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Atypical Mechanism of Regulation of the Wrch-1 Rho Family Small GTPase

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Summary

Rho family GTPases are GDP/GTP-regulated molecular switches that regulate signaling pathways controlling diverse cellular processes [1, 2, 3]. Wrch-1 was identified as a *W*nt-1 *r*egulated *C*dc42 *h*omolog, upregulated by Wnt1 signaling in Wnt1-transformed mouse mammary cells [4], and was able to promote formation of filopodia and activate the PAK serine/threonine kinase. Wrch-1 shares significant sequence and functional similarity with the Cdc42 small GTPase. However, Wrch-1 possesses a unique N-terminal 46 amino acid sequence extension that contains putative Src homology 3 (SH3) domain-interacting motifs. We determined the contribution of the N terminus to Wrch-1 regulation and activity. We observed that Wrch-1 possesses properties that distinguish it from Cdc42 and other Rho family GTPases. Unlike Cdc42, Wrch-1 possesses an extremely rapid, intrinsic guanine nucleotide exchange activity. Although the N terminus did not influence GTPase or GDP/GTP cycling activity in vitro, N-terminal truncation of Wrch-1 enhanced its ability to interact with and activate PAK and to cause growth transformation. The N terminus associated with the Grb2 SH3 domain-containing adaptor protein, and this association increased the levels of active Wrch-1 in cells. We propose that Grb2 overcomes N-terminal negative regulation to promote Wrch-1 effector interaction. Thus, Wrch-1 exhibits an atypical model of regulation not seen in other Rho family GTPases.

Results and Discussion

Wrch-1 Possesses Rapid Intrinsic Guanine Nucleotide Exchange Activity Independent of N-Terminal Sequences

In contrast to Cdc42, human Wrch-1 contains N- and C-terminal extensions of 46 and 21 amino acids, respectively. We have examined the role of the N terminus in Wrch-1 GDP/GTP regulation and effector interaction. First, we determined the in vitro guanine nucleotide binding and hydrolysis activities of wild-type (WT) and N-terminally truncated Wrch-1 (ΔN) in vitro. Surprisingly, both WT and ΔN Wrch-1 completely exchanged mantGDP rapidly within 2 min (both $k_{obs} = 0.01\ s^{-1}$) (Figure 1A). Exchange with mantGMPPNP was even more rapid and occurred completely within 30 s (data not shown). In contrast, WT Cdc42 exchange occurred at a 10-fold slower rate ($k_{obs} = 0.001\ s^{-1}$) than Wrch-1, and a fast-cycling mutant of Cdc42 (C18A) [5] showed an exchange rate that was approximately 50% slower (C18A $k_{obs} = 0.004\ s^{-1}$) than Wrch-1. Therefore, Wrch-1 has a significantly greater exchange rate than Cdc42, and the N-terminal extension of Wrch-1 does not play a role in this difference.

The mechanism of Rho guanine nucleotide exchange factor (GEF) action involves displacement of the GTPase-associated Mg^{2+} ion. This effect is mimicked in vitro by addition of excess EDTA to the solution [6], and we observed that EDTA treatment of Cdc42 resulted in a 10-fold increase in the rate of nucleotide exchange ($k_{obs} = 0.01\ s^{-1}$). Unexpectedly, Wrch-1 and ΔN Wrch-1 showed decreases in fluorescence intensity of approximately 75% from their initial levels ($k_{obs} \approx 0.05\ s^{-1}$) (Figure 1B), suggesting that the nucleotide was released rather than exchanged from the protein. Fluorescent anisotropy measurements confirmed this hypothesis (Figure 1C), showing an increase of nucleotide binding to Wrch-1 and Cdc42; yet, upon addition of EDTA, Wrch-1-related anisotropy decreased, whereas Cdc42-related anisotropy increased. Therefore, the release of protein-bound Mg^{2+} caused a loss of Wrch-1 nucleotide binding and exchange activity. Consequently, Cdc42 and Wrch-1 exhibit distinct properties of intrinsic and GEF-mediated nucleotide exchange and that the usual method of nucleotide-loading recombinant small GTPases results in inactive Wrch-1.

