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ARAP3 Is a PI3K- and Rap-Regulated GAP for RhoA

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Summary

Rho and Arf family small GTPases [1] are well-known regulators of cellular actin dynamics. We recently identified ARAP3, a member of the ARAP family of dual GTPase activating proteins (GAPs) for Arf and Rho family GTPases [2, 3], in a screen for PtdIns(3,4,5)P_3 binding proteins. PtdIns(3,4,5)P_3 is the lipid product of class I phosphoinositide 3OH-kinases (PI3Ks) [4] and is a signaling molecule used by growth factor receptors and integrins in the regulation of cell dynamics [5]. We report here that as a Rho GAP, ARAP3 prefers RhoA as a substrate and that it can be activated in vitro by the direct binding of Rap [6] proteins to a neighbouring Ras binding domain (RBD). This activation by Rap is GTP dependent and specific for Rap versus other Ras family members. We found no evidence for direct regulation of ARAP3's Rho GAP activity by PtdIns(3,4,5)P_3 in vitro, but PI3K activity was required for activation by Rap in a cellular context, suggesting that PtdIns(3,4,5)P_3-dependent translocation of ARAP3 to the plasma membrane may be required for further activation by Rap. Our results indicate that ARAP3 is a Rap-effector that plays an important role in mediating PI3K-dependent crosstalk between Ras, Rho, and Arf family small GTPases.

Results and Discussion

ARAP3 Is a GAP for RhoA

We have previously shown that ARAP3 is a PtdIns(3,4,5)P_3-dependent Arf6 GAP and that it has a separate, functional Rho GAP domain [3]. To address the substrate specificity of this Rho GAP domain in vitro, we performed Rho GAP assays under conditions of limiting substrate concentration. The substrates were [γ-^{32}P]GTP preloaded recombinant GST-RhoA, Rac, or Cdc42 fusion proteins. Figure 1A shows that ARAP3 is a potent GAP for RhoA, while it is much less effective on either Rac or Cdc42. To further define the substrate specificity of ARAP3's Rho GAP activity, we also performed Rho GAP assays against RhoB and RhoC, but neither of these served as good substrates for ARAP3 in vitro (data not shown).

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Further titration of the relative levels of ARAP3 and RhoA in these in vitro assays suggested that 5 nM ARAP3 could stimulate the GTPase activity of 10 nM GTP-RhoA approximately 3-fold (not shown). While such a high enzyme to substrate ratio is not unusual in these types of assays, to confirm the ability of ARAP3 to act as a RhoA GAP in vivo, we performed "pull-down" assays (Figure 1B) from Sf9 cells expressing Rac1 or RhoA alone or together with ARAP3. Figure 1B shows that relatively low amounts of ARAP3 were sufficient to cause a significant decrease in the amount of RhoA-GTP in the Sf9 cells lysates, while Rac1-GTP levels were not affected by the presence of larger amounts of ARAP3. We saw a similar substrate specificity when ARAP3 was transiently cotransfected with Rac or Rho in COS-7 cells (Figure S1). We conclude that RhoA is a likely physiological substrate of ARAP3.

Rap Binds to ARAP3 and Activates Its Rho GAP Activity In Vitro

Just C-terminal to ARAP3's Rho GAP domain lies a weakly conserved Ras binding domain (RBD; Figure 2A). To investigate whether ARAP3 binds to Ras family proteins, we immobilized ARAP3 protein on nitrocellulose and overlayed with [γ-^{32}P]GTP preloaded GST-fusion proteins of several Ras family members. We detected an interaction with Rap1B and 2B and Rheb but not with H-Ras, R-Ras, TC21, M-Ras, or RalA (Figure 2B). All analyzed Ras family members interacted with the Raf kinase RBD but not with the Rac/Cdc42 binding domain of PAK (PAK CRIB). To map binding of Rap-GTP to ARAP3's putative RBD, a three-dimensional model of the ARAP3 RBD was constructed manually based on the structures of the complex of p110γ PI3K with Ras [7] and the complex of the RalGDS RBD with Ras [8]. This suggested that Arg1155 would be central to the Rap binding interface of ARAP3. A R1155E point mutation indeed abolished the interaction between ARAP3 and Rap1B-GTP (Figure 2B). We conclude that the ARAP3 RBD can interact with the small GTPases of the Rap family and also Rheb.

