ArhGAP15, a novel human RacGAP protein with GTPase binding property[1]

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Abstract **We have previously described a partial cDNA sequence encoding a RhoGAP protein, GAP25 that is homologous to the recently reported ArhGAP9 and ArhGAP12. We now describe a related new member ArhGAP15 that shares a number of domain similarities, including a pleckstrin homology (PH) domain, a RhoGAP domain and a novel motif N-terminal to the GAP domain. This novel motif was found to be responsible for nucleotide-independent Rac1 binding. Using swop mutants of Rac/Cdc42, we have established that the binding is through the C-terminal half of Rac1. The GAP domain of ArhGAP15 showed specificity towards Rac1 in vitro. The PH domain is required for ArhGAP15 to localize to cell periphery and overexpression of the full-length ArhGAP15, but not the mutant with a partial deletion of the PH domain, resulted in an increase in actin stress fibers and cell contraction. These morphological effects can be attenuated by the co-expression of dominant negative Rac1^{N17}. HeLa cells expressing ArhGAP15 were also resistant to phorbol myristatate acetate treatment, suggesting that ArhGAP15 is a potential regulator of Rac1.**
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Key words: Rac1; GAP; Rac1-binding; Actin cytoskeleton

1. Introduction

The Rho GTPases such as Cdc42, Rac1 and RhoA regulate diverse biological processes including actin cytoskeletal dynamics, cell adhesion, cell polarity, cell cycle progression and transcriptional activation in response to external stimuli [1–3]. In adherent cells such as fibroblast and HeLa cells, activation of RhoA causes stress fiber and focal adhesion formations, whereas Rac1 and Cdc42 correspondently induce lamellipodia and filopdodia respectively [3]. The co-ordinated interplay of RhoA, Rac1 and Cdc42 is crucial for proper cell attachment and motility [4]. Like Ras oncoproteins, the Rho family GTPases cycle between the active GTP-bound form and the inactive GDP-bound form through intrinsic GTPase activity. Exchange of GDP for GTP is enhanced by guanine nucleotide exchange factors, whereas the hydrolysis of GTP- to GDP-bound form can be catalyzed by a variety of GTPase activating proteins (GAPs), thereby resulting in down-regulation of the prospective GTPase. A recent genome-wide survey of human RhoGAP proteins has uncovered at least 53 distinct gene sequences potentially encoding GAP proteins [5]. Phylogenetic analysis of the GAP domain has revealed that these proteins can be categorized into a distinct family that may be functionally related. Apart from the RhoGAP domains, these proteins also contain regulatory regions consisting of diverse functional modules known to be involved in cytoskeletal regulation and protein–protein interactions, suggesting that they may also participate in molecular complexes specific for their cellular activities.

Here we describe a novel ArhGAP15 with N-terminal pleckstrin homology (PH) domain and C-terminal GAP domain with specific activity towards Rac1. Structurally and phylogenetically, it is most closely related to ArhGAP9 [6] and ArhGAP12 [7]. These RhoGAP proteins also share a common motif that interacts with Rac1. We also show that both PH and GAP activity towards Rac1 are essential for its cytoskeletal effects.

2. Materials and methods

2.1. Construction of expression vectors

Full length human ArhGAP15 cDNA was derived from EST clone R20038. The coding region was obtained by polymerase chain reaction (PCR) using two adapter primers, 5′-CGGGATCCATGCAGAAATCTACAAATTC-3′ (forward) and 5′-TCCCCCGGGCATCAAGACAGATGTG-3′ (reverse). The 1.5 kb PCR fragment was digested with *Bam*H1 and *Sma*1 and cloned into pXJ40-FLAG and pGEX4T1 vectors for sequencing and expression studies. Partial PH domain deletion mutant (residues 128–475) was obtained by PCR using 5′-CGGGATCCATGAAAACTGGGCACAAACC-3′ as forward primer and the PCR product was similarly subcloned into pXJ40-FLAG and pGEX4T1 vectors. The GAP domain (residues 263–475) construct was obtained by subcloning an internal *Xmn*1 fragment into *Sma*I-digested pGEX4T1 for expression. The ArhGAP15-BD (encoding residues 240–332) construct in pGEX4T1 was

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[1] The nucleotide and derived amino acid sequences reported in this paper have been submitted to GenBank/EMBL data banks with accession number AY219338.