Wrch-1 Hydrolyzes GTP Independent of the N-Terminal Sequence

To assess the Wrch-1 intrinsic GTP hydrolysis activity, we utilized the MDCC-phosphate binding protein (PBP) probe [7] to report on P_i produced from single-turnover GTP hydrolysis in vitro and a glutathione S-transferase fusion protein containing the GTP-dependent binding domain of PAK (GST-PAK-RBD) for pull-down of GTP-bound Wrch-1 for in vivo analysis. Cdc42 and Wrch-1, as well as ΔN Wrch-1, showed similar exponential rates of hydrolysis ($k_{obs} = 0.001\ s^{-1}$ at 25°C) (Figure 2A). Thus, Wrch-1 hydrolyzes GTP with similar kinetics to Cdc42, and this activity is not influenced by the N-terminal extension. We extended these analyses in vivo and evaluated the GTP-bound state of WT and a putative GTPase-deficient mutant of Wrch-1 in vivo. We generated a Q107L mutant of Wrch-1 (analogous to the Q61L GTPase-deficient activating mutant of Cdc42). WT and Wrch-1 (Q107L) proteins were expressed in NIH 3T3 cells (Figure 2B). Under starved conditions, the amount of Wrch-1 precipitated by GST-PAK-RBD was considerably less than that seen with Wrch-1 (Q107L). In the presence of serum, the amount of GTP-bound WT Wrch-1 was increased but was still lower than the level seen with the Q107L mutant. Therefore, Wrch-1 is an active GTPase

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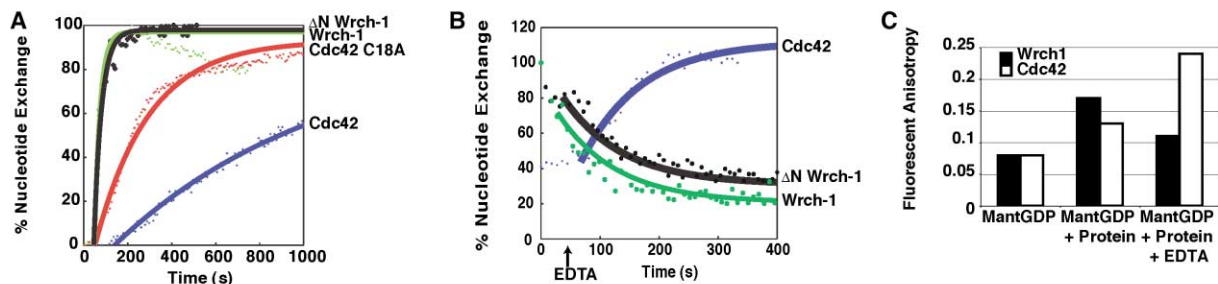

Figure 1. Wrch-1 and Cdc42 Exhibit Very Distinct Interactions with Guanine Nucleotides In Vitro

(A) Wrch-1 possesses a rapid guanine nucleotide exchange rate in vitro. GST-Wrch-1 or GST-Cdc42 was added to a solution of excess mant-GDP, and the changes in mant fluorescent intensity followed.

(B) Wrch-1 demonstrates unusual properties in the absence of bound Mg^{2+}. GST-Wrch-1 or GST-Cdc42 protein was incubated with excess mant-GDP before excess EDTA was added (arrow). The subsequent changes in fluorescent intensity followed.

(C) Wrch-1 releases nucleotide on loss of Mg^{2+}. GST-Wrch-1 or GST-Cdc42 protein was added to a solution containing excess mant-GDP and the fluorescent anisotropy of the Mant-GDP measured after 2 min of incubation. Excess EDTA was added to the solution and the fluorescent anisotropy was then measured after another 2 min of incubation. Data shown are representative of three separate determinations.

in vivo, and the Q107L substitution renders Wrch-1 both GTPase-deficient and GTP-bound. A serum factor may also stimulate Wrch-1 activation.