To investigate a regulatory role of Rap binding on ARAP3's Rho GAP activity, we added GTPγS- or GDPβS-preloaded Ras family proteins to in vitro Rho GAP assays. While addition of GTPγS-Rap1B or Rap2B reproducibly increased ARAP3 Rho GAP activity 2-fold, GDPβS-Rap1B had no effect on ARAP3 Rho GAP activity (Figure 2C and data not shown). Adding GTPγS- or GDPβS-preloaded H-Ras, R-Ras, M-Ras, TC21, or RalA did not affect ARAP3 Rho GAP activity (Figure S2A and not shown). Perhaps surprisingly, despite being able to interact with ARAP3, GTPγS-Rheb had only a negligible stimulatory effect on ARAP3 Rho GAP activity (Figure S2A).

In line with the interaction assays, the ARAP3 RBD point mutant (R1155E) was not activated as a Rho GAP by addition of GTPγS-Rap1B (Figure 2D), indicating that Rap's ability to regulate ARAP3's Rho GAP activity is

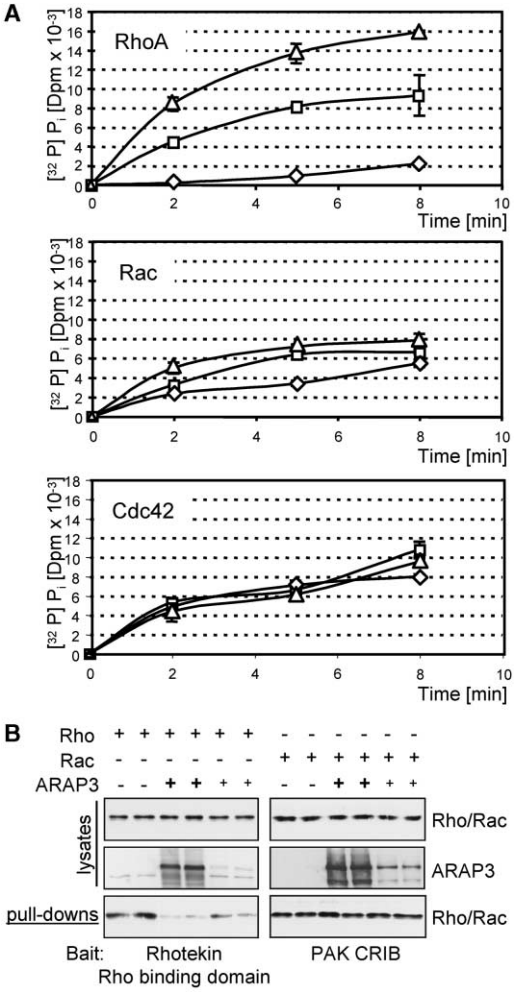

Figure 1. ARAP3 Is a GAP for RhoA In Vitro and In Vivo

(A) 1.25 nM RhoA, Rac1, or Cdc42 [γ-^{32}P]GTP preloaded GTPases were incubated with 10 nM (squares) or 25 nM (triangles) ARAP3 or with its vehicle (diamonds) at 30°C for the indicated times, at which point hydrolyzed $^{32}P_i$ was extracted and quantified by scintillation counting. All assays were performed at least four times and data represent means ± SD.

(B) For each lane, 5×10^6 Sf9 cells were infected with 1% baculovirus for RhoA or Rac1 alone or with 1% or 0.1% baculovirus for ARAP3 (indicated by size of plus symbol). 2% of the total lysates were used for a Western blot to determine the total amounts of the expressed proteins they contained. The remainder of the lysates were incubated with PAK-CRIB or rhotekin Rho binding domain immobilized on sepharose beads, washed, and subjected to SDS-PAGE and Western blotting to visualize the amounts of GTP-loaded Rac and RhoA, respectively. Experiments were repeated three times (Rac) and four times (Rho); a representative example is shown.