Abbreviations: ABR, active breakpoint cluster region gene product related protein; BD, p21-binding domain; GAP, GTPase activating protein; PCR, polymerase chain reaction; PAK, p21-activated kinase; PH, pleckstrin homology; PMA, phorbol myristatate acetate

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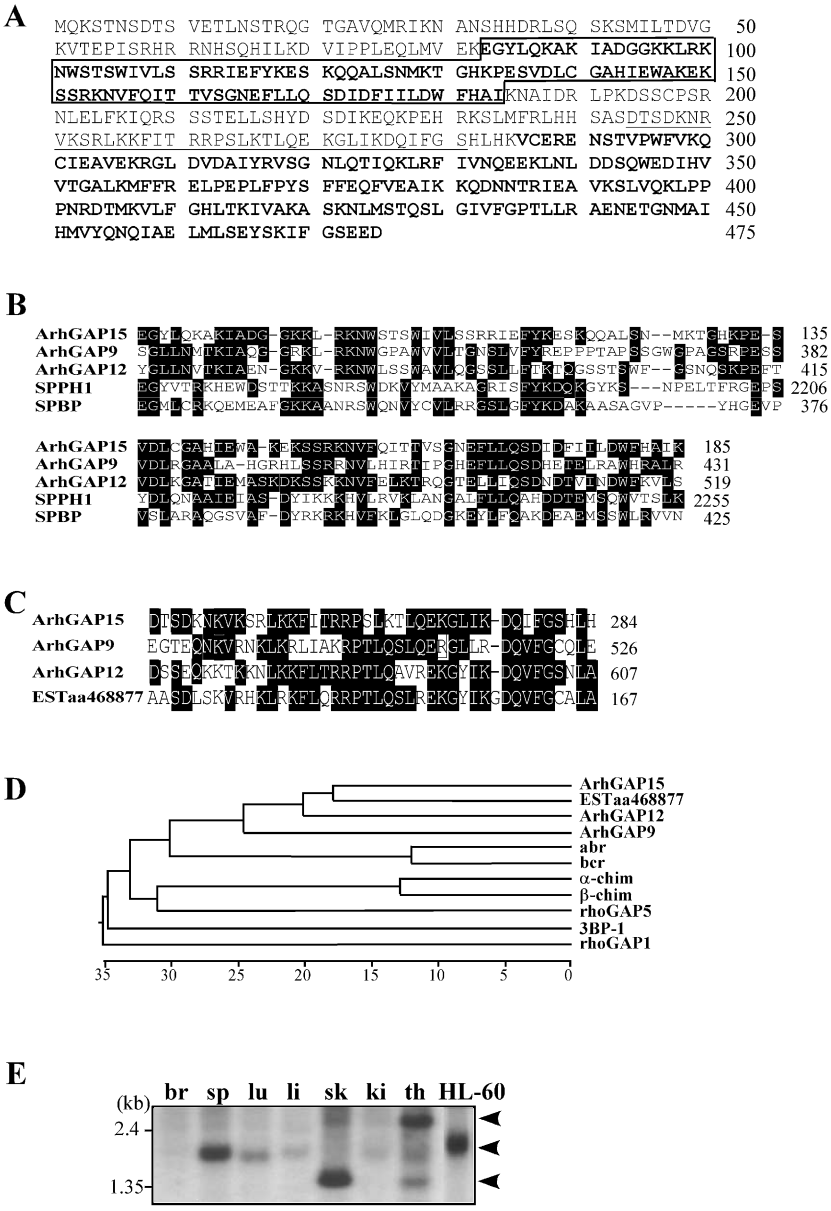

Fig. 1. ArhGAP15 is a member of related RhoGAP proteins. A: Amino acid sequence of the human ArhGAP15. The N-terminal PH domain is in bold and boxed and the C-terminal RhoGAP domain is in bold letters. The conserved central motif (see also C) is underlined. B: Comparison of PH domain of ArhGAP15 and other PH containing related proteins. The PH domain of human ArhGAP15, ArhGAP9, ArhGAP12, spectrin (SPPH-1) and spectrin binding protein (SPBP) were aligned with Clustal Method from DNASTAR. Identical amino acid residues are highlighted in black squares. C: Comparison of a conserved central motif from ArhGAP15 and related GAP proteins. D: Phylogenetic tree of ArhGAP15 with some RhoGAP proteins. The numbers denote the number of residues that are varied from each other as derived from DNASTAR program. E: Expression of ArhGAP15. Human mRNA blot was purchased from Clontech and total RNA from HL-60 cells (20 μg) were separated on 1% agarose-formaldehyde gel and transferred onto Hybond (Amersham) for probing with full-length ArhGAP cDNA labeled with [^{32}P]dCTP by random priming [12]. Br, brain; sp, spleen; lu, lung; sk, skeletal muscle; ki, kidney; th, thymus. Arrows indicate the position of the different mRNA sizes.

obtained by subcloning a *Bam*H1/*Hin*cII fragment from PCR reaction using oligonucleotide 5′-CAGGATCCAGTGCTTCCGATACAAGCG-3′ as forward primer. The PH domain construct (encoding residues 1–262) in pXJ40-FLAG was obtained by ligating the *Bam*H1/*Xmn*I fragment from the full-length clone to *Bam*H1/*Sma*1 digested vector. All PCR subclones were verified by DNA sequencing.