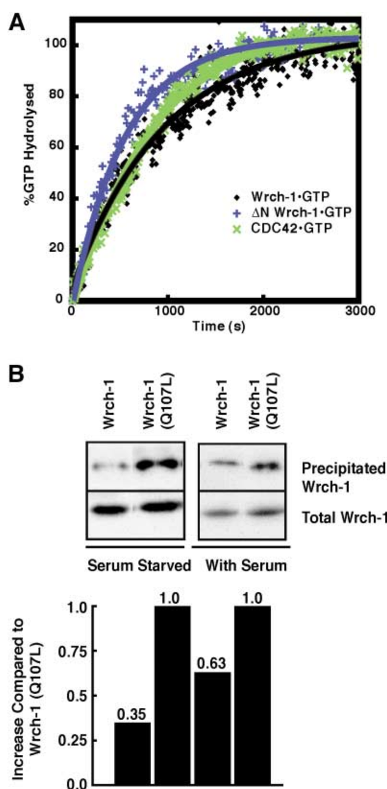

Figure 2. Wrch-1 Is an Active GTPase

(A) Wrch-1 GTPase activity in vitro is not altered by deletion of the N terminus. GST-Wrch-1, GST-ΔN Wrch-1, or GST-Cdc42, preloaded with GTP, was incubated with MDCC-PBP. Increases in fluorescent intensity of the MDCC-PBP then followed.

(B) The Q107L substitution impairs Wrch-1 GTPase activity in vivo. NIH 3T3 cells transfected with HA epitope-tagged Wrch-1, Wrch-1 (Q107L), and Myc epitope-tagged Cdc42 and Cdc42 Q61L were maintained in serum-starved or complete-growth media for 24 hr. Wrch-1 or Cdc42 proteins were precipitated with GST-PAK-RBD and detected by Western blot analysis; quantitation is shown below. Data shown are representative of three individual experiments.

N-Terminal Truncation of Wrch-1 Increases Effector Association and Activation and Transforming Activity

We next determined if the N terminus was able to influence the activation of Wrch-1 in vivo. Full length and ΔN versions of WT and Wrch-1 Q107L were expressed in serum-starved cells, and the amount of GTP-bound Wrch-1 was assessed (Figure 3A). ΔN Wrch-1 (Q107L) showed the highest level of precipitated protein, followed by Wrch-1 (Q107L), ΔN Wrch-1, and, finally, WT Wrch-1. Because our earlier analyses indicated that exchange was extremely rapid and independent of the N terminus, the increased level of precipitated protein seen with both ΔN protein forms suggests that the N terminus may serve as a negative regulator of effector association, partially independent of nucleotide state. This suggests that Wrch-1 may be regulated by other, non-GEF, mechanisms, such as protein-protein interactions with its N terminus.

To confirm that the N terminus influences effector association, we compared the ability of the different Wrch-1 proteins to cause activation of endogenous PAK1 (Figure 3B). Using a phosphospecific antibody that specifically recognizes activated PAK1 auto-phosphorylation at serine 144 [8], we found that PAK phosphorylation was dependent on both the nucleotide state and the presence of the N-terminal extension—mirroring our pull-down analyses. Wrch-1 (Q107L) caused a higher level of PAK phosphorylation than wild-type Wrch-1, and each N-terminally truncated protein caused more PAK phosphorylation than its full length counterpart. Therefore, the N-terminal extension of Wrch-1 plays an inhibitory role in the activation of PAK1.

