This RhoA increase is the result of the WIP-dependent translational upregulation of neutral sphingomyelinase (NSM), whose activity reduces sphingomyelin (SM) levels at synapses. This alteration in membrane lipid composition enhances RhoA membrane binding, raft partitioning, and activation in steady state, but prevents changes in these RhoA features in response to stimuli. WIP thus has an essential role in connecting actin cytoskeleton and synaptic membrane lipid composition.[41] The distinct physiological effects should be noted of local RhoA activation, which leads to increased actin filaments in dendritic spines, and general RhoA activation, which can cause dendritic retraction and growth cone collapse.[42,43]

In contrast to the effect in neurons, in which lack of WIP increases RhoA levels, silencing WIP expression reduces RhoA levels and attenuates the tumorigenic and metastatic abilities of A549 lung adenocarcinoma cells. WIP overexpression enhances cell invasion and proliferation as well as their anchorage-dependent growth.[25] In this human epithelial cell system, and at difference from WIP-deficient neurons, WIP reduction does not affect RhoA mRNA expression or RhoA levels downstream of effectors such as ROCK-II and mDia1, or other Rho GTPases such as Cdc42 and Rac1. The overexpression effect is counteracted when cells are treated with the proteasome inhibitor MG132, which produces a time-dependent increase in RhoA expression.[25] This WIP protective capacity against proteasomal degradation is not restricted to RhoA, as WIP levels correlate with those of WASP, Syk.[44] or YAP/TAZ.[31] in a proteasome-dependent manner. We recently reported that loss of proliferation, anchorage-dependent growth and cell invasion in glioma cells are due to decreased WIP expression,[45] which leads to reduced RhoA levels (unpublished data), a result of p53 silencing.

Some GTPases might regulate WIP levels. We observed that expression of constitutive active Rac-V12, by increasing WIP levels, can rescue proliferation of WIP-deficient gliomas.[31] This strongly suggests Rac-WIP crosstalk, and requires further analysis. Data differ with respect to Ras, however; complementary RNA-sequencing analysis identified increased WIP expression in Ras-transformed human keratinocytes compared to controls,[25] whereas we found that direct transformation of an astrocytoma with Ras-V12 increased the proliferation rate, but not WIP levels (our unpublished data).

Future perspectives

The picture of how WIP participates in GTPase regulation is still far from complete. Nonetheless, research in recent years has begun to show that WIP is not a simple structural bystander, but has relevant roles in GTPase protein stability and subcellular distribution, as well as transcriptional control. As a key component of postsynaptic membranes, WIP modifies some lipid content.

At the neuron level, lack of WIP leads to altered levels of SM and its catabolic enzyme NSM at the synaptic membranes, which might affect trafficking and membrane components. All these lipid and protein arrangements could alter neuron synaptic functions and plasticity. Our data from WIP-deficient murine neurons clarify a role in

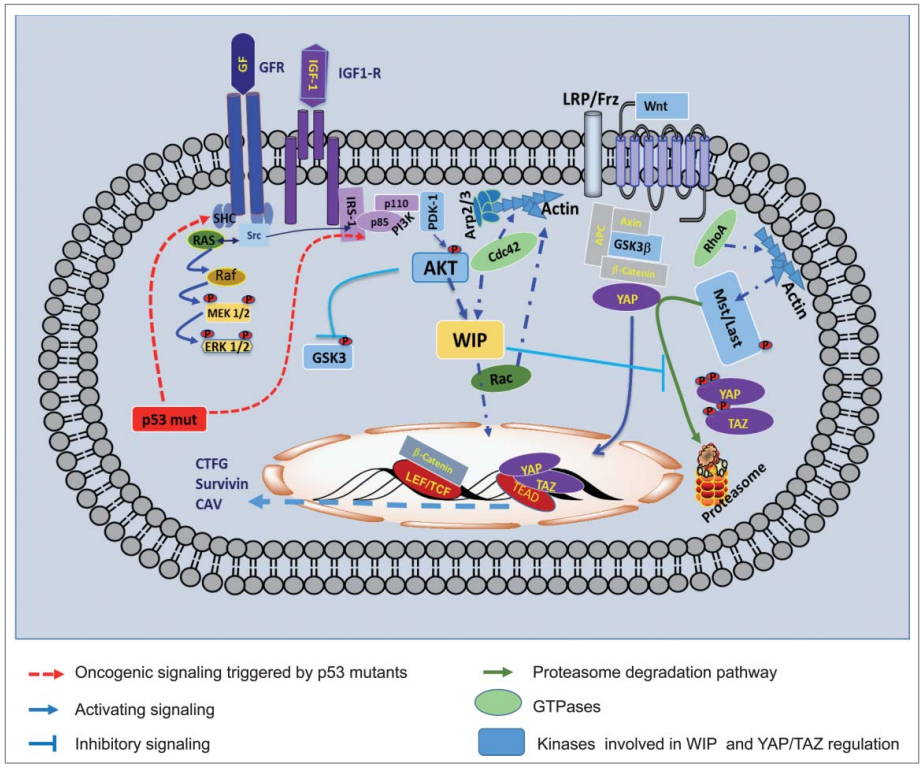

Figure 1. Scheme summarizing several pathways that control cell proliferation during glioma progression, including the recently identified position of WIP. Representative groups are shown of membrane receptors such as tyrosine kinase receptors (IGFR, GFR) or seven-transmembrane receptors (such as Wnt receptor). Through various mechanisms, these receptors can trigger generic PI3K-Akt signaling, which controls WIP activity. In many tumor cell types, certain proto-oncogenic proteins, such as the mutant versions of p53, enhance these membrane receptor activities. The oncogenic function of WIP operates by controlling YAP/TAZ stability/degradation. In some tumor cell types, YAP/TAZ can work together with beta-catenin; this collaboration enhances the relevance of this regulatory pathway, as an abundance of genes could be upregulated to ensure proliferation and survival. Levels of active GTPases such as RhoA are modified in some WIP-deficient cells, whereas in other cases Rac activity can compensate WIP-deficient function and/or WIP levels.

neuron differentiation for WIP, which, by modulating F-actin levels, has an important regulatory function in dendritic spines. We observed dysfunction of some Akt downstream elements in WIP-deficient neurons, although further work is needed to link mTORC1 dysfunction, F-actin polymerization and RhoA in these cells.[39-41]

Many data support an essential role for WIP and GTPases, not only in tumor cell migration but also in cancer stem cell proliferation. These data allow the proposal of a molecular mechanism involved in cell proliferation, differentiation and actin cytoskeleton dynamics, all instrumental features in cell transformation and invasiveness. Our working hypothesis is that, as seen in gliomas, in other tumors WIP is under the control of mutant p53; through Akt activity, WIP regulates protein stability and subcellular distribution of regulatory proteins such as the co-transcription factors YAP/TAZ (Fig. 1).

These findings highlight WIP versatility, which enables it to modulate GTPase-dependent actin cytoskeleton reorganization in different ways depending on cell type, status and site, as well as the partners with whom it associates.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

All authors wrote and revised the manuscript.

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