Rac1, Cdc42, RhoA, Abr GAP domain and αPAK (p21-activated kinase) p21-binding domain (PAK-BD) in pGEX vectors, and Rac1^{N17} in pXJ40-HA vector were obtained as previously described [8,9]. Swop mutants of Rac1/Cdc42 were obtained by two-rounded PCR protocol [9]. Rac1 was also subcloned into pGEX4T1-BirA vector to obtain in vivo biotinylation of the fusion protein in *Escherichia coli* (unpublished data).

2.2. Preparation of recombinant proteins, GAP and GTPase-binding assays

GST-fusion proteins with ArhGAP15$^{1-475}$, ArhGAP15$^{263-475}$ (ArhGAP15-GAP), ArhGAP15$^{240-332}$ (ArhGAP15-BD), GAP domain of ABR (active breakpoint cluster region gene product related protein) and RhoGAP190, αPAK, RhoA, Cdc42, Rac1 and its swop mutants (Rac1^{64}Cdc42, Cdc42^{64}Rac1 and Cdc42^{64}Rac1^{107}Cdc42) were obtained as fusion proteins according to standard protocol. For the GST-biotinylated Rac1 (containing a biotin acceptor site after the thrombin cleavage site), the protein was cleaved from GST with thrombin according to recommended protocol.

For GAP activity measurement, both filter and solution assays were used as previously described [10]. A filter assay was used for determin-

ing the interaction of ArhGAP15 with various p21 proteins [11]. When biotinylated Rac1 was used, the detection was with streptavidin-HRP and ECL kit (Amersham) and subsequent exposure to X-ray film.

2.3. Cell culture, transfection, microinjection, cell staining and immunofluorescence microscopy and cell fractionation

HeLa cells were maintained and transfected using lipofectamine as described [9]. For microinjection of HeLa cells, subconfluent cells were plated on coverslips 48 h before being microinjected with the different ArhGAP15 constructs (50 ng/μl). Two to four hours after injection, cells were fixed with 4% paraformaldehyde and stained with anti-FLAG (M2; IBI)/FITC anti-mouse antibodies alone or double-stained with TRITC–phalloidin. Stained cells were analyzed with a Bio-Rad Radiance 2000 Confocal Imager adapted to a Nikon microscope. For phorbol myristatate acetate (PMA) treatment, HeLa cells were treated with PMA (30 ng/ml) for 30 min before fixation and double staining with anti-FLAG/FITC anti-mouse and TRITC–phalloidin. For cell transfections, subconfluent HeLa or COS-7 cells were transfected with various constructs in lipofectamine (Life Technologies, Inc.). Cells were harvested 24 h after transfection and soluble cell extracts were obtained with lysis buffer containing 25 mM HEPES, pH 7.7, 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM sodium vanadate, 20 mM β-glycerol phosphate, 5% glycerol, 0.1% Triton X-100 and 1× inhibitor mix (Roche Molecular Biochemicals). Immunoprecipitations and protein analyses were performed as described previously [9].

For preparation of soluble and pellet fractions for cellular localization of ArhGAP15, COS-7 cells transfected with the various constructs of ArhGAP15 were harvested with lysis buffer containing 25 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.25 M sucrose and 0.05% Triton. After removing cell debris by centrifuging at 1000×*g* for 10 min, the supernatant was further centrifuged at 100 000×*g* for 30 min to obtain the soluble (supernatant) and membrane pellet fractions for further analysis.

3. Results

3.1. ArhGAP15 is a member of related RhoGAP proteins

Using degenerate primers to the conserved nucleotide sequence of the RhoGAP cDNAs, we have previously isolated a number of cDNAs including β-Chimaerins [12] and ABR [8]. We also detected a short cDNA fragment termed GAP25 [13] that represents a less abundant mRNA in the rat brain, which bears resemblance to the recently reported human ArhGAP9, a multi-domained RhoGAP protein with SH3, PH and WW domains [6]. More recently, a related ArhGAP12 that has similar domain arrangement was reported [7] and has been categorized as phylogenetically related RhoGAP proteins [5]. Two other related RhoGAP proteins that have similar GAP domain were also detected. The unreported ArhGAP15 has a related N-terminal PH domain and a C-terminal GAP domain most similar to ArhGAP9 and ArhGAP12 (Fig. 1A–D). A less characterized EST cDNA clone aa468877 also contains a related GAP domain (Fig. 1C,D),