We anticipated that N-terminal truncation may also enhance Wrch-1 biological activity. Like Cdc42, Wrch-1 causes growth transformation of NIH 3T3 mouse fibroblasts (A.C.B., C.J.D., and A.D.C., unpublished data), so we evaluated the ability of NIH 3T3 cells stably expressing the different Wrch-1 proteins to proliferate in an anchorage-independent environment (Figure 3C). Cells expressing WT Wrch-1 did not increase the number of colonies formed compared to vector-transfected cells (Figure 3D). ΔN Wrch-1-expressing cells demonstrated an approximately 3-fold increase in the number of colo-

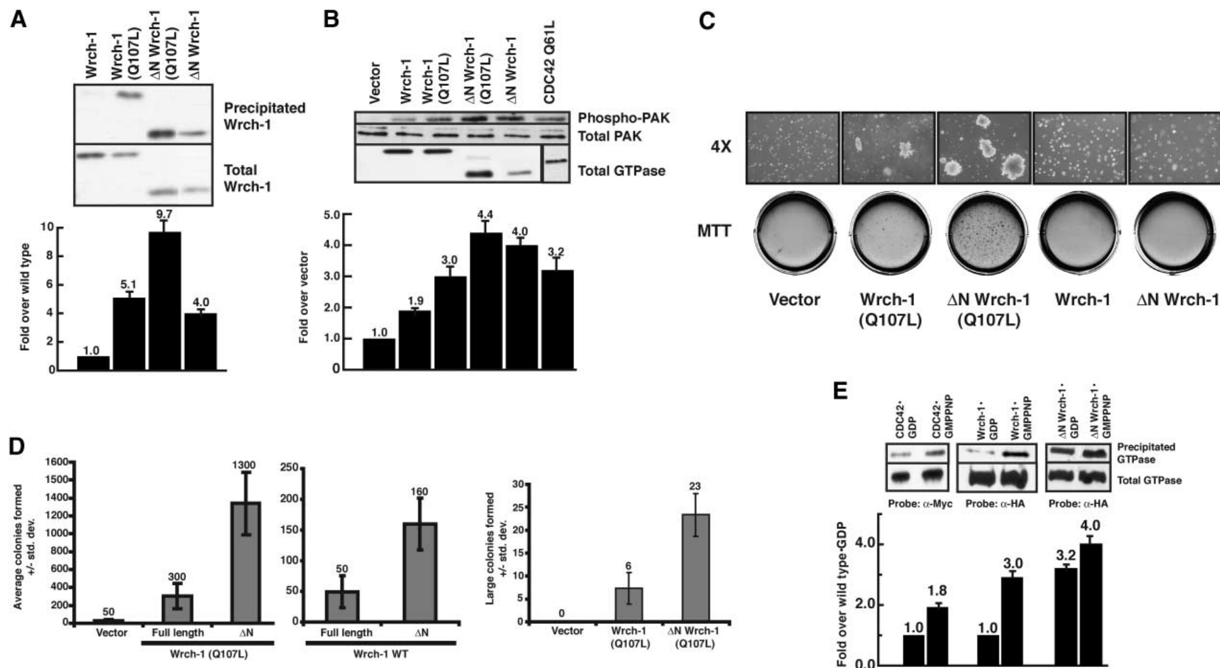

Figure 3. Deletion of the N Terminus Enhances Wrch-1 Effector Binding and Activation, and Transforming Activity

(A) Deletion of the N terminus enhances association with the PAK-RBD in vitro. Expression vectors encoding HA epitope-tagged Wrch-1, Wrch-1 (Q107L), ΔN Wrch-1, or ΔN Wrch-1 (Q107L) were transiently expressed in serum-starved cultures of NIH 3T3 cells for 24 hr. The GST-PAK-RBD-precipitated proteins were subjected to Western blot analysis; quantitation is shown below.

(B) N-terminal deletion enhances Wrch-1 activation of PAK in vivo. Cell lysates of NIH 3T3 cells stably expressing the indicated Wrch-1 proteins were subjected to SDS-PAGE and Western blot analysis using antibodies for anti-PAK1/2/3 or anti-phospho-PAK1(Ser 144)/PAK2(Ser 141). Quantitation is shown below.