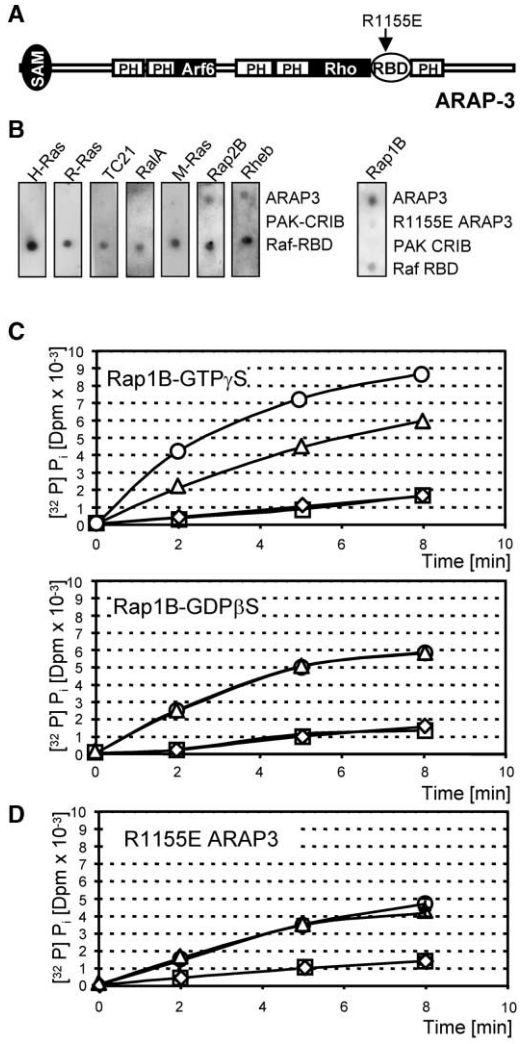

Figure 2. ARAP3 Binds to and Is Activated by the Ras Small GTPase Rap In Vitro

(A) A schematic diagram of ARAP3 domain structure. The GAP domains are marked Rho and Arf6. The RBD lies just C-terminal to the Rho GAP domain.

(B) 10 μg of ARAP3 protein and R1155E ARAP3 or equivalent molar amounts of Raf RBD and Pak CRIB were spotted onto nitrocellulose membrane (Schleicher and Schüll) using a dot blot apparatus (Hoefer). Membranes were overlaid with 0.5 μg active, [γ-^{32}P]-preloaded Ras family proteins as indicated, followed by washing. Any bound GTPases were detected by autoradiography.

(C) 1.25 nM [γ-^{32}P]GTP preloaded RhoA was incubated with 10 nM ARAP3 or its vehicle and with GTPγS (top panel) or GDPβS (bottom panel) preloaded Rap1B (30 nM) or their respective vehicles (as detailed in Supplemental Experimental Procedures) at 30°C for the indicated times. Hydrolyzed $^{32}P_i$ was extracted and quantified by scintillation counting. The symbols used are: Rho only, diamonds; Rho and Rap, squares; Rho and ARAP3, triangles; Rho, ARAP3, and Rap, circles. Data are means ± SD; error bars in this experiment are within the size of the symbols.

(D) Assays were carried out as in (C, top), except that wt ARAP3 was substituted with the R1155E point mutant. Symbols used are as in (C). All of these GAP assays were performed four times.

through its direct interaction with the ARAP3 RBD. While there are several examples of the regulation of GTP loading of a Rho family member (Rac) by a Ras family member (Ras) via GEFs [9], to our knowledge there is no precedent for the regulation of GTPase activity of Rho family members by Ras family members via GAPs.

Since a possible Rap GAP activity of ARAP3 could have confounded our interpretation of the RhoGAP assay, we performed some Rap GAP assays. These showed that ARAP3 is not a Rap GAP (not shown).

We have seen that PtdIns(3,4,5)P_3 is essential for ARAP3's Arf6 GAP activity and drives a plasma membrane recruitment of GFP-ARAP3 in pig aortic endothe-

lial (PAE) cells [3]. To see whether PtdIns(3,4,5)P_3 regulates ARAP3 Rho GAP activity, we did in vitro Rho GAP assays with or without GTPγS-Rap1B in the presence of lipid vesicles that did or did not incorporate small molar fractions of PtdIns(3,4,5)P_3. As was previously observed, lipid vesicles in the Rho GAP assays caused a decrease in the intrinsic GTPase activity of Rho and in the Rho GAP activity of ARAP3 independently of the presence or absence of PtdIns(3,4,5)P_3 [3]. However, neither "control" lipid vesicles nor those containing PtdIns(3,4,5)P_3 had any significant effect on the observed activation of the ARAP3 Rho GAP activity by GTPγS-Rap (Figure S2B). We conclude that the direct interaction of Rap-GTP with ARAP3 can cause an increased Rho GAP activity of ARAP3, but we could find no evidence for an effect of PtdIns(3,4,5)P_3 in vitro.