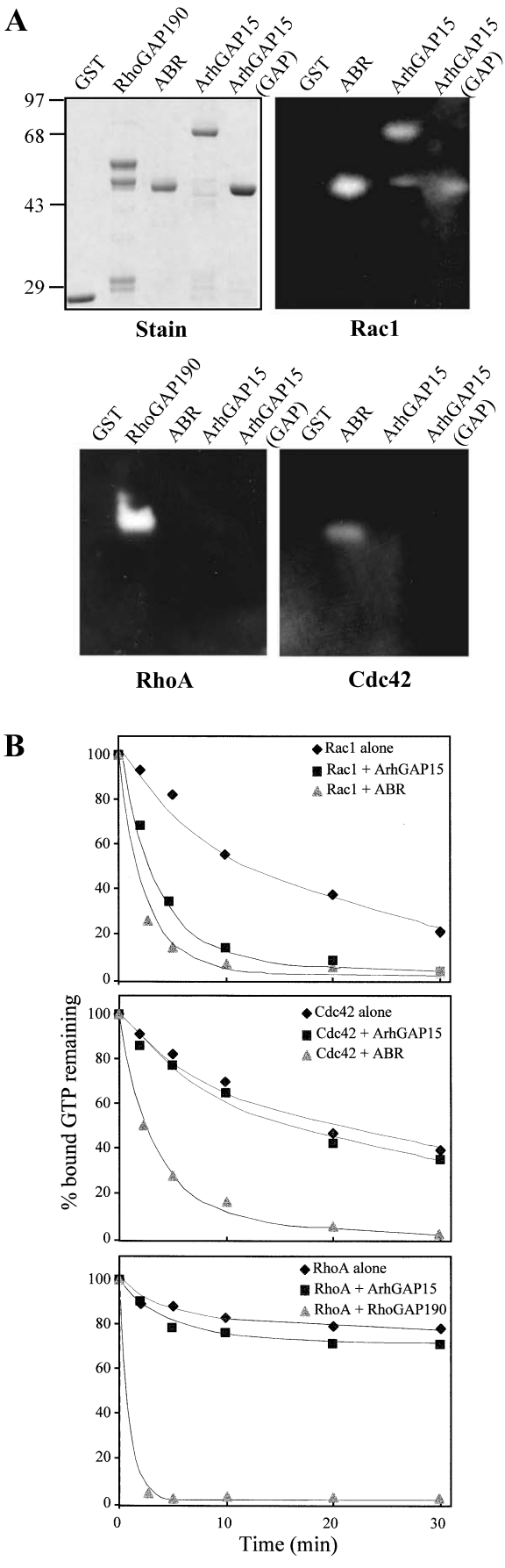

Fig. 2. ArhGAP15 has specific Rac1 GAP activity. A: Filter overlay assay for GAP activity. Negative control GST and positive controls of GAP domain of RhoGAP190 (RhoGAP) and ABR together with various GST-fusion proteins of ArhGAP15 were separated on 10% polyacrylamide gel and transferred to PVDF filter for Coomassie blue staining, renaturation and probing for GAP activity with various GTPases exchanged with [γ^{32}P]GTP as previously described [13]. Band with clear area indicates active GTP hydrolysis and GAP activity. B: Solution GAP assay for ArhGAP15. GAP activity of ArhGAP15 towards Rac1 was assayed in solution with [γ^{32}P]GTP-bound Rac1, Cdc42 and RhoA. A comparison was made with the GAP domain of ABR (for Rac1 and Cdc42) and RhoGAP190 (RhoA) as controls.

although the PH domain is not typical (data not shown). ArhGAP9, ArhGAP12, ArhGAP15 and ESTaa468877 can therefore be considered as structurally related RhoGAP proteins, with various regulatory domains at their N-termini (Fig. 1D). Most strikingly, these RhoGAP proteins share sequence homology immediately N-terminal to the GAP domains (Fig. 1C), the function of which has not been previously studied.

Expression of a 1.7 kb mRNA of ArhGAP15 was detected in human spleen, lung, liver and lymphoid cells such as HL-60 (Fig. 1E). Additional mRNA of different sizes were also detected in skeletal muscle (1.4 kb) and thymus (3 kb).

3.2. *ArhGAP15 has specific GAP activity to Rac1*

It has now been established that many of these RhoGAPs have characteristic activities towards specific GTPases [5,13]. All Chimaerin members are more active towards Rac1, whereas the related BCR and ABR proteins are active towards both Rac1 and Cdc42 [8,12]. As the RacGAP members are phylogenetically closer to abr and bcr proteins (Fig. 1D), it is of interest to determine the substrate specificity of ArhGAP15. When ArhGAP15 was expressed as full-length or GAP domain GST-fusion proteins, they showed GAP activities towards Rac1, but not Cdc42 and RhoA in both the filter assay (Fig. 2A) and solution assay (Fig. 2B). They therefore behave more like the Chimaerin proteins in term of substrate specificity towards Rac1, although they are structurally more related to ABR/BCR proteins. However, a recent report has shown that the related ArhGAP9 has GAP activity toward both Rac-1 and Cdc42. It should be noted that ArhGAP9 has an extended loop (residues 576–594; [6]) between the GAP homology 1 and GAP homology 2 regions. Whether this extra loop region affects substrate specificity remains to be determined.