(C) N-terminal deletion enhances activated Wrch-1 induction of anchorage-independent growth. Stable NIH 3T3 cell lines expressing Wrch-1 (Q107L), ΔN Wrch-1, and ΔN Wrch-1 (Q107L) were seeded in soft agar. After 14–21 days, colonies formed were stained, photographed, and quantified. The number of large colonies (greater than 16 cell diameters) were also counted and captured photographically after MTT staining.

(D) Quantitation of data shown in panel (C). Data shown are representative of two separate experiments performed in triplicate.

(E) N-terminal truncation of Wrch-1 contributes to the nucleotide independent binding to PAK in vitro. NIH 3T3 cells transiently expressing either full-length Wrch-1 or ΔN Wrch-1 proteins were maintained in growth medium (0.5% calf serum) for 24 hr. Cell lysates were incubated with GST-PAK-RBD with excess of desired nucleotide (1 mM GDP or 1 mM GMPPNP). Samples were then subjected to SDS-PAGE and Western blot analysis.

nies compared to WT Wrch-1 cells. Wrch-1 (Q107L)-expressing cells readily formed a 6-fold increase in colonies, and ΔN Wrch-1 (Q107L)-expressing cells formed a ten-fold increase in total colony number over WT Wrch-1 expressing cells, (and a 5-fold increase in the number of large colonies compared to Wrch-1 [Q107L]) (Figure 3C). These data suggest that Wrch-1-transforming activity is regulated by both GDP/GTP cycling and the presence of the N terminus.

To confirm the roles of bound nucleotides and N-terminal extension in effector binding, cells expressing Wrch-1 and ΔN Wrch-1 were precipitated with GST-PAK-RBD in the presence of excess GDP or GMPPNP (Figure 3E). Consistent with previous observations, Cdc42 demonstrated nucleotide-dependent binding. Wrch-1•GDP was precipitated in significantly lower amounts than Wrch-1•GMPPNP, consistent with the GTP-bound form of Wrch-1 being the activated form. ΔN Wrch-1•GDP and ΔN Wrch-1•GMPPNP associated with the PAK-RBD in high amounts (Figure 3E), although the GMPPNP form consistently associated at a slightly higher level. These results suggest that the regulation of effector association with Wrch-1 is complex and regulated by both the N terminus of Wrch-1 and binding of GTP.

Together, the PAK1 activation, transformation, and saturated-nucleotide precipitation data indicate that the binding of Wrch-1 to PAK (both the isolated RBD and full length protein) is affected directly by two factors: the presence of the N-terminal extension (important in effector binding) and the bound nucleotide state (which may be important in effector activation). Deletion of the N terminus partially activates Wrch-1, although both N-terminal deletion and GTP binding act synergistically to produce the most active form of Wrch-1. Based on our analyses with the PAK-RBD, Wrch-1 exists predominantly as a GDP-bound protein in vivo, and because the N terminus of Wrch-1 inhibits effector interaction, this assay may provide a misleading indication of the nucleotide state of Wrch-1.

The N Terminus of Wrch-1 Associates with the SH3 Domain-Containing Grb2 Adaptor Protein

The data above suggest that the N terminus of Wrch-1 may serve as a novel intramolecular regulator of Wrch-1 function. The presence of proline-rich motifs in the N terminus suggests that SH3 domain-containing proteins