Rap and PtdIns(3,4,5)P_3 Activate ARAP3 Rho GAP Activity In Vivo

To test whether a Rap family member activates ARAP3's Rho GAP activity in vivo, we performed more Rho-GTP pull-downs from Sf9 cells expressing Rho, ARAP3, and Rap1A. We carefully titrated ARAP3 expression levels such that there was only a minimal effect on Rho-GTP. Under these conditions, expression of Rap1A caused a significant increase in ARAP3-dependent RhoA GAP activity (Figure 3A); Rap by itself did not affect the amount of Rho-GTP found in the Sf9 cell lysates (Figure S3).

To address a potential involvement of PI3K in ARAP3's Rho GAP activity in vivo, we expressed p110γ together with ARAP3 and Rho in Sf9 cells. We previously showed that expression of p110γ in this context substantially elevates PtdIns(3,4,5)P_3 [10]. Contrasting our in vitro observations, we detected a substantial activation of ARAP3 Rho GAP activity in the presence of elevated PtdIns(3,4,5)P_3 (Figure 3A). By itself, p110γ had no effect on Rho-GTP levels (not shown). In experiments where ARAP3, p110γ, and Rap levels were so low that only a minor effect of any two on ARAP3 GAP activity was detected, they had an additive effect (not shown).

To determine whether the effect of Rap on ARAP3's Rho GAP activity was mediated by a direct interaction in vivo, we expressed Rho, Rap, and the R1155E ARAP3 point mutant and performed further Rho-GTP pull-downs. R1155E ARAP3 was not activated by Rap as a Rho GAP, but it was significantly activated by p110γ (Figure 3B). These results strongly suggest that Rap can directly regulate ARAP3's Rho GAP activity in vivo. Moreover, the effect of p110γ on ARAP3's Rho GAP activity is not mediated entirely via an effect on Rap.

To see whether PtdIns(3,4,5)P_3 is required for activation of ARAP3 by Rap, we pretreated Sf9 cells with the PI3K inhibitor wortmannin such that little PtdIns(3,4,5)P_3 would be present in the Sf9 cells [10]. Inhibition of PI3K in this manner interfered with the activating effect of Rap on ARAP3 Rho GAP activity (Figure 3C). Similarly, R307,8A ARAP3, which cannot interact with PtdIns(3,4,5)P_3 [3], is not significantly activated by Rap in the pull-down assays (not shown). We conclude that PI3K activity is required for Rap-GTP to activate ARAP3 Rho GAP activity in vivo and that this effect requires direct interaction