3.3. *A conserved motif responsible for Rac1 binding*

Apart from having RacGAP activities, an apparent observation was an additional GTPase binding property of ArhGAP15 on the filter assay (Fig. 3A). ArhGAP15 containing the central conserved motif N-terminal to the GAP domain is required for interaction with Rac1 but not with Cdc42 (Fig. 3A). The weak interaction of the GAP domain alone was not detectable with this assay (Fig. 3A). We have mapped the BD of ArhGAP15 to the conserved domain since the expression of this motif alone is equally effective in binding to Rac1 (Fig. 3B).

To characterize the nature of the interaction, we have used the swop mutants of Rac1/Cdc42 for the binding experiments. Here we showed that Rac-1 binding to ArhGAP15 requires the C-terminal half of Rac1 (Fig. 3C). Furthermore, binding of ArhGAP15 to mutant Cdc42^{64}Rac1^{107}Cdc42 showed much weaker binding, suggesting a large part of the C-terminus may be involved in interaction (Fig. 3C). To see if the interaction was nucleotide-dependent, as has been documented for CRIB domain proteins such as that of αPAK, we tested the binding with GTP-bound and GDP-bound forms of Rac1. As shown in Fig. 3D, ArhGAP15 interacted equally well with both GTP- and GDP-bound forms of Rac-1, suggesting that the GTPase binding is not dependent on the nucleotide state of Rac-1. Similar Rac-1 binding was also obtained with the related ArhGAP12 (data not shown) indicating that this binding property is shared amongst these members of GAP proteins. Rac-1, but not Cdc42, was also able to interact with ArhGAP15 over-expressed in COS-7 cells (Fig. 3E).

3.4. *PH domain is required for ArhGAP15 localization to cell periphery*

Both full-length ArhGAP15 and PH domain proteins were detected in both cytosolic and membrane fractions (Fig. 4A, top panel), whereas the N-terminal deleted ArhGAP15 protein (residues 128–475) was predominantly cytosolic. In HeLa cells expressing full-length ArhGAP15, punctate structure at the cell periphery was observed (Fig. 4A, lower panel). The expression of the mutant ArhGAP15 (ArhGAP15$^{128-475}$) with partial deletion of PH domain showed more diffused distribution in cytoplasm (Fig. 4A, b). This distribution of ArhGAP15 to the cell periphery is dependent on the PH domain as the expression of this domain alone clearly showed typical cell peripheral staining (Fig. 4A, c).

3.5. *ArhGAP15 effects on cellular morphology depend on both PH and GAP domain*

Overexpression of ArhGAP15 in HeLa cells by transfection and prolonged expression generally resulted in cell rounding (data not shown). To minimize this effect, we microinjected ArhGAP15 constructs into HeLa cells and observed morphological effects 2 h after injection. As shown in Fig. 4B, expression of ArhGAP15 caused rapid cell contraction and a moderate increase in actin stress fibers (a and b). These effects were not observed with the PH domain truncated mutant (c and d), indicating that an intact PH domain is required not only for localization of ArhGAP15, but also morphological consequences. Expression of the PH domain alone was not sufficient to give similar effects (data not shown), suggesting that both the PH and the C-terminal including the BD and GAP domain are required for ArhGAP15 biological activities. These effects of ArhGAP15 can be abrogated when Arh-