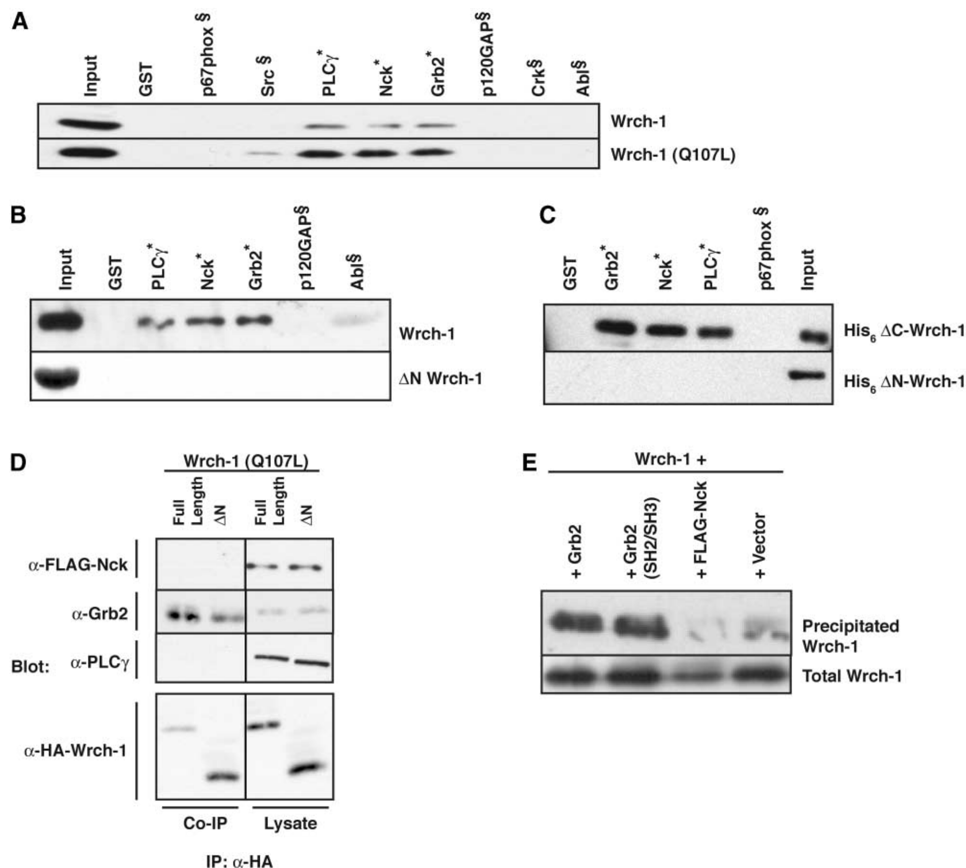

Figure 4. Wrch-1 Associates with Grb2, Nck and PLCγ In Vitro but Only Grb2 In Vivo

(A) Wrch1 associates with specific SH3 domains. Wrch-1 and Wrch-1 (Q107L) were transiently expressed in NIH 3T3 cells for 24 hr. Cell lysate was incubated with agarose-GST fusion proteins containing full-length (*) or isolated SH3 domains (§), and the precipitate was subjected to Western blot analysis.

(B) The N terminus of Wrch-1 is required for SH3 domain association. Wrch-1 and ΔN Wrch-1 were transiently expressed in NIH 3T3 cells. The lysate was incubated with agarose-GST-SH3 proteins before Western blot analysis of the precipitate with anti-HA antibody.

(C) The association of the SH3 domains of Grb2, Nck, and PLCγ to the N terminus of Wrch-1 is direct. His_6-ΔC or ΔN Wrch-1 was incubated with the GST-SH3 domain constructs of Grb2, Nck, PLCγ, and p67, and the precipitate was subjected to Western blot analysis.

(D) Wrch-1 associates with Grb2 but not Nck or PLCγ in vivo. Wrch-1 (Q107L) or ΔN Wrch-1 (Q107L) were transiently expressed in NIH 3T3 cells, either alone or coexpressed with FLAG-Nck. Cells were lysed and HA-tagged proteins were immunoprecipitated before Western blot analysis.