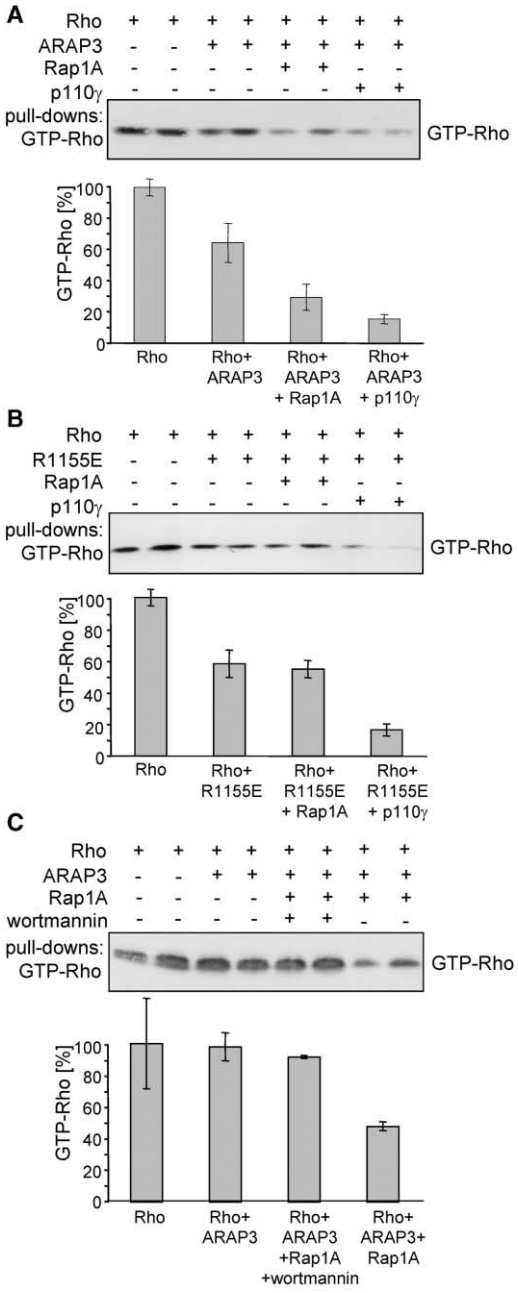

Figure 3. ARAP3 Rho GAP Activity Is Regulated by Rap and PtdIns(3,4,5)P_3 In Vivo

(A) Sf9 cells were infected with baculoviruses to produce RhoA and a small amount of ARAP3 alone or together with Rap1A or p110γ as indicated (0.2% baculoviral stock for both ARAP3 and Rap1A and 1% for RhoA and p110γ were used). Lysates were used for pull-down experiments with rhotekin Rho binding domain as in Figure 1B.

(B) Experiment was as in (A) except that cells were infected with a baculovirus encoding the R1155E point mutant of ARAP3.

(C) Experiments were carried out essentially as in (A), except that the indicated dishes were treated with 200 nM wortmannin 40 min prior to the start of the assay to inhibit Sf9 cell PI3K and deplete cells of PtdIns(3,4,5)P_3. The pull-downs were repeated two (C), three (B), or seven (A) times. Representative examples are shown and quantification of the shown blots by densitometric scanning are plotted in the graphs below.

of PtdIns(3,4,5)P_3 with ARAP3. (It is also possible that PI3K activity is additionally required for maximal activation of Rap, but this cannot be assessed from these experiments.) Given that the stimulation of PI3K leads to the plasma membrane translocation of GFP-ARAP3 in transfected cells [3], the most likely role of PI3K with respect to ARAP3 Rho GAP regulation is to recruit ARAP3 from a cytosolic pool to the plasma membrane where its substrates, RhoA-GTP and Arf6-GTP, are localized and where its Rho GAP activity can be further activated by Rap-GTP. There are reports that Rap-GTP [11], Arf6-GTP [12, 13], and RhoA-GTP [14, 15] localize to the plasma membrane. Such a "translocation plus subsequent activation" model of ARAP3 activity also then provides an explanation for the apparently high basal activity of ARAP3 toward RhoA combined with the low fold-activation observed by Rap-GTPγS in the in vitro assays. It is also likely that this "colocalization model" determines the precise Rap family member that is involved in regulating ARAP3 in a particular cell response.

Rap Does Not Affect ARAP3 Arf6 GAP Activity

We looked also at any possible role of Rap in regulating ARAP3 Arf6 GAP activity. Incorporation of GTPγS-Rap into Arf6 GAP in vitro assays did not activate ARAP3 Arf6 GAP activity alone or cause an increase in the activation of ARAP3 Arf6 GAP activity seen by PtdIns(3,4,5)P_3 (Figure S4). We were unable to address this question in vivo, since effective Arf6-expressing baculoviruses have yet to be created.