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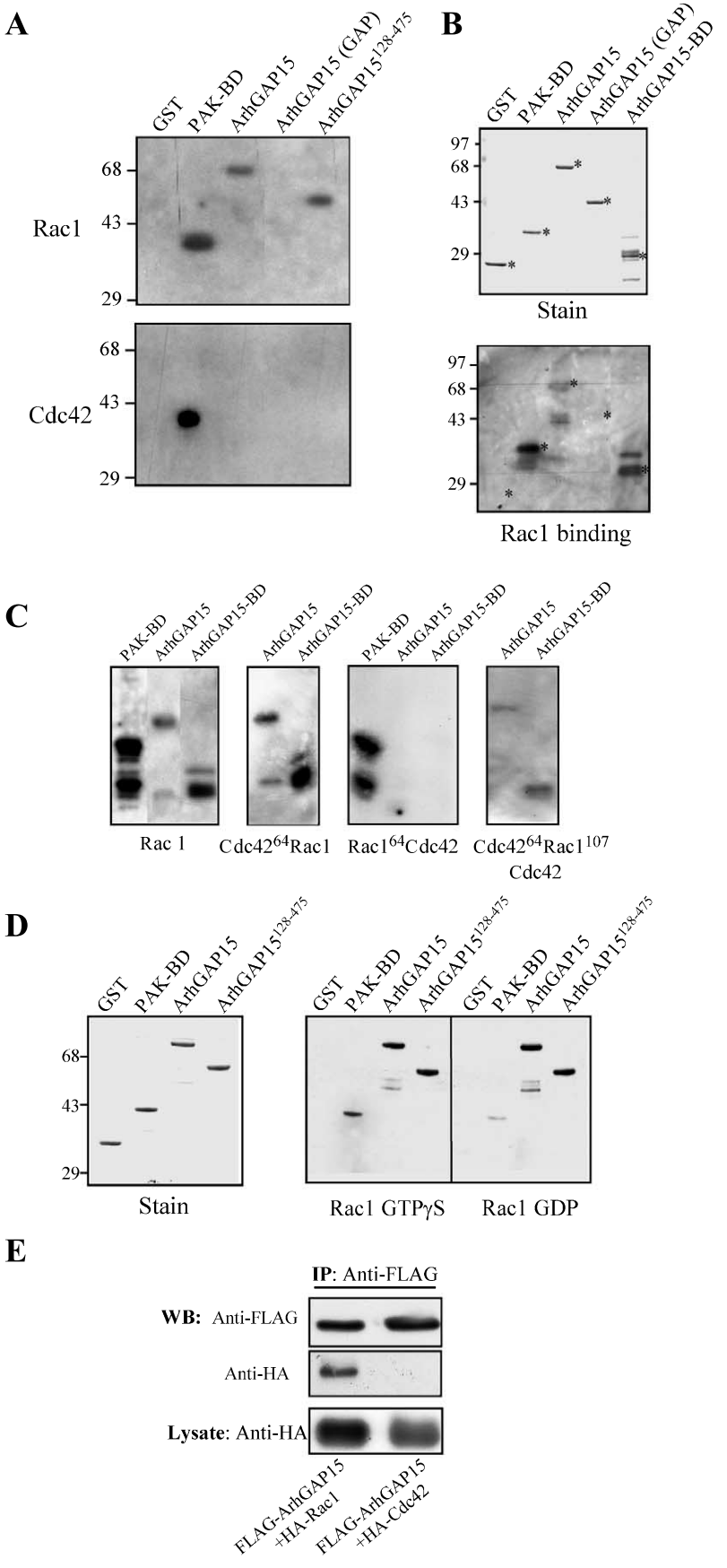
Fig. 3. ArhGAP conserved central motif binds the C-terminus of Rac1. A: Filter binding assay was carried out with [γ^{32}P]GTP-bound Rac1 according to Manser et al. [11]. Fusion proteins of GST, αPAK-BD, and various ArhGAP15 proteins were separated on 10% PAGE. After transferring to PVDF and renatured with renaturation buffer, the filter was incubated with radiolabelled Rac1 for 10 min at 25°C. After extensive washing, the filter was exposed to X-ray film for 2 h at −80°C. B: Mapping of the Rac1 binding to the conserved central motif. Various deletion mutants were produced as GST-fusion proteins for Rac1 binding as described in A. The minimal construct containing the conserved central motif showed intense binding indicating this motif is responsible for Rac1 interaction. C: ArhGAP15 binding requires the C-terminus of Rac1. Swop mutants of Rac1 and Cdc42 (Cdc42^{64}Rac1, Cdc42^{64}Rac1^{107}Cdc42 and Rac1^{64}Cdc42) were used for analysis of region on Rac1 that interact with ArhGAP15. D: Binding of ArhGAP15 to Rac1 is nucleotide independent. Biotinylated Rac1 exchanged with either GDP or the non-hydrolyzable GTP-γS was used for filter binding assay to ArhGAP15. E: ArhGAP15 expressed in Cos-7 cells interacted with Rac1. FLAG-tagged ArhGAP15 construct was co-transfected with either HA-tagged Rac1 or Cdc42 construct. Immunoprecipitation (IP) of ArhGAG15 was carried out with anti-FLAG antibody and Western blot (WB) analysis was carried out with either anti-FLAG (for ArhGAP15) or anti-HA antibodies (for Rac1 and Cdc42) on immunoprecipitates (top two panels) and cell lysates (bottom panel).