(E) Coexpression of Grb2 increases Wrch-1 binding to PAK-RBD in vivo. Cell lysate from NIH 3T3 cells transiently expressing either Wrch-1 alone or with full-length Grb2, the SH2/SH3 domains of Grb2, or full-length Nck were incubated with GST-PAK-RBD before Western blot analysis.

may interact with the N terminus. These sequences (PAFPDR at residue 8, PPVPPRR at residue 20, and PPGEPGGR at residue 38) closely match the consensus binding motifs of the SH3 domains of Grb2 (PXXPXR), phospholipase C γ (PLCγ) (PPVPPRP), and Nck (PXXPX RXXS), respectively [9]. To evaluate whether these or other SH3 domain-containing proteins associate with the N terminus of Wrch-1, we expressed HA epitope-tagged versions of full-length or ΔN Wrch-1 in NIH 3T3 cells. Cell lysates were incubated with GST fusion proteins containing isolated SH3 domains or SH3 domain-containing, full-length proteins. As expected, Wrch-1 was precipitated by the SH3 domains of Grb2, Nck, and PLCγ, and activated Wrch-1 was precipitated to a slightly greater extent (Figure 4A), and no association of ΔN Wrch-1 with the GST-SH3 domains was observed (Figure 4B). Association was not dependent on the nucleotide bound state, although there was a greater level of binding to GTP-bound Wrch-1, which suggests that GDP/GTP cycling may somehow regulate the N terminus. Using His_6-tagged ΔC and ΔN Wrch-1, we performed in vitro association analyses with the GST-SH3 domains (Figure 4C). Together, these data show that the interaction between Wrch-1 and Grb2 and Nck and PLCγ is direct and absolutely requires the N terminus.

To validate that these interactions occur in vivo, we immunoprecipitated HA-Wrch-1 proteins, followed by western blot analyses to detect coimmunoprecipitated endogenous Grb2, PLCγ or exogenously co-expressed Nck. Endogenous Grb2 was co-immunoprecipitated from cells expressing Wrch-1; surprisingly, ΔN Wrch-1, exogenous Nck, and endogenous PLCγ showed no association with either form (Figure 4D). The precipitation of Grb2 with ΔN Wrch-1 is most likely indirect via Grb2

association with PAK because we found that Grb2 association with Wrch-1 in vitro required the N terminus.

We then speculated that coexpression of Grb2 and Wrch-1 may enhance Wrch-1 effector interaction. To test this, full-length Grb2 or Nck was coexpressed with Wrch-1 in NIH 3T3 cells, and GST-PAK-RBD was used to precipitate Wrch-1 from the cell lysate. Coexpression of Grb2 but not Nck caused a significant increase in the levels of precipitated Wrch-1 (Figure 4E), supporting our proposal that Grb2 relieves the negative regulatory function of the N terminus to promote effector interaction and Wrch-1 activation.

In conclusion, we suggest that the N terminus of Wrch-1 acts as a negatives regulator of Wrch-1 interaction with PAK and other effectors, and Grb2 interaction with the N terminus relieves this inhibition to promote Wrch-1 effector activation and potential GDP/GTP exchange. Whereas the classical Rho family GTPases are regulated by primarily by GAP- and GEF-stimulated GDP/GTP cycling, Wrch-1 function is regulated by an intramolecular auto-inhibitor mechanism that regulates effector association and potentially rapid exchange, although regulation by a GAP (such as the Rheb GTPases [10]) remains a likely possibility. We have also determined that a Wrch-1-related protein, Chp/Wrch-2 [11], exhibits a similar mechanism of regulation (Chenette et al., personal communication) – deletion of its proline-rich N-terminal extension, rather than GTPase-deficiency, caused potent activation of Chp transforming activity. While this mechanism of regulation may be unique for Rho family of GTPases, it may not be unique to other GTPases. Like Wrch-1, the PIKE [12, 13] and dynamin [14] GTPases contain proline-rich flanking sequences, which may be regulated by SH3 domains. PIKE, a nuclear GTPase, contains proline-rich regions that are N-terminal of the GTPase domain, which associate with the SH3 domain of PLCγ and increase PIKE activity. We suggest that the SH3 domain of PLCγ acts by relieving steric hindrance and promoting a more active state. Future studies to determine the structure of Wrch-1 will be required to establish the mechanism by which the N terminus regulates effector interaction.

Supplemental Data

Supplemental Data including Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/14/22/2052/DC1/.

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