PI3K and Rap Regulate ARAP3 Rho GAP Activity in Transiently Transfected PAE Cells

To address the regulation of ARAP3 by PtdIns(3,4,5)P_3 and Rap in mammalian cells, we exploited the fact that overexpressed ARAP3 in pig aortic endothelial cells interferes with their normal ruffling response to PDGF stimulation [3]. We expressed GFP fusions of wild-type ARAP3 or of full-length protein incorporating single point mutations in its GAP domains or regulatory regions, stimulated the starved cells with PDGF, and observed membrane ruffling. Control transfected PAE cells produce several large, spread lamellipodia with smooth ruffles when stimulated with PDGF (Figure 4A). However, stimulated PAE cells overexpressing wild-type ARAP3 cannot produce normal lamellipodia/ruffles, but display numerous filopodia and striking retractions indicating a loss of adhesion to the substratum (Figure 4B; this was confirmed by time-lapse imaging; not shown). Cells expressing ARAP3 point mutants in the RBD (R1155E; Figure 4C) or in the Rho GAP domain (R982A; Figure 4D) have similar phenotypes: they retain a similar shape to control cells, but on PDGF stimulation their lamellipodia are characteristically "ragged" with filopodia-like protrusions. In contrast, cells expressing the ARAP3 Arf GAP domain point mutant (C504A; Figure 4E) can produce smooth ruffles when stimulated but their cell bodies are severely retracted. Mutations in the N-terminal PH domain of ARAP3 (R307,8A; Figure 4F) largely abolish any effect of overexpressing ARAP3, as do mutations in both the Arf GAP and Rho GAP (C504A, R982A; Figure 4G) or Arf GAP and RBD (C504A, R1155E; Figure 4H) domains. Taken together, these results support our conclusions from the in vitro and Sf9 cell assays that the RBD governs ARAP3's Rho GAP activity while N-terminal PH domain binding to PtdIns(3,4,5)P_3 is an overriding regulatory feature for both ARAP3's Arf and Rho GAP activities.

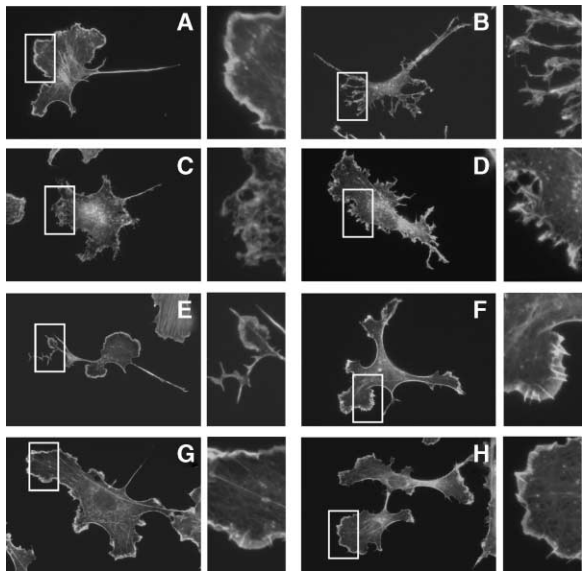

Figure 4. ARAP3 Is Regulated by PI3K and Rap in Transiently Transfected PAE Cells

Pig aortic endothelial cells were transiently transfected by electroporation [17] with pEGFP vector as control (A) or with full-length pEGFP-ARAP3 fusion constructs: wt (B), R1155E RBD mutant (C), R982A Rho GAP mutant (D), C504A Arf GAP mutant (E), R307,8A PH domain mutant (F), R982A, C504A double GAP domain mutant (G), and R1155E RBD and C504A Arf GAP mutant (H). Transfected cells were serum starved for 12 hr and then stimulated with 10 ng/ml PDGF for 5 min. Cells were fixed and filamentous actin was visualized by staining with phalloidin.

With a protein as multifunctional as ARAP3, it is difficult to ascribe the individual regulatory contributions it may make to a physiological cell response. However, its PtdIns(3,4,5)P_3-regulated Arf6 GAP activity and PtdIns(3,4,5)P_3- and Rap family-regulated RhoA GAP activity clearly place this protein in a regulatory web downstream of receptors that control the cell cytoskeleton/adhesion/motility via the activation of class I PI3Ks and Raps. The role of class I PI3Ks in these cellular responses is well documented [4], but that of Raps less so, though there are clear examples of their role in signaling downstream of integrin and growth factor receptors [6, 16]. The challenge now is to establish the molecular links between endogenous ARAP3's control of Rho and Arf family proteins and their targets with their relative importance in cellular responses such as cell motility and phagocytosis.

Supplemental Data

Supplemental Data including Experimental Procedures and several figures may be found online at http://www.current-biology.com/cgi/content/full/14/15/1380/DC1.

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