A
GST
PAK-BD
ArhGAP15
ArhGAP15 (GAP)
ArhGAP15128-475
Rac1
Cdc42
68
43
29
B
GST
PAK-BD
ArhGAP15
ArhGAP15 (GAP)
ArhGAP15-BD
97
68
43
29
Stain
Rac1 binding
C
PAK-BD
ArhGAP15
ArhGAP15-BD
Rac 1
Cdc4264Rac1
Rac164Cdc42
Cdc4264Rac1107
Cdc42
D
GST
PAK-BD
ArhGAP15
ArhGAP15128-475
Stain
Rac1 GTPγS
Rac1 GDP
E
IP: Anti-FLAG
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Anti-HA
Lysate: Anti-HA
FLAG-ArhGAP15 +HA-Rac1
FLAG-ArhGAP15 +HA-Cdc42

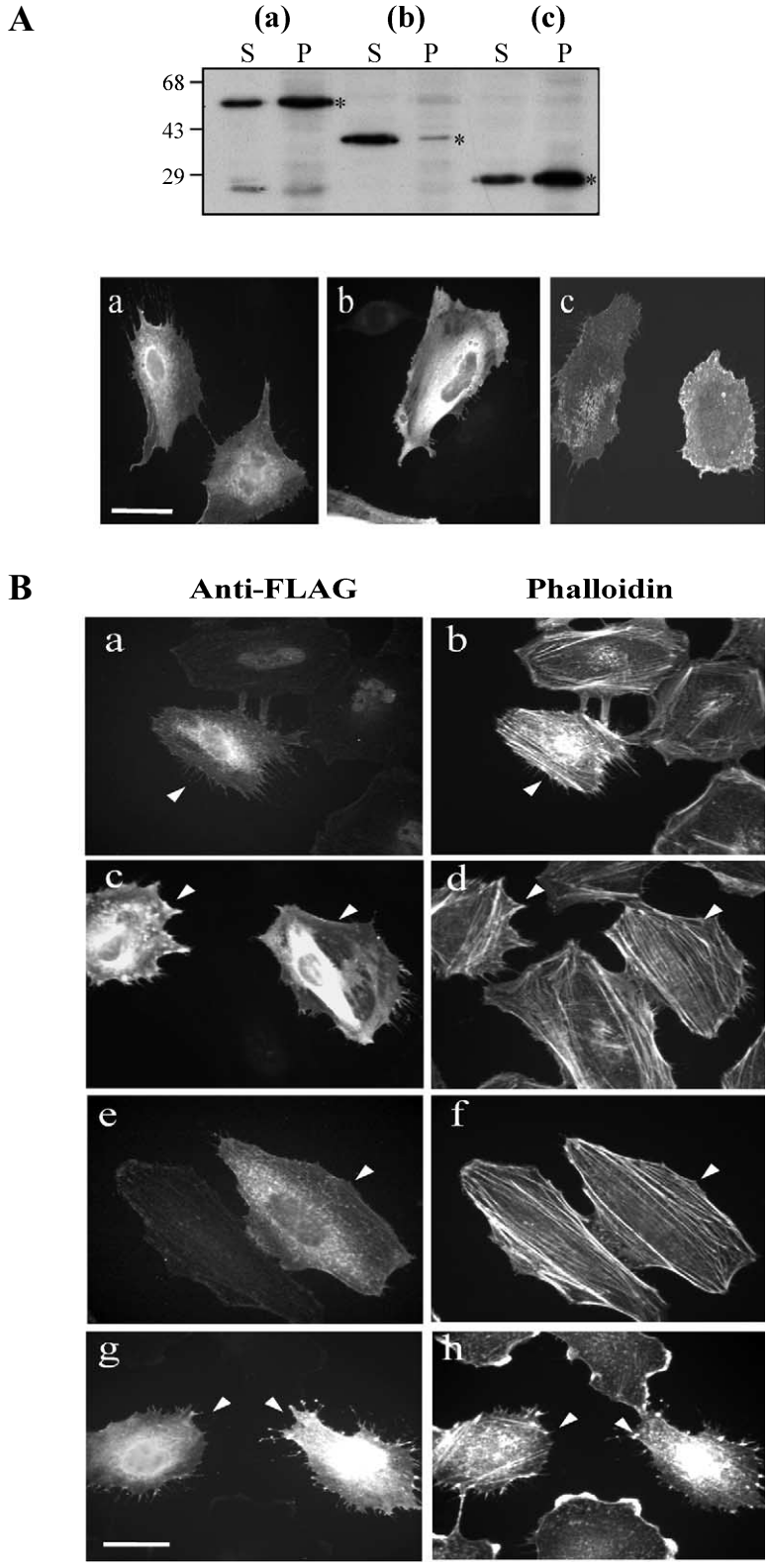

Fig. 4. The membrane targeting PH domain and RacGAP domain are both essential for the morphological effects of ArhGAP15. A: Membrane localization of ArhGAP15 is dependent on PH domain. COS-7 cells (top panel) or HeLa cells (lower panel) were transfected with (a) pXJ40-FLAG ArhGAP15$^{1-475}$, (b) pXJ40-FLAG ArhGAP15$^{128-475}$ (GAP domain) or (c) pXJ-FLAG ArhGAP15$^{79-262}$ (PH domain). For COS-7 cells, soluble and pellet fractions were isolated and 25 μg of each was separated on a 12% polyacrylamide gel and immunoblotted with mouse anti-FLAG antibody. For transfected HeLa cells, cells were fixed with 4% paraformaldehyde and immunostained with mouse anti-FLAG/FITC-rabbit anti-mouse antibodies. Bar = 10 μM. B: Cell morphological changes with ArhGAP15 expression depend on both PH and GAP domain. HeLa cells were microinjected with either pXJ40-FLAG ArhGAP15$^{1-475}$ (a and b), or pXJ40-FLAG ArhGAP15$^{128-475}$ (c and d). In e and f, cells were co-injected with full-length ArhGAP15 and pXJ40-HA Rac1^{N17}. In g and h, cells microinjected with full-length ArhGAP15 were serum starved and treated with 30 ng/ml PMA for 30 min. Cells were fixed with 4% paraformaldehyde and co-stained with mouse anti-FLAG/FITC anti-mouse and TRITC-conjugated phalloidin. Bar = 10 μM.

GAP15 was co-expressed with the dominant negative Rac1^{N17} (Fig. 4B, e and f), suggesting that recycling of Rac1 may be essential for the morphological phenotype.

Cells showing the morphological effects by the over-expression of ArhGAP15 were also resistant to Rac1 activation induced by PMA treatment (Fig. 4B, g and h). This further supports the notion that ArhGAP15 may be an essential regulator of Rac1.

4. Discussion

Here we have identified ArhGAP15 as a closely related member of a phylogenetically related RhoGAP subfamily that includes ArhGAP9, ArhGAP12 and an uncharacterized RhoGAP encoded by an EST cDNA aa468877. ArhGAP15 has a PH domain, which is also common to ArhGAP9 and ArhGAP12. In addition ArhGAP9 and ARhGAP12 have SH3 and WW domains in their regulatory N-terminus [5–7]. Most strikingly, all four members have a conserved central motif N-terminal to the GAP domain that is unique to this family of GAP proteins. Biochemically we have detected a specific interaction of this motif with Rac1. This interaction requires the C-terminus of Rac-1. This is different from the known CRIB motif binding which primarily involves the N-terminal effector loop (N-terminal residues 30–40) of Rac1/Cdc42 for interaction with a variety of regulatory effectors [14]. Furthermore unlike the CRIB motif, the binding is nucleotide independent as both GTP- and GDP-bound forms of Rac1 can interact equally well with ArhGAP15. The significance of this binding is currently not known. A similar binding of *Arabidopsis* Rop1 GTPase through a central CRIB motif has been reported for the unique plant RopGAP proteins [15], where the GTPase binding resulted in an enhancement of the GAP activity. It is therefore possible that for ArhGAP15, the binding to Rac1 may also affect GAP activity, resulting in an increase in GTP hydrolysis, thereby down-regulating Rac1. In this respect, it is interesting to know that ArhGAP15 has a GAP activity towards Rac1, but not Cdc42 and RhoA.

In general, RhoGAP proteins function in down-regulation of specific GTPase signals, although some effector functions have been assigned to some known GAPs. For example, the expression of n-Chimaerin, a RacGAP protein, gave rise to multiple rounds of lamellipodia and membrane protrusions, suggesting that an intact GTPase cycle is required locally for the cytoskeletal reorganization to occur [16]. Some of these spatial requirements for effector functions most likely depend on their distinctive regulatory domains for the various cellular GAP proteins. In the case of ArhGAP15, over-expression in general resulted in cell retraction and subsequent cell rounding. Within a short period of expression after microinjection, a gradual cell contraction with concomitant increases in actin stress fibers was observed. The PH domain, which is required for cell periphery distribution of ArhGAP15, is also essential for the morphological effects as deletion of the first 127 amino acid residues abolished these effects. The PH domain alone is sufficient for cellular localization, but is not effective in producing similar cellular effects, indicating that both the PH and GAP domains are necessary for such cytoskeletal changes. Cells showing this cytoskeletal arrangement are also resistant to PMA treatment, which in general, activates Rac1 [17]. It is not known if this is due to the involvement of both the cell contraction event, which counteracts the induction of lamellipodial formation and/or the direct result of down-regulation of Rac1. As resistance to PMA treatment was also observed with the GAP domain of ArhGAP15 alone (data not shown), down-regulation of Rac1 may play a more important role for the resulting phenotypic effects observed with the full-length protein upon PMA treatment. Taken together, these results suggest that ArhGAP15 could be a genuine regulator of Rac1 signaling.

The N-terminus of ArhGAP15 is also a good in vitro substrate for a number of Rac1 effector kinases including αPAK and MRCKα (data not shown). It is currently not known what are the biological consequences of these phosphorylation events. Phosphorylation of the regulatory domain of RhoGAP190 by Src is required for regulating the RhoGAP activity [18]. Future experiments should address the in vivo modifications and the physiological roles of these structurally and phylogenetically related RhoGAP proteins